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N^{α}-Acetylcarnosine is a prodrug of L-carnosine in ophthalmic application as antioxidant

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Abstract

The naturally occurring compound N^{α}-acetylcarnosine (NAC) is proposed as the prodrug of L-carnosine (C) resistant to enzymatic hydrolysis by human serum carnosinase. Rabbit eyes were treated with 1% NAC, C or placebo and extracts of the aqueous humor from the anterior eye chamber were analyzed for imidazole content by reverse phase analytical high performance liquid chromatography (HPLC), thin-layer (TLC) and ion-exchange chromatographic techniques. The topical administration of pure C to the rabbit eye did not lead to accumulation of this compound in the aqueous humor over 30 min in concentration exceeding that in the placebo-treated matched eye. NAC showed dose-dependent hydrolysis in its passage from the cornea to the aqueous humor, releasing C after 15–30 min of ocular administration of prodrug in a series of therapeutical modalities: instillation $\leq$ subconjunctival injection $\leq$ ultrasound induced phoresis. Different treatment techniques showed excellent toleration of 1% NAC by the eye. Once in the aqueous humor, C might act as an antioxidant and enter the lens tissue when present at effective concentrations (5–15 mmol/l). The advantage of the ophthalmic prodrug NAC and its bioactivated principle C as universal antioxidants relates to their ability to give efficient protection against oxidative stress both in the lipid phase of biological membranes and in an aqueous environment. NAC is proposed to treat ocular disorders which have the component of oxidative stress in their genesis (cataracts, glaucoma, retinal degeneration, corneal disorders, ocular inflammation, complications of diabetes mellitus, systemic diseases).

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1. Introduction

L-Carnosine (β-alanyl-L-histidine), first identified in beef extract [1], has been found to be one of the most abundant (1–20 mmol/l) nitrogenous compounds present in the non-protein fraction of vertebrate skeletal muscle [2–4] and certain other tissues, including olfactory epithelium, bulbs (0.3–5.0 mmol/l) [5] and also the crystalline lens [6,7]. Some related compounds, e.g. anserine (β-alanyl-3-methyl-L-histidine), homocarnosine (α-amino-butyryl-L-histidine) and carcinine (β-alanylhistamine) have been reported [8,9] to be present at millimolar concentrations in several mammalian tissues, including skeletal muscles, cardiac tissue and brain, although there are interesting differences in their tissue distribution, activities and metabolic transformation [10,11].

Previously published data suggest that L-carnosine has excellent potential to act as a natural antioxidant with hydroxyl-radical- and singlet oxygen-scavenging and lipid peroxidase activities [10,12,13]; thus, it may be useful to prevent, or partially re verse, lens cataracts [6,12]. Exogenous carnosine entering the organism intravenously, intraperitoneally, with food or topically to the eye, is not accumulated by the tissues but is excreted in the urine or destroyed by carnosinase, a dipeptidase present in blood plasma, liver, kidney and other tissues, except muscle and, probably, lens [6,7,12,14,15].

The *N*-acetyl derivatives of histidine, carnosine and anserine exist in the cardiac and skeletal mammalian muscles and the total concentration of these imidazoles may lie within the measured range of that of L-carnosine in skeletal muscle (i.e. ≈ 10 mmol/l) [11]. pharmaceutical compositions containing NAC aluminium salt have been reported for the treatment of gastric ulcers [16]. Among 29 dipeptides of the carnosine family tested as potential substrates for a highly purified human serum carnosinase preparation, NAC and few other compounds were not hydrolysed [14] thus promising a prolongation of physiological responses to the therapeutical treatments. A knowledge of corneal and iris/ciliary body esterase activity, in particular, acetylesterase (EC 3.1.1.6) and, in addition to esterase, the identified *N*-acetyltransferase activities [17,18] prompted the development of L-carnosine for ophthalmic application as an antioxidant, e.g. as the chemically characterized N^{α}-acetylated form of the dipeptide. Due to its relative hydrophobicity compared to L-carnosine,

NAC might cross the cornea of the treated eye gradually and maintain longer the concentration of the active principle reaching the aqueous humor. In the present study we considered whether NAC topically administered to the eye acts as a pro-drug of L-carnosine.

2. Materials and methods

2.1. Chemicals and biological reagents

L-Carnosine was obtained from Neosystems Laboratories (France). The chemicals and biological reagents were purchased from Sigma Chemical Co (St. Louis, MO) except for the *N*-acetylated form of L-carnosine which was prepared by acetylation of the parent compound [19,20]. Solvents were of HPLC grade and other chemicals were reagent grade or purer.

2.2. NAC

To ascertain the physicochemical characteristics of NAC as a pure compound, NMR spectra were recorded in D_2O on a Bruker MSL 200 spectrometer operated at 200 mHz frequency. Tetramethylsilane was used as the internal reference. Analytical TLC was performed on silica gel 6OH plates (Merck) in chloroform/methanol/25% ammonia (3:3:1, v/v/v). The plates were developed with chloro-tolidene reagent. The product was collected to give $R_f = 0.73$; $[\alpha]_D^{27} + 17.0$ (CO.5, H_2O) measured on a Digital Polarimeter DIP-360 JASCO (Japan). Elementary analysis: calculated (%) — C, 41.0; H, 6.80; N, 17.40; found (%) — $C_{11}H_{16}N_4O_4 \cdot 3H_2O$; C, 41.17; H, 6.97; N, 17.70.

2.3. Experimental design

Fourteen grey Chinchilla rabbits (male, 28 eyes) 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. Fifteen or 30 min prior the ocular incision right eyes of rabbits were instilled with 80 μl of 1% NAC or 1% L-carnosine solutions and the control left eyes of the animals were similarly instilled with their vehicles. In the separate experimental groups of eyes, the tested solutions of compounds were administered to the examined or control eyes with a single subconjunctival injection (0.2 ml) or using a therapeutic ultrasound-induced (phonophoresis) procedure.

2.4. Ultrasound administration procedure

The application of therapeutic ultrasound was performed under topical ocular anaesthesia with trimecaine (dicaine) solutions (0.5%, 1 or 2 drops) within 5 min of the exposure time at an ultrasound intensity of 0.2 W/cm^2 and 880 kHz frequency with the ultrasound device UZT-104 (Russia). The matched (control) rabbit eyes were treated with the vehicle (placebo) solution as described above.

2.5. Surgical procedure

Topical anaesthesia of the rabbit eyes was performed with instillations of 5% trimecaine solution (three times at 1.5–2.0 min intervals). Also, 0.1 ml of the 0.5% novocaine solution was introduced by a subconjunctival injection, and infection in the extraocular tissues to induce lid anaesthesia and anaesthesia of the two vertical recti muscles. 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 a 25-gauge needle connected to an insulin syringe and immediately introduced into an Eppendorf tube with addition of ethanol (0.8 ml), keeping the sample on ice before extraction.

2.6. Preparation of the testing solutions

NAC and L-carnosine were prepared fresh by dissolving to 1% solution in Dulbecco's standard phosphate-buffered saline (pH 7.2–7.4) without divalent cations of Ca^{2+} and Mg^{2+} (Gibco Lab., USA) with the contents described earlier for use with peptides [21].

2.7. Lenses

Transparent rabbit lenses were obtained from freshly enucleated eyes of intact animals. In all cases the integrity of the lens capsule was preserved. The lenses removed were briefly rinsed in Hanks' medium for 20–30 s and then immediately placed in a moist chamber. The lenses were either used directly after the extraction procedure, or at least 1–3 h was allowed to elapse between the dissection from the eye and the start of the experiment. This delay did not influence the results.

2.8. 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 mmol/l (or higher concentration) L-carnosine solution in Hanks' salt medium (without bicarbonate, pH 7.4), containing 7 mmol/l glucose [12] and incubated at room temperature (20°C). After 1 h, the extraction procedure was utilized [12] 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 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 nm/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 [6] to evaluate the L-carnosine level in eye lenses.

2.9. Incubation of lenses with liposomes

To control effects of metal ions or potential $OH^{\cdot}$ scavengers (such as glucose present at scavenging concentrations in standard culture media), the lens culture medium was composed of 10 mmol/l Tris–HCl, 140 mmol/l NaCl (pH 7.4), phosphate and glucose-free (Medium A) or glucose-containing medium nutritious to the lens comprising 50 mmol/l Tris–HCl, 5.5 mmol/l glucose, 4.0 mmol/l KCl, 102.5 mmol/l NaCl, 1.0 mmol/l K_2HPO_4 (pH 7.2–7.4) (Medium 3) [22]. 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 [23]. 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 lipid peroxidation (LPO) products. Generally, the total incubation time was 3 h.

2.10. Extraction of imidazoles from aqueous humor

Extractions of imidazole-containing compounds from the aqueous humor

aliquots were performed according to O'Dowd et al. [11]. 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 and evaporated to dryness under reduced pressure and heating below 40°C before being resuspended in phosphate buffer (Na_2HPO_4, 0.1 mol/l, pH 7.0, 2.0 ml) prior to analytical HPLC.

2.11. Analytical HPLC

Reverse phase analytical HPLC was performed using a Gilson 714 liquid chromatography system (Gilson, Villiers le Bel, France). Samples dissolved in phosphate buffer as described above were injected (20 μl) onto a column (250 × 4.6 mm) packed with Partisil 5 μm ODS-3 (Anachem Ltd., Luton, UK). The column was eluted isocratically at 20°C with phosphate buffer (Na_2HPO_4, 0.1 mol/l, pH 2.1) over 25 min at a flow rate of 1.0 ml/min. Eluates were monitored for absorbance at 210 nm. The output was relayed to a data processor and areas under the peaks were used to provide data for calibrating the system. Subsequent analyses utilized stored chromatographic calibration data for comparison. Figures quoted are means ± S.D. after adjustment for extraction efficiency (see Section 3). Standard solutions of L-carnosine, *N*α-acetylcarnosine or other compounds were used for calibration.

2.12. Visualization with thin layer chromatography (TLC)

After removal of the water-ethanol solution under vacuum, the extracts from the aqueous humor were concentrated and the products of NAC biotransformation were identified by TLC on silica gel 6OH plates (Merck). The plates were developed with chloroform/methanol/25% water ammoniac (3:3:0.5 or 3:3:1, v/v/v) and stained with ninhydrin solution in acetone. Appropriate control lanes with standard peptides or constituent amino acids or pharmaceuticals were run simultaneously.

2.13. Ion-exchange chromatography

After deproteinisation of the aqueous humor samples, solubilisation in ethanol and drying, approximately 100–200 μl of the extracted probes were used for ion-exchange chromatography on a Chromaspec (Rank Hilger, UK) amino acid analyzer equipped with Dowex ion-exchange resin column and a two-channel ninhydrin detector. Each run was calibrated by the standard mixture of all amino acids (100 nmol/ml, each) and an internal standard of β-thienylalanine. The detection limit was approxi-

mately 14 nmol/ml for each amino acid. The amino acids were eluted in a standard regime with buffers in the pH range from 2.2 to 11.5, under pressure, at 40°C.

2.14. Peroxidation reaction system

The techniques for phospholipid extraction, purification and preparation of liposomes (reverse-phase evaporation technique) have been described previously [12,22]. Peroxidation of phosphatidylcholine (PC, derived from egg yolks) was initiated by adding 2.5 μmol/l $FeSO_4$ and 200 μmol/l ascorbic acid to the suspension of liposomes (1 mg/ml) in 0.1 mol/l 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 mmol/l 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 μmol/l or by the addition of 2.0 ml of ice-cold 0.25 mol/l HCl containing 15% (w/v) TCA. TBA (0.125% w/v) was then added to the mixture and followed by boiling for 15 min. The TBA assay was described previously [22]. The differential absorbance of the condensation product, malonyl dialdehyde (MDA), at 535 and 600 nm was measured spectrophotometrically ($\epsilon_{535} = 1.56 \times 10^5\ M^{-1} \cdot cm^{-1}$). 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 [22]. This protocol removes any water-soluble secondary oxidation products, leaving them in the methanol-aqueous phase. 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, v/v). Accumulation of net diene conjugates corresponding to the level of lipid hydroperoxides was assessed from characteristic absorbance of diene conjugates at ≈ 230 nm ($\eta CD = 2.8 \times 10^4\ M^{-1} \cdot cm^{-1}$), in a Shimadzu UV-260 spectrophotometer (Japan) [22]. 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 [22]. An average MW of phospholipid was assumed to be ≈ 730 Da.

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

3. Results

3.1. Pharmacokinetics of NAC and L-carnosine in rabbit eyes (in vivo studies)

Histidyl compounds of aqueous humor have been examined by reverse phase analytical HPLC (see Section 2) as used in separation of imidazole-containing amino acids and dipeptides from muscles [11]. Amino acids can be detected by the absorbance of carboxylate (≈ 200 nm) and peptides by absorbance of carboxylate and the peptide bond (200–220 nm). Chromatograms of solutions of L-carnosine and its putative *N*-acetyl derivative (Fig. 1a) show that these compounds are well separated. The elution order of the compounds was compared to a predicted order based upon their relative hydrophobicities as outlined by Rekker [24]. The chromatographic system is suitable to monitor the behaviour of other histidine containing derivatives of L-carnosine (Fig. 1b). The calibrating chromatograms show the predicted elution order and the average elution times for each standard of L-carnosine, anserine and NAC in mixtures. Peaks were unequivocally identified by comparison of their retention times to those of the authentic standard compounds or of putative acetylated compound run singly (Fig. 1). Tests for specific chemical reactivity [11] provided additional evidence for the identification of L-carnosine, anserine and acetylcarnosine. The HPLC pattern of an extract of the aqueous humor obtained after instillation of 1% NAC to the rabbit eye confirms that the peak characteristic of L-carnosine (C) has a retention time clearly distinct from the dead time of the column (peak S) and relates to the extended peak in the region of 3.59 min (Fig. 2). The identified L-carnosine peak overlaps with several smaller peaks of unidentified compounds (presumably of amino acid or peptide nature): the data processor integrates for the overall extraction efficiency at retention times of 3.58–3.62 min. The data on the L-carnosine-related product (Crp) were blanked with the control placebo data applied to the paired eyes of the animals. The peak in the region of 3.59 min shows asymmetry in integrated concentrations of the Crp (mean $\pm$ S.D. adjusted for extraction efficiency, $n = 5$ measurements) between the 1% NAC-treated and placebo matched eyes, indicating that the major imidazole of the 1% NAC-treated eye is L-carnosine. Traces of NAC are detectable in the chromatogram of aqueous humor from the 1% NAC-treated eye (Fig. 2). Quantitative HPLC analysis of the peptide moieties in the aqueous humor shows significant differences in concentrations of Crp in the 1% NAC-treated eyes and placebo eyes (Table 1). Concentrations of imidazole products in the aqueous humor of intact rabbit eyes and the notation 'if any' (Table 1) refer to baseline values of

Crp variously detected in extracts from normal animals. The difference between the value for the 15-min time-point after the instillation of 1% NAC and the corresponding 30 min value (Table 1) indicate that the

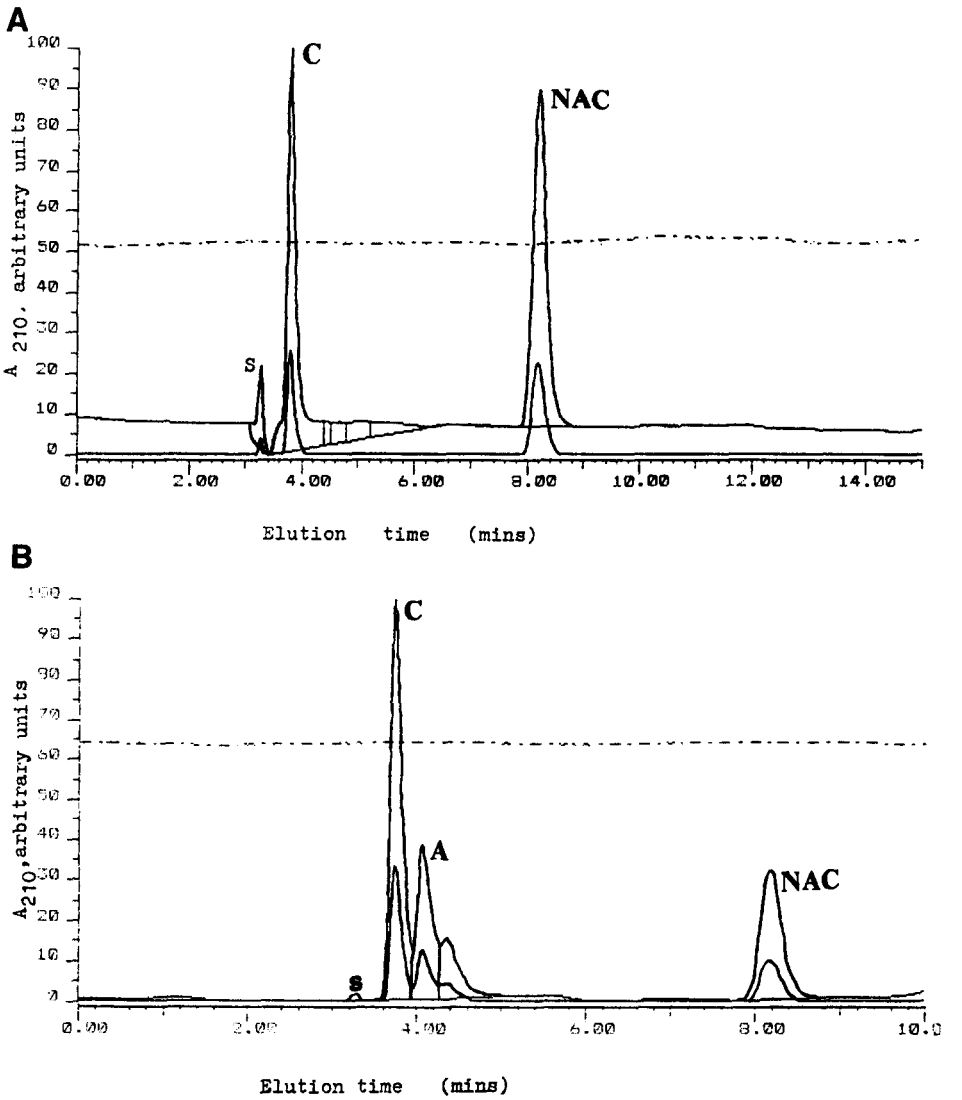

Fig. 1. HPLC of a mixture of L-carnosine (C), anserine (A) and NAC. Peak fractions were examined for specific chemical reactivity. The integrated calibrating concentrations for the standard peaks were: (a) 5.25×10^{-2} mg/ml, 3.77 min for C; 9.75×10^{-2} mg/ml, 8.17 min for NAC; (b) 3.89×10^{-2} mg/ml, 3.73 min for C; 3.28×10^{-2} mg/ml, 4.06 min for A; 3.33×10^{-2} mg/ml, 8.16 min for NAC. The retention times of compounds in mixtures did not vary significantly from those run singly. Peak S is the solvent front.

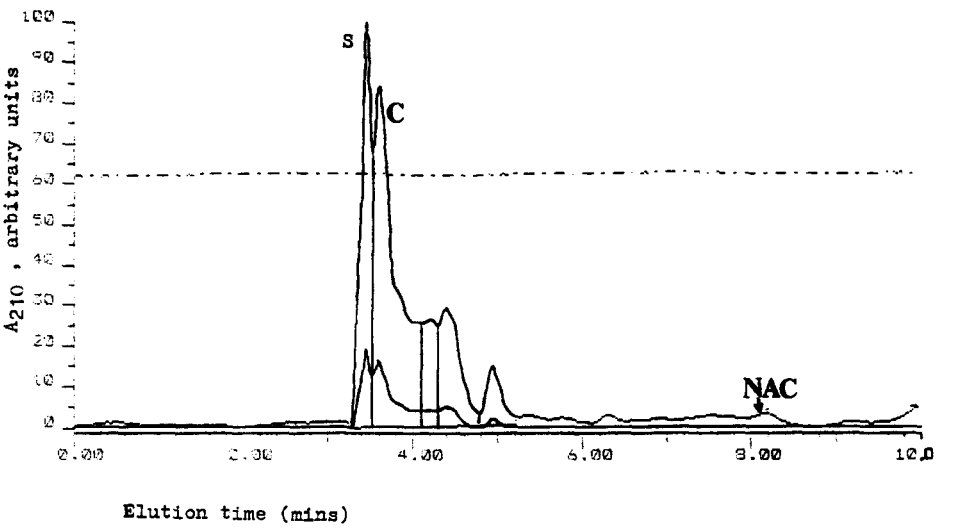

Fig. 2. HPLC of extract of aqueous humor aspirated 30 min after the instillation of 1% NAC to the rabbit eye. The difference between the integrated concentration of the C-related product (3.63×10^{-2} mg/ml, 3.59 min) and that in the matched placebo-treated eye (2.83×10^{-2} mg/ml, 3.58 min) is attributed to accumulation of C in the NAC-treated eye. Traces of NAC are detectable with a retention time of 8.16 mind Peak S is the solvent front.

released L-carnosine does not accumulate in the aqueous humor during the prolonged time intervals, probably due to its uptake by the adjacent ocular (lens) tissues. The small but significant variation in concentration of L-carnosine in the aqueous humor with administration technique is consistent with differences in effectiveness of administration of NAC, in decreasing in order of effectiveness: ultrasound-induced phoresis $\geq$ subconjunctival injection $\geq$ instillation (Table 1). Thirty minutes after administration of 1% L-carnosine or place concentrations of the product with the elution time predicted for L-carnosine was noted in the matched treated eyes (Tables 1 and 2). This indicates that L-carnosine fails to appear in the aqueous humor in concentrations above those in placebo (vehicle) treatments. Some of the placebo values of Crp are even greater than the integrated concentrations in L-carnosine-treated eyes (Tables 1 and 2). Possibly part of the L-carnosine in aqueous humor is rapidly hydrolysed (within 30 min) by serum carnosinase.

The L-carnosine-related product contains highly polar short-time retained material which may be integrated with other hydrophillic molecules and co-eluted with medicaments used for topical anaesthesia of the eyes and potentially contaminating the extract of aqueous humor. Dose-dependent HPLC analysis of trimecaine (dicaine) and novocaine solutions in the system utilized to detect imidazoles showed retention times of 3.51 min for trimecaine (dicaine) molecules and over 9 min for novocaine moieties.

Although trimecaine (dicaine) ingredients were eluted with retention time close to the Crp, they were not co-eluted with L-carnosine, so that this imidazole compound in aqueous humor could be chromatographed separately against other blanks.

Extracts of aspirated aqueous humor were examined by TLC and visualization techniques designed to indicate reactive functional groups. The

Table 1
Elution order and concentration of imidazole-containing compounds extracted from aqueous humor samples and separated by HPLC

Characteristic of the treatment to the matched eyes	Time after administration to the eye (min)	Predicted product	Elution time (mean ± S.D., min)	Concentration (μg/l)
Intact rabbit eye	None	Crp	3.59 ± 0.01	29.9 ± 7.9
Instillation (80 μl)				
1% NAC (OD)	15	Crp	3.63 ± 0.01	49.2 ± 1.3**
Placebo (OS)	15	Crp	3.60 ± 0.02	30.2 ± 1.9
1% NAC (OD)	30	Crp	3.59 ± 0.01	33.1 ± 1.1*
Placebo (OS)	30	Crp	3.58 ± 0.01	27.7 ± 1.7
1% L-Carnosine (OD)	30	Crp	3.58 ± 0.01	27.0 ± 5.6‡§
Placebo (OS)	30	Crp	3.58 ± 0.01	33.2 ± 5.8
Subconjunctival infection (0.2 ml)				
1% NBC (OD)	30	Crp	3.60 ± 0.00	35.7 ± 2.7*
Placebo (OS)	30	Crp	3.59 ± 0.01[a]	25.7 ± 2.6[a]
1% L-Carnosine (OD)	30	Crp	3.59 ± 0.01	32.6 ± 5.7‡§
Placebo (OS)	30	Crp	3.59 ± 0.01	33.5 ± 7.2
Ultrasound-induced phoresis				
1% NAC (OD)	30	Crp	3.60 ± 0.01	41.5 ± 1.8†
		NAC	8.22 ± 0.05	2.4 ± 0.5
Placebo (OS)	30	Crp	3.58[a]	31.0 ± 2.0[a]

Each value represents mean ± S.D. of at least five measurements. OD, right eye; OS, left eye; Crp, L-carnosine-related product.

[a]If any.

*$P < 0.05$ (non-significant difference), compared to administration of placebo to the matched eye.

**$P < 0.001$ (non-significant difference), compared to administration of placebo to the matched eye.

†$P < 0.02$ (non-significant difference), compared to administration of placebo to the matched eye.

‡$P > 0.1$ (non-significant difference), compared to administration of placebo to the matched eye.

§$P > 0.1$ (non-significant difference), compared to other route of administration.

Table 2
Concentration of the predicted L-carnosine product in extracts of aqueous humor after amino acid analysis

Characteristic of the treatment to the matched eyes	Time after the administration to the eye (min)	Concentration	
		μg/ml	nmol/ml
None, intact rabbit eye	–	8.4 ± 2.0	19.0 ± 7.9
Instillation (80 μl)			
1% NAC (OD)	30	13.7 ± 1.3*	28.5 ± 2.8*
Placebo (OS)	30	7.7 ± 1.9	17.4 ± 3.1
1% L-Carnosine (OD)	30	10.5 ± 2.1**	23.7 ± 4.2**
Placebo (OS)	30	11.9 ± 4.1	26.8 ± 7.2
Subconjunctival injection (0.2 ml)			
1% NAC (OD)	30	22.4 ± 1.7†	50.6 ± 7.1*
Placebo (OS)	30	10.8 ± 3.5	22.1 ± 7.9
1% L-Carnosine (OD)	30	12.6 ± 2.0**	25.5 ± 3.0**
Placebo (OS)	30	18.1 ± 4.3	26.1 ± 5.7
Ultrasound-induced phoresis			
1% NAC (OD)	30	28.0 ± 3.4*	63.2 ± 8.5*
Placebo (OS)	30	14.7 ± 3.5	33.2 ± 7.5

Each value represents mean ± S.D. of at least three measurements.
*$P < 0.05$, difference with the placebo administration.
**$P > 0.1$, difference with the placebo administration.
†$P < 0.02$, difference with the placebo administration.

results of staining with ninhydrin to visualize both the chromatographic fractions of authentic compounds and their putative *N*-acetyl derivatives suggest that TLC verifies the information obtained by HPLC, but with lower sensitivity. The free histidine impurity was absent from extracts of aqueous humor according to the TLC data. The reactivity of L-carnosine can be found occasionally in the aqueous humor from intact rabbit eyes. In eyes treated 30 min previously with 1% NAC (instillation, subconjunctival injection, ultrasound-induced administration), the L-carnosine compound appeared as a mauve spot ($R_f = 0.10$) after TLC separation with chloroform/methanol/25% aqueous ammonia (3:3:0.5, v/v/v) as a developing solvent. The asymmetry in appearance of the Crp in aqueous humor or 1% NAC-treated eyes and placebo-treated eyes agrees with the analytical HPLC data. Similar treatments

with 1% L-carnosine or placebo eventually showed weak ninhydrin staining close to the L-carnosine reference ($R_f = 0.27$) and the faster and slower contaminants of amino acid or protein character after concentration of water-ethanol extracts and TLC separation with a modified developing solvent (3:3:1, v/v/v). No distinct differences in the presence of the Crp or the intensity of its staining were detected between the matched eyes, indicating lack of specific persistence of the dipeptide product in the aqueous humor of the L-carnosine- and placebo-treated eyes. The novocaine standard showed an R_f-value greater than 0.50 during the concomitant staining with chlorotolidene reagent.

The L-carnosine and free amino acids contents of extracts of aqueous humor were also estimated by amino acid analysis (Table 2). In the 1% NAC-treated and placebo eyes the levels of free amino acids in the aqueous humor were comparable with the amounts of L-carnosine. L-Histidine, L-carnosine and the mixture thereof had coinciding retention times during amino acid analysis. Since TLC suggested the absence of appreciable amounts of free histidine in extracts of aqueous humor, the sum of histidyl-containing compounds in the samples mainly consisted of the L-carnosine product. Table 2 shows that persistence of L-carnosine in the aqueous extracts after ocular treatment with 1% NAC tends to increase in ascending order of application modalities as follows: instillation $\leq$ subconjunctival injections $\leq$ ultrasound-induced phoresis. The discrepancy in absolute values of the Crp concentration between the HPLC and amino acid analyses is due to the overall integration of areas with a Chromatopac (HPLC) data processor corresponding to the peaks of compounds which have retention times (3.58–3.62 min) almost equalling that of L-carnosine (Fig. 2). The significant differences in the Crp concentrations between the aqueous humor from the 1% NAC-treated and placebo matched eyes were in agreement by both the amino acid and HPLC analyses adjusted for monitoring of L-carnosine. Amino acid analysis also showed that the level of L-carnosine in the aqueous humor does not increase over 30 min of ocular treatment with 1% L-carnosine, independently of the administration technique used, in good agreement with the chromatographic results.

3.2. Studies with isolated lenses of oxidative stress and the antioxidative capacity of L-carnosine and its derivative NAC

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 mmol/l). Incubation of lens preparations with histidine depeptide led to its accumulation in the lens tissue (Fig. 3). 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 its concentration in this case was about 25 μmol/l in normal human lenses and 0.89 ± 0.1 mmol/l ($n = 4$), rabbit lenses [6,7]. At different stages of cataract development, the level of L-carnosine fell, reaching about 5 μmol/l in ripe human cataracts [6]. Thus, L-carnosine that finds its way into the aqueous humor can accumulate in the lens tissue for a reasonable period of time.

The comparative antioxidant activity of NAC and L-carnosine was assessed in the liposome peroxidation system, catalyzed by Fe^{2+} + ascorbate (Fig. 4). The accumulation kinetics of molecular LPO products such as MDA, liposomal-conjugated dienes and trienes, are shown in Fig. 4a–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 mmol/l. Data on the biological effectiveness of L-carnosine as antioxidant preventing PC liposomal or linoleic acid peroxidation in physiological concentration ranges of 5–25 mmol/l have

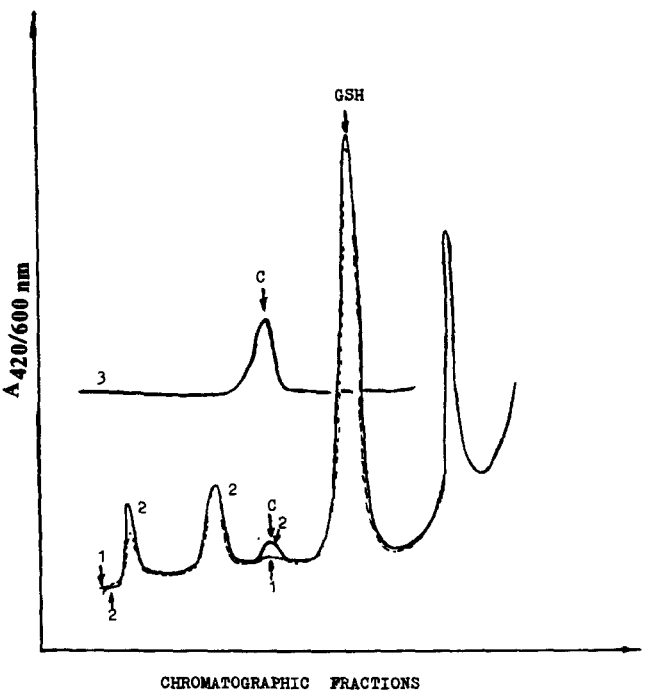

Fig. 3. Absorbances of the extracted rabbit lens components after TLC separation. Curve 1 (broken line), extract of the control lens incubated for 60 min in Hank's medium. Curve 2, extract of the lens incubated for 60 min in a medium containing L-carnosine (5 mmol/l). Curve 3, a standard preparation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

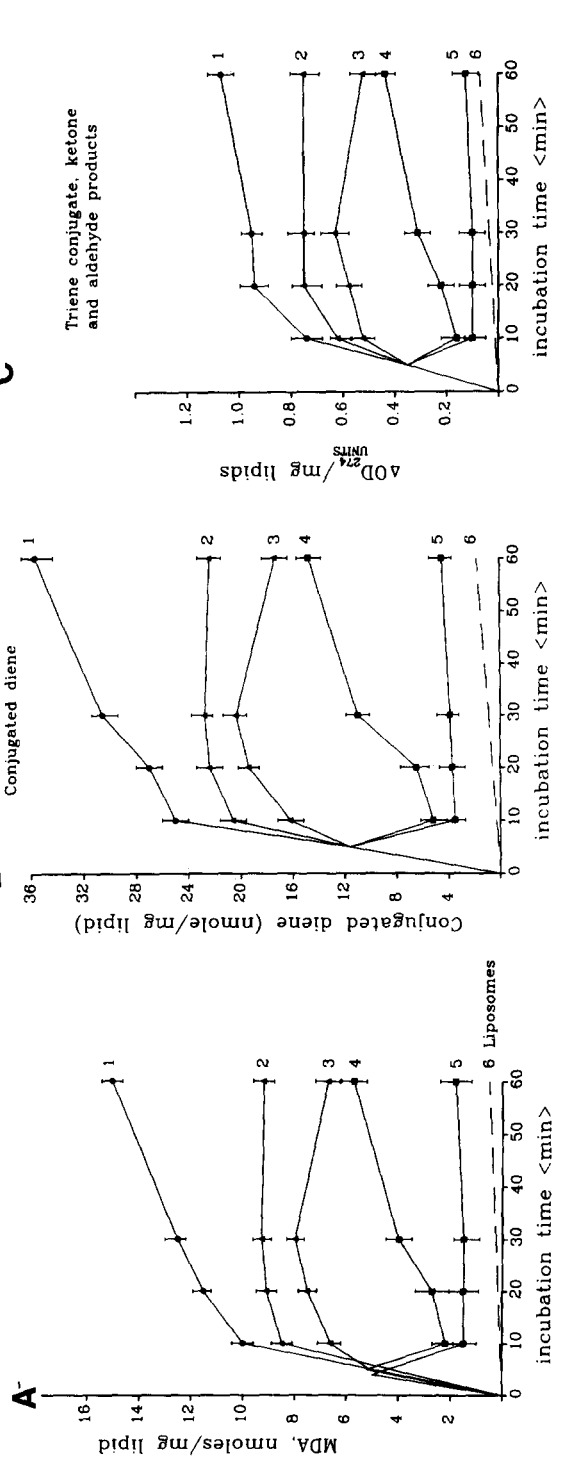

Fig. 4. The accumulation of lipid peroxidation products (TBARS, measured as MDA; (A) conjugated diene, (B) triene conjugates, ketone and aldehyde products (274 nm absorbing material, (C) in the liposomes (1 mg/ml) incubated alone (6, dotted line) for 60 min and with addition of the peroxidation-inducing system of Fe^{2+} + ascorbate (1). Antioxidants NAC (10 mmol/l or 20 mmol/l) (2,3) or L-carnosine (10 mmol/l or 20 mmol/l) (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 ThARS (Section 2). A similar amount of sample was partitioned through chloroform and used for detection of conjugated diene and trienes dissolved in 2–3 ml of methanol-heptane mixture (5:1, by volume).

already been published [10,12,22]. Fig. 4a shows that the level of TBA reactive substances (TBARS) reached at 5 min incubation, decreases in the presence of L-carnosine (10 or 20 mmol/l) at 10 min and at later time points (20 mmol/l), which must be due to a loss of existing TBARS or peroxide precursors of MDA and not due to a decreased formation of peroxide compounds. The ability of the histidine-containing compound NAC to inhibit the Fe^{2+} + ascorbate-induced oxidation or PC liposomes was compared with that of equimolar concentrations of L-carnosine. The antioxidant activities of 10 and 20 mmol/l NAC corresponded to 38% and 55% for each concentration after 60 min incubation. NAC exhibited less antioxidant protection than L-carnosine, corresponding to 60% and 87% of the equimolar (10 or 20 mmol/l) L-carnosine inhibition percentage. However, since NAC can cross the cornea into the aqueous humor but is readily metabolized into L-carnosine in this process, the antioxidant activity of NAC in vivo is significantly increased. Once in the aqueous humor, L-carnosine might act against peroxidation of the lens.

The biological effectiveness of L-carnosine as specific scavenger for activated oxygen species was assessed in the lens-induced LPO system. Trans-

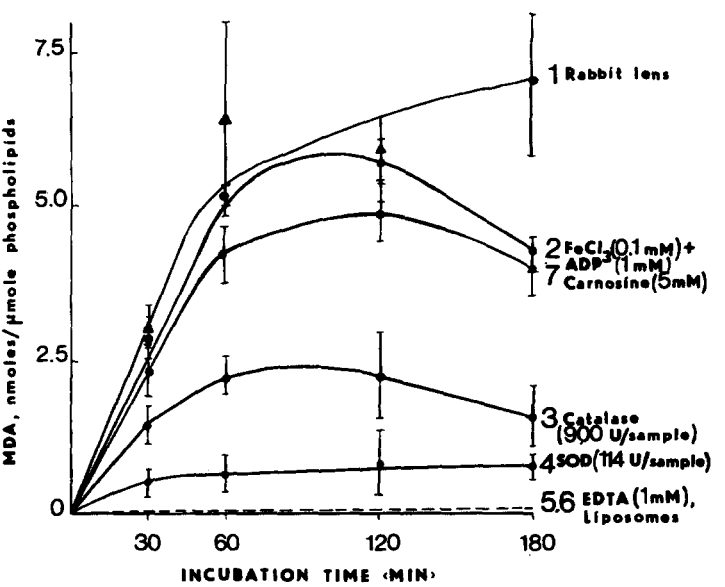

Fig. 5. Effect of various oxygen radical scavengers on lipid peroxide formation in liposomes added to the incubation medium of the normal rabbit lens. In a total volume of 3.0 ml the incubation mixture of the lens contained medium A or B, 0.5 mg/ml liposome suspension and concentrations of scavenger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

parent 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 auto-oxidation [22]. In a background study in the absence of the lens, virtually no oxidation of liposomes took place during 180 min (Fig. 5, curve 6). In the presence of the lens, however, a marked increase in concentration of different molecular LPO products was demonstrated for the appropriate time of incubation both in glucose-free (A) or glucose-containing (B) culture media ([22]; Fig. 5, curve 1). The level of MDA accumulation after incubation of rabbit lenses for 3 h was 3.5- and 5.3-fold higher than of normal human or mouse lenses. The larger normal lenses (rabbit or human) have more epithelial cells rich in GSH, which are primarily metabolically active, and these lenses can generate the active oxygen species and lipid peroxides more rapidly than cataractous lenses, with their exhausted pool of reductants, or tiny mouse lenses [22]. 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 [22]). A considerable reduction in the accumulation rate of the liposomal LPO products was found after the addition of catalase (900 U/sample) (Fig. 5, 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 (Fig. 5, curve 4), suggesting that the lens could generate $O_2^{\cdot -}$ 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 mmol/l EDTA which eliminates free and accessible metal ions from the peroxidizing system (Fig. 5, curve 5). L-Carnosine has recently been shown to act as a good scavenger of the lipid peroxyl ($LOO^{\cdot}$) and hydroxyl ($OH^{\cdot}$) radicalals [10,25]. The presence of 5 mmol/l L-carnosine in the rabbit lens/liposome-containing medium decreased the TBA-reactivity by approximately 25% at 2 h incubation (Fig. 5, curve 7).

4. Discussion

L-Carnosine appeared to be suitable for therapy and prophylaxis of

cataracts as a water-soluble antioxidant inhibiting oxidative modification of proteins, accumulation of DNA damage and utilizing lipid peroxides in the lens as precursors to opacification [6,10,12]. However, the potential physiological and antioxidant activities of L-carnosine in vivo are limited by its susceptibility to hydrolysis by human cytosolic and serum carnosinases, the latter being a unique dipeptidase specific for a dipeptide with the amino group of one residue in the unusual β-carbon position. The present study relates to the suitability of the *N*α-acetylated form of L-carnosine to act as a prodrug resistant to hydrolysis by human serum carnosinase in pharmaceutical compositions for ophthalmic application such as solutions, emulsions, ophthalmic gels, collyriums or ointments. Because the level of carnosinase increases in serum with age, hydrolysing the deacetylated forms of carnosine and anserine in the blood stream, the NAC is essential to treat senile cataract as the ophthalmic prodrug and not as the parenteral solution applied ophthalmically. Because of the physiological and antioxidant activities of L-carnosine, other forms of its pro-drugs including those obtained by the acylation of functional groups can be suggested to treat ophthalmic disorders in order to prolong and even to increase the inherent antioxidant characteristics of this dipeptide. However, the penetration of the putative prodrugs into the aqueous humor and their accumulation in any tissue for any reasonable period of time are still not clear.

The data reported here demonstrate that topical administration of pure L-carnosine (1% sol) to the rabbit eye (instillation, subconjunctival injection) does not lead to accumulation of this natural compound in the aqueous humor over 30 min in concentrations exceeding that in the placebo-treated matched eyes and its effective concentration is exhausted more rapidly. The data also establish the ability of NAC to become hydrolysed and to release L-carnosine and its passage from the cornea to the aqueous within 15–30 min of topical application to the eye. The techniques used for ocular administration ensured penetration across the cornea and biotransformation of NAC (pro-drug) into L-carnosine (active principle) which in this way does enter the aqueous humor. Different treatment techniques showed excellent toleration of the 1% NAC by the eye. The data suggest that, once in the aqueous humor, L-carnosine might act as an antioxidant and actually enter the lens tissue when present at effective concentrations (5–15 mmol/l). Although L-carnosine and the related compounds anserine and homocarnosine are considered to be present and to function predominantly in human muscle and olfactory epithelium and bulbs, we have found appreciable levels of L-carnosine in transparent human lenses which are markedly depleted in mature cataracts [6]. This suggests that the crystalline lens represents a specific site for L-carnosine as a defence against free radical damage to its tissues when subjected to

oxidative stress. The present experiments and other studies [6,12] show that L-carnosine affords strong protection against peroxidizing factors in the area surrounding the crystalline lens and the lens tissues when present at millimolar concentrations and this antioxidant protection potentially increases at higher L-carnosine concentrations. The *N*-acetyl form of L-carnosine showed a moderate antioxidant activity in vitro, less pronounced than that of the L-carnosine active principle. However, the pro-drug form may play an important role in de fence against lipid membrane damage induced by oxidative stress in vivo by ophthalmic biotransformation into L-carnosine. Most known biological antioxidants that can prevent oxidative damage to lipids, proteins, DNA and other essential macro-molecules show some specificity in their mechanism of action and so they can provide only one type of protection [26]. The experiments presented here on the Fe^{2+} + ascorbate-induced LPO system indicate that L-carnosine may exert its antioxidant properties by removing high reactive peroxide compounds from the lipid phase. The advantage of NAC and L-carnosine as universal antioxidants relates to their ability to give efficient protection against LPO both in the lipid phase of biological membranes and in an aqueous environment. Various protective antioxidant enzymes such as SOD or catalase can only react with their substrates in the aqueous environment.

The antioxidant activity shown by L-carnosine and its acetylated derivative as well as the data presented on the pharmacokinetics of these compounds suggest the benefits for ophthalmic application of the proposed L-carnosine prodrug in treating ocular disorders which have a component of oxidative stress in their genesis, such as senile and age-related cataracts, primary open-angle glaucoma, retinal degeneration, corneal disorders, ocular inflammation and complications of diabetes mellitus or other systemic diseases [27–30]. 1% NAC can be effectively combined for ophthalmic application with other medicines such as β-blockers to treat primary open-angle glaucoma [19,20,31]. We conclude, therefore, that further attention should be directed to the prospects of NAC in ophthalmology.

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