L-Carnosine: multifunctional dipeptide buffer for sustained-duration topical ophthalmic formulations

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Abstract

Objectives The use of L-carnosine as an excipient in topical ophthalmic formulations containing gellan gum, a carbohydrate polymer with in-situ gelling properties upon mixing with mammalian tear fluid, was developed as a novel platform to extend precorneal duration. Specific utilisation of L-carnosine as a buffer in gellan gum carrying vehicles was characterised.

Methods Buffer capacity was evaluated using 7.5, 13.3, and 44.2 mM L-carnosine in a pH range of 5.5–7.5. Accelerated chemical stability was determined by HPLC at L-carnosine concentrations of 5–100 mM. Combinations of 7.5 mM L-carnosine with 0.06–0.6% (w/v) gellan gum were characterised rheologically. L-Carnosine-buffered solutions of gellan gum were tested for acute topical ocular tolerance *in vivo* in pigmented rabbits. A unique formulation combining timolol (which lowers intraocular pressure) in L-carnosine-buffered gellan gum was compared with Timoptic-XE in normotensive dogs.

Key findings L-Carnosine exhibited optimal pharmaceutical characteristics for use as a buffer in chronically administered topical ocular formulations. Enhancement trends were observed in solution-to-gel transition of L-carnosine-buffered vehicles containing gellan gum vs comparators. Topical tolerability of L-carnosine-buffered gellan gum formulations and lowering of intraocular pressure were equivalent with timolol and Timoptic-XE.

Conclusions Functional synergy between excipients in gellan gum formulations buffered with L-carnosine has potential for topical ocular dosage forms with sustained precorneal residence.

Keywords controlled release; gels; glaucoma; inflammation; ocular drug delivery

Introduction

Development of efficient topical ophthalmic drug delivery systems remains a formidable challenge because of the anatomical characteristics of the eye.[1] Topical drug delivery to the eye is hindered by practical, mechanical and anatomical factors, including patient compliance, reflex blinking, the superficial tear film and the corneo–conjunctival epithelial barrier. In-situ forming gels have shown clinical success in improving corneal contact time and tolerability of ophthalmic solutions, and have resulted in marketed topical ophthalmic products such as Timoptic-XE.[2] In-situ gelation can be triggered by a variety of molecular and thermodynamic mechanisms, including changes in temperature (e.g. poloxamers),[3] pH (cellulose derivatives e.g. acetate phthalate or carbomers)[4] or ionic strength (e.g. gellan gum).[2,5–10]

Our current work characterises a broadly applicable synergistic topical ophthalmic delivery system using the established concept of cation-activated in-situ gelation mediated by gellan gum, an anionic heteropolysaccharide derived from *Pseudomonas elodea*,[11] which is complemented by in-vitro buffering capabilities and (bio)chemical properties of L-carnosine. Following the acceptance of timolol maleate formulated using highly purified gellan gum (Timoptic-XE), a once-daily derivative of the twice-daily parent product Timoptic, for the management of intraocular pressure (IOP) in glaucoma, the heteropolysaccharide was commercialised in the USA as an inactive pharmaceutical excipient under the label

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Gelrite.[2,12] Conventionally, gellan gum has been dissolved in aqueous non-ionic, anionic or diffusely cationic buffered media to prevent gelation before instillation of eye drops. Reported embodiments of topical ocular drugs containing gellan gum showing enhanced duration of action have been achieved in tromethamine maleate, mesylate and acetate buffers, in addition to simple, ultrapure, deionised or distilled water solutions.[2,6–8,10,11] Formulation of emerging novel active chemical entities with gellan gum often proves challenging in terms of solubility, chemical stability and bioequivalency – three biopharmaceutic characteristics taken into account at early stages of drug discovery. Once the optimal formulation pH of a putative ocular pharmaceutical drug product is determined, a mechanism for adjusting and maintaining it is essential. Use of common conjugate acid/base systems (e.g. alkali metal salts of phosphates or citrates) that buffer within the physiological pH range acceptable for topical ocular dosing is restricted in the presence of gellan gum because of the inherent mechanism of gelation.

L-Carnosine is an endogenous water-soluble dipeptide composed of amino acid residues β-alanine and L-histidine (Figure 1). In its pure form, L-carnosine occurs as a white solid powder with a melting point of 257°C. The solubility of L-carnosine in 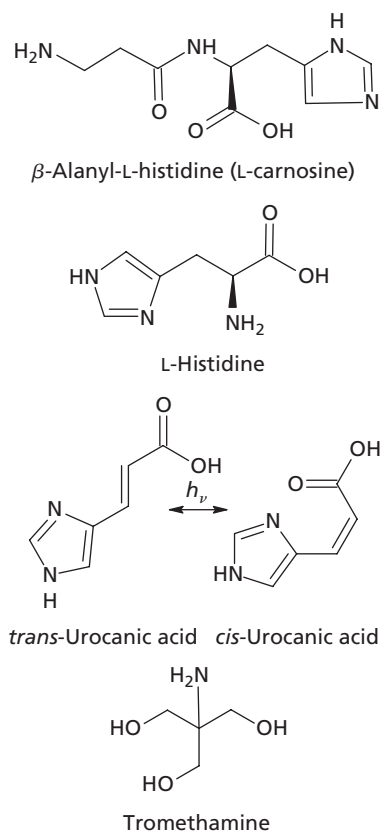unbuffered water at 25°C is 0.20 mol/l, with an equilibrium pH of 8.38. The molar solubility of L-carnosine is 4.42 mol/l at pH ≤6, and 0.91, 0.23, 0.28 and 1.46 mol/l at pH 7, 8, 9 and 10, respectively. The presence of carboxylic acid, imidazole and primary amine functional groups in the molecular structure of L-carnosine correspond to the three acid dissociation constants (i.e. pK_a) of 2.76, 6.78 and 9.36, respectively.[13] L-Carnosine was discovered in 1900 by Gulevitch and Amiradgibi in Russia.[14] The skeletal muscle L-carnosine content in mammals is 150–200 mg per % wet weight, and its presence has also been reported in brain, cardiac muscle, kidney, stomach, olfactory bulb and lens tissues. Many biological functions of this natural metabolite are recognised, including activity as a buffer, neurotransmitter, immune enhancer and antioxidant.[15] L-Carnosine exhibits anti-oxidative properties in cytosolic domains of reported cell types and tissues, primarily as a free-radical scavenger.[16–19] L-Carnosine is also thought to modulate immune response in human neutrophils by increasing interleukin-1β production and suppressing apoptosis.[15] An N-acetyl derivative of L-carnosine is the putative active ingredient in Nu-Eyes, marketed as an eye-drop treatment for cataract prophylaxis.[20] Other compositions of L-carnosine have also been investigated for management of schizophrenia and bipolar disorder, and as an anti-ulcer remedy that protects the gastrointestinal mucosa.[15,21] L-Carnosine has significant buffering action at physiological pH: in muscle tissue it effectively prevents acidification of the intracellular environment during vigorous exercise.[15]

In topical ocular dosage forms, rapid in-situ gel formation is important to resist elimination from the tear film. Rate of active electrolyte absorption from tear fluid into the microenvironment of gellan gum polymer dispersions depends on osmotic gradients across gel–aqueous surfaces.[2,6,12,22] As a result, the ionic strength of formulations may influence the solution–gel transition rate. To develop L-carnosine as a functionally synergistic excipient for topical ophthalmic use compatible with pharmaceutical dosage forms containing gellan gum, we investigated its in-vitro buffering action, chemical stability in the relevant pH range, rheology after mixing with simulated tear fluid (STF), topical acute tolerance *in vivo*, and pharmacodynamic equivalence to Timoptic-XE in normotensive dogs.

Materials and Methods

Chemicals and reagents

All chemicals and reagents were of highest purity available. Formulation excipients used for in-vivo safety and efficacy evaluation met standard compendia requirements (except for L-carnosine). D-Mannitol (USP/NF), NaCl (USP/NF), KCl, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $NaHCO_3$, NaH_2PO_4, L-carnosine (~99%, crystalline, CAS#305-84-0), L-histidine (≥99%), urocanic acid (3-(4-imidazolyl)acrylic acid, 99%), tromethamine (USP/NF), Gelrite (gellan gum, USP/NF), concentrated HCl (USP/NF; 36.5–38% w/v), timolol maleate (CAS#26921-17-5), 0.1N HCl and NaOH standard solutions for titration, and HPLC-grade methanol (>99.9%), water (ultrapure) and acetonitrile (>99.9%) were purchased from

Figure 1 Chemical structures of proposed and evaluated buffer agents (L-carnosine, tromethamine) and their degradation products (L-histidine and urocanic acid). Urocanic acid exists in equilibrium between its *cis* and *trans* photoisomers, an interconversion that can be catalysed by exposure to a quanta of light.

Sigma-Aldrich (St Louis, MO, USA). Timolol hemihydrate (CAS#91524-16-2) was a generous gift from PCAS (Turku, Finland). Tetracaine HCl and Timoptic-XE for non-human research use were purchased from Henry Schein, Inc. (Melville, NY, USA). Test solutions and formulations for each study were prepared in sterile water for injection (SWFI) (Baxter I.V. Solutions, USP, West Chester, PA, USA).

Animals

Female pigmented Dutch belted rabbits (1.5–2 kg) were obtained from Myrtle's Rabbitry (Thompson Station, TN, USA) and were acclimated to the testing facility for a minimum of 6 days before starting the acute topical ocular tolerance study. Conditions included free access to food (Certified High Fiber diet #5325, Newco Inc., Rancho Cucamonga, CA, USA) and water, with standard housing conditions (12 h light–dark cycle).

For the IOP studies, Beagle dogs (male and female, weighing 9–13 kg) were purchased from Marshall Farms (North Rose, NY, USA) and were housed in kennels with access to food and water *ad libitum*. Before inclusion in the studies, all animals underwent physical examinations including tonometry, slit lamp and ophthalmoscopy. These tests did not reveal any relevant abnormalities, and the IOP was characterised as normotensive (~20 mmHg). At the time of study, the dogs were aged 24–30 months.

All animal-related work was conducted under a protocol approved by the Institutional Animal Care and Use Committee in compliance with Animal Welfare Act regulations and the Guide for the Care and Use of Laboratory Animals. In addition, recommendations outlined by the Association for Research in Vision and Ophthalmology regarding animal research were strictly adhered to.

Preparation of test solutions

Buffers of L-carnosine at different pH values for use in rheological and in-vivo studies were prepared by dissolving L-carnosine (7.5 mM final concentration) in SWFI and the pH adjusted to 5.5, 6.0, 6.5 and 7.0 using concentrated HCl. The ionic strength of test formulations was adjusted to 167–571 mOsm/kg (assessed with an Advanced Model 2020 multisample osmometer, Advanced Instruments Inc., Norwood, MA, USA), using 2.5–7.5% (w/v) D-mannitol. Gellan gum solutions at three different concentrations (0.06%, 0.1% and 0.6% w/v) were prepared in each of the four pH buffers described above (i.e. 12 different test formulations, Table 1). For 0.6% gellan gum, additional formulation variations that were hypotonic and hypertonic with respect to tear fluid were prepared, summarised in Table 1. The required amount of Gelrite was weighed using an analytical balance and slowly added to L-carnosine buffers at 80% of final target volume in a volumetric flask while stirring at 200–300 rpm using a magnetic stir bar on a hotplate at 50–60°C. The dispersion of gellan gum in L-carnosine buffer solution was allowed to heat to 80–90°C, until it became clear. Buffer solutions were used to adjust to final volume, followed by hot filtration (ensuring > 50°C) through a 0.45 μm cellulose acetate bottle-top membrane filter (Corning, NY, USA). Although sterility was not assessed in these studies, it is vital to maintain

Table 1 Final composition of various test formulation parameters evaluated.

	pH 5.5	pH 6.0	pH 6.5	pH 7.0
L-Carnosine (mM)	7.5	7.5	7.5	7.5
Gellan gum (% w/v)	0.06	0.06	0.06	0.06
	0.1	0.1	0.1	0.1
	0.6	0.6	0.6	0.6
D-Mannitol (% w/v)	2.5[a]	ND	ND	2.5[d]
	5[b]			5[e]
	7.5[c]			7.5[f]

[a]187 ± 4.0 mOsm/kg; [b]359 ± 4.0 mOsm/kg; [c]561 ± 11 mOsm/kg; [d]169 ± 1.5 mOsm/kg; [e]315 ± 0.6 mOsm/kg; [f]470 ± 6.1 mOsm/kg; all mean ± SEM of at least three determinations. ND, not determined.

aseptic conditions during compounding of ophthalmic solutions. Alternatively, a variety of topical ophthalmic preservatives with antimicrobial effectiveness can be used (e.g. benzododecinium bromide[12]); however, their potential interaction with L-carnosine would need to be examined.

L-Carnosine formulations containing 7.5 mM buffer, prepared as described above, were used in accelerated stability studies for method development and optimisation. L-Histidine, a common amino acid buffering agent, was used for comparative purposes. Acid- and base-catalysed decomposition of 7.5 mM L-carnosine formulations was achieved by preparing 50 : 50 (v/v) mixtures with 0.1 N HCl and 0.1 N NaOH, respectively, which were heated under constant stirring with a magnetic stir bar on a hotplate at 85°C for 60 min (cooled to room temperature before HPLC analysis).

For evaluation of chemical stability, L-carnosine and L-histidine were dissolved at 5, 10, 15, 25, 50 and 100 mM in SWFI only, adjusted to a final pH of 6 using concentrated HCl.

STF was prepared based on a modification of a method reported by Rozier and colleagues[2], where the original recipe was supplemented with naturally occurring cations found in mammalian tears. Additional salts included KCl, MgCl$_2$ and NaH$_2$PO$_4$, based on reported levels of K$^+$, Mg^{2+} and PO$_4^{3-}$ content in resting and stimulated tear secretions.[23] The composition defined as STF used in rheological studies reported herein included 116 mM NaCl, 18.8 mM KCl, 0.435 mM CaCl$_2$·2H$_2$O, 0.18 mM MgCl$_2$·6H$_2$O, 25.9 mM NaHCO$_3$ and 0.775 mM NaH$_2$PO$_4$ dissolved in SWFI, having an ionic strength of 270–300 mOsm/kg and pH 7–7.4 (adjusted with 0.1 N HCl or NaOH as needed).

Titrations and buffer capacity

The buffer capacities of L-carnosine, L-histidine and tromethamine at three predetermined concentrations (7.5, 13.3 and 44.2 mM) were estimated using a digital autotitrator (DL55, Mettler Toledo, Columbus, OH, USA) equipped with DG115 type electrodes and burettes of 10 ml capacity containing 0.1N HCl as a standardised titrant. A preprogrammed method for equivalence point titration (Mettler Toledo, ID# 20063) was used to generate curves of titrant amount vs pH; the data were subsequently fitted to the Van Slyke equation: $\beta = \Delta$[equivalents acid]$/\Delta$pH = (2.3 × K$_a$ × C × [H$^+$]) / (K$_a$ + [H$^+$])^2, where buffer capacity β is

expressed as a function of the acid dissociation constant, K_a, total buffer concentration, C, and the equivalents of H^+ derived from pH.[24] Titrations of buffer solutions were carried out under thermostatic conditions at 25°C. The volume of the titrated buffer solutions was 50 ml, with an initial equilibrium pH of approximately 7.7 for L-histidine, 8.4 for L-carnosine and 10.2 for tromethamine. Titrations encompassed the range ~5–10 pH units, capturing the relationship between equivalents of HCl added to buffer solutions and the resultant incremental change in pH.

Chemical stability

Chemical stability of L-carnosine and L-histidine was evaluated as a function of two endpoints: overall changes from initial pH (e.g. time 0), and chemical degradation of buffering molecules in solution at pH 6.0, as a function of time. Bulk solutions of L-carnosine and L-histidine were prepared at 5, 10, 15, 25, 50 and 100 mM and the pH adjusted to 6.0 using concentrated HCl. Changes from initial pH and chemical degradation of buffers were estimated weekly, in triplicate 20 ml samples stored in borosilicate glass scintillation vials sealed with pulp-backed metal-foil-lined polypropylene caps (Thermo Fisher Scientific Inc., Houston, TX, USA), upright, in the dark at 4, 25 and 40°C for 6 weeks. SWFI was used as an unbuffered control. A digital pH meter (MP230, Mettler Toledo) was used to determine the pH of L-carnosine solutions. Chemical stability was determined in a quantitative manner using an Agilent 1100 HPLC system (Santa Clara, CA, USA) equipped with a G1311A quaternary pump, G1316A column thermostat and G1315B diode array detector. A specific HPLC method was developed to resolve L-carnosine from the potential chemical degradation byproducts L-histidine and urocanic acid. A Waters Symmetry 4.6×250, 5 μm C_{18} column was used (Waters, Milford, MA, USA), maintained at $20.0 \pm 0.8°C$. Samples of buffer (10 μl) were injected onto the column, and the percentage of L-carnosine and L-histidine remaining was estimated by comparing the concentration at predetermined time intervals with initial levels set prior to storage under the selected conditions. Solvents used in the mobile phase consisted of HPLC-grade methanol, water and acetonitrile, run at a flow rate of 1 ml/min using the following linear gradient: 0–0.5 min, 60 : 40 (v/v) methanol : water; changing to 100% acetonitrile by 7.5 min and maintained to 7.99 min; changing to 60 : 40 methanol : water by 8 min, and maintained to 13 min. Analytes were resolved under the prescribed chromatographic conditions (retention times of 2.2, 2.4 and 5.5 min for L-histidine, L-carnosine and urocanic acid, respectively) and detected by selective UV absorbance. A diode array detector was programmed for estimation of concentrations against reference standards at 215 and 268 nm for L-carnosine/L-histidine and urocanic acid, respectively, while collecting UV spectral data within each peak at a 2 nm resolution.

The first-order rate constant and half-lives ($t_{1/2}$) for buffer consumption in test samples were estimated using the equation, $\log [S] = (-k/2.3) \times t + \log [S_0]$, where [S] represents the concentrations of L-carnosine or L-histidine at various time points t, k denotes the reaction (i.e. decomposition) rate and $[S_0]$ is the initial concentration.

A plot of log[buffer] versus t is linear, with a slope of $-k/2.3$; $t_{1/2}$ is the time at which 50% of the initial buffer concentration remains. The reaction rate k can be estimated from the equation $0.693/k = t_{1/2}$.

Rheological evaluations

The viscosity and rheological behaviour of L-carnosine-buffered formulations containing gellan gum were compared with commercial Timoptic-XE using a Brookfield cone and plate type R/S Rheometer (Middleboro, MA, USA) connected to a circulating water bath for temperature control. A C50-1 spindle-cone assembly was used in all viscosity determinations. Rheological properties were evaluated in 7.5 mM L-carnosine-buffered solutions as a function of gellan gum concentration (0.06, 0.1 and 0.6% w/v), formulation pH (5.5, 6.0, 6.5 and 7.0) and adjusted ionic strength (with respect to STF, using D-mannitol at 2.5% (w/v) for hypotonic, 5% (w/v) for isotonic and 7.5% (w/v) for hypertonic). Formulation samples and STF were stored at room temperature before testing, and were allowed to equilibrate to either 25 or 37°C for 5 min on the centre of the stationary plate of the rheometer. Rheological analysis was carried out using preprogrammed parameters of Brookfield software program RHEO 2000 (version 2.6), in which the shear rate was increased from 0 to 216/s in 10 min. Intrinsic viscosities of 0.5 ml formulation aliquots were determined at 25°C. Changes in viscosity upon combining with STF were characterised at 37°C, where STF and test formulation were added in a specific sequential fashion to simulate mixing with tears (i.e. 0.07 ml STF initially smeared on the stationary plate, followed by 0.5 ml formulation and overlaid with another 0.07 ml aliquot of STF). Volume ratios of formulation to STF were maintained at 3.6, to mimic the situation *in vivo*. (Clinical settings customarily deliver a 0.027 ml eye drop to a resident tear pool of 0.0075 ml.) Shear-dependent rheological changes over time were obtained for different formulations, and observed viscosity values plotted as a function of applied shear stress. In order to establish a dynamic response range and validate our rheology assay, studies were performed in the absence of test buffers using fixed volume mixtures of STF with 0.06, 0.1, 0.25, 0.4 and 0.6% (w/v) gellan gum solutions in SWFI. Concentration-dependent viscosity shear profiles established agreed with those previously reported for Gelrite.[22,25]

Acute in-vivo topical safety in rabbits

Clinical and ophthalmic evaluations were performed before dosing to assess the general health and condition of each animal. Ophthalmic observations were performed for approximately 3 hours after application of each topical dose, three-times daily for 3 days. Specifically, eyes were examined for corneal abnormalities and signs of redness in the surrounding episcleral and conjunctival tissue. The test protocol was carried out on six rabbits, after a single instillation of 50 μl of the formulation; the untreated contralateral eye in each animal was used for comparison. Findings were scored according to the Draize eye irritancy scale of + (slight), ++ (mild), +++ (moderate) or ++++ (severe). Severity scores increase with the intensity of colour

(redness) and size of the affected area, swelling that pushes the lid away from the eye (chemosis) and the size of any observed opacities. The option of fluorescein staining of the cornea was available (although not exercised in the studies described) for cases where treatment was suspected to have caused corneal abnormalities. IOP was measured in formulation-treated and control/contralateral eyes using a Model 30 classic pneumatonometer (Medtronic, Minneapolis, MN, USA).

IOP modulation studies

Eight dogs were used for the IOP studies. The animals were acclimated to the dosing IOP procedures before initiation of the studies. The dogs had a minimum of 1 week washout period before dosing with L-carnosine-buffered gellan gum formulations of timolol maleate or Timoptic-XE (2 weeks for studies with timolol hemihydrate formulations). On the study day, each animal received a single 50 μl topical administration of timolol maleate/hemihydrate formulated using L-carnosine-buffered gellan gum vehicle or commercial Timoptic-XE in their right eye (treated eye). The contralateral eye was treated with either matching vehicle for experimental L-carnosine buffered formulations, or commercial Timoptic-XE. All test and control articles were administered at 8–9 a.m. IOP was measured using a Model 30 classic pneumatonometer (Medtronic) before administration of test solutions (baseline, 0 h), and at 1, 2, 4, 6 and 24 h after administration. Animals received a 30 μl drop of topical anaesthetic (0.5% tetracaine HCl) before each IOP measurement.

Statistical analysis

All data are expressed as means ± SEM for 3–6 determinations. The unpaired two-tailed Student's *t*-test was used to determine the statistical difference between two group means, where applicable. Comparisons among three or more group means were performed by one-way analysis of variance (ANOVA) using Statgraphics Plus 5.0 (StatPoint Inc., Warrenton, VA, USA). Statistical significance among the group ($\geq$3) means was determined by the modified Fisher's least-squares difference approach. $P < 0.05$ was considered statistically significant.

Results

Comparative buffer capacity ranges

pK$_a$ values of L-carnosine, L-histidine, and tromethamine were confirmed from the buffer capacity spectra by empirically mapping putative acid–base equilibria of the imidazoles and amine functional groups (Figure 1). These pK$_a$ values roughly agree with published data (i.e. pK$_a$ ~6.9 for L-carnosine, ~6 for L-histidine and ~8.3 for tromethamine). Overall peak buffer capacity (β) increased in a predictable manner with increasing molar concentration of each buffering agent in solution (β of 1.86–2.01 × 10^{-3} for 7.5 mM, 3.31–3.51 × 10^{-3} for 13.3 mM, 9.69 × 10^{-3}–1.01 × 10^{-2} for 44.2 mM). During all titrations, buffer capacity maxima corresponding to imidazole groups of L-carnosine and L-histidine, and the amine of tromethamine,

maintained their positions on the pH axis (e.g. β_{max} when pH = pK$_a$). However, when each agent was compared with its matching theoretical prediction of β according to the method of Van Slyke, using previously reported pK$_a$ values,[13] the observed peak β values appeared 2.5–3 times lower. The buffer capacity range of L-histidine solutions that was most effective compared with L-carnosine and tromethamine appeared at pH values of 5.5–6.5, whereas for L-carnosine and tromethamine ranges were 6.5–7.6 and 7.6–8.8, respectively.

Chemical stability

Buffer stability was assessed as a function of overall pH change (Figure 2), with concomitant specific monitoring of L-carnosine and L-histidine levels over time. Deviations from initial pH value were most dramatic in 5, 10 and 15 mM buffered solutions, while the extent was less pronounced at the higher concentrations (25, 50 and 100 mM) of L-carnosine and L-histidine (Figure 2). Apparent trends in pH drift over time as a function of temperature were greater in magnitude at 4°C than at 25 or 40°C storage (Figure 2). Most prevalent changes in pH were 0.08–0.13 pH units observed in 5, 10 and 25 mM buffers of L-carnosine at 4°C, and 5–10 mM buffers of L-histidine at 25°C (Figure 2). Furthermore, the pH of unbuffered SWFI (solvent used to make the L-carnosine and L-histidine samples) varied between values of 5 and 8 during weekly measurements (data not shown). Chromatographic analysis of accelerated thermal acid/base-catalysed decomposition samples of 7.5 mM L-carnosine and L-histidine solutions established chemical conversion from L-carnosine to L-histidine and urocanic acid (identity of peaks confirmed using commercially available reference standards).

Buffer solutions at pH 6 containing 5, 10, 15, 25, 50 and 100 mM L-carnosine and L-histidine stored in the dark at 4, 25 and 40°C were analysed at weekly time intervals for concentration of starting material remaining. All samples
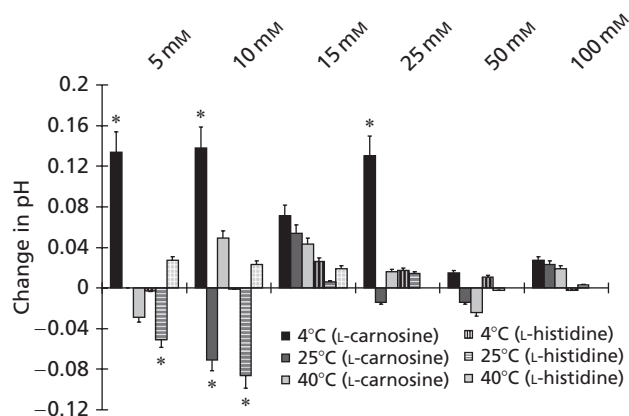
Figure 2 Buffer stability of L-carnosine and L-histidine solutions. Change in pH after 6 weeks from an initial value of pH 6 is shown for L-carnosine- and L-histidine-buffered solutions stored at 4, 25 and 40°C. Values above the graph are the concentration of each buffer. *$P < 0.05$ vs initial pH value in samples stored at same temperature (one-way analysis of variance). Values represent means ± SEM of at least three determinations.
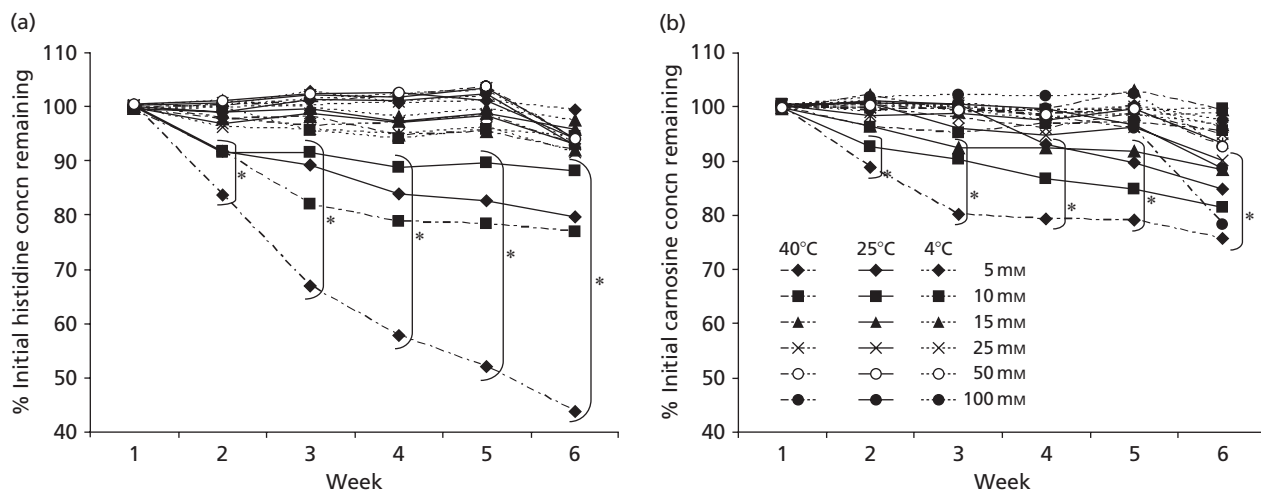
Figure 3 Chemical stability of (a) L-histidine and (b) L-carnosine buffers. The buffer solutions were stored at 4°C - - -, 25°C **- - -** and 40°C ⋯ for 6 weeks, and the graphs shows the remaining amount of L-histidine and L-carnosine, expressed as adjusted % of the initial signal (time 0) measured by HPLC peak area. *$P < 0.05$ vs initial concentration from samples stored at same temperature (one-way analysis of variance). Values represent means for at least three determinations; error bars have been omitted for clarity.

stored at 4°C retained at least 95% of the initial concentration over 6 weeks (Figure 3). Statistical analysis showed there were no significant differences in the stability of L-carnosine and L-histidine between any of the concentrations stored at 4°C. Significant decreases from starting L-histidine concentrations were found in the 5 and 10 mM samples stored at 40°C from 2 weeks (only for 5 mM) through to 6 weeks, where approximately 45% of the starting buffer amount remained for 5 mM L-histidine at week 6 (Figure 3). Similar trends were recorded for L-carnosine samples stored at 40°C but for the lowest (5 mM) and highest (100 mM) concentrations only. While L-carnosine concentrations from the 5 mM samples were significantly lower at 3–6 weeks, the 100 mM samples appeared comparably decreased at the 6 week time point only, with approximately 80% of initial amount remaining (Figure 3). Both 5 and 10 mM L-histidine and L-carnosine buffer samples stored at 25°C displayed trends of gradual decrease with time, whereas only the 10 mM L-histidine samples at 4 and 5 weeks, and 5 mM at 4–6 weeks were significantly lower (82–90% remaining; Figure 3). First-order buffer degradation rate constants and $t_{1/2}$ values for L-carnosine and L-histidine were determined from the semi-log plots of the integrated first-order velocity equation. Slopes of -0.02 ($r^2 = 0.82$) for L-carnosine disappearance and -0.07 ($r^2 = 0.99$) for L-histidine disappearance were estimated. The $t_{1/2}$ for L-carnosine and L-histidine at 40°C was estimated to be approximately 15 and 4 weeks, respectively.

Rheological characteristics

Pseudoplastic behaviour of gellan gum formulations buffered with L-carnosine was observed. Tested compositions showed time-dependent decrease in viscosity under stepwise increases in shear stress (e.g. angular velocity) rates. Viscosity curves of L-carnosine-buffered gellan gum formulations following mixing with STF were similar to the profile of Timoptic-XE controls (Figure 4). The curves were largely superimposable,
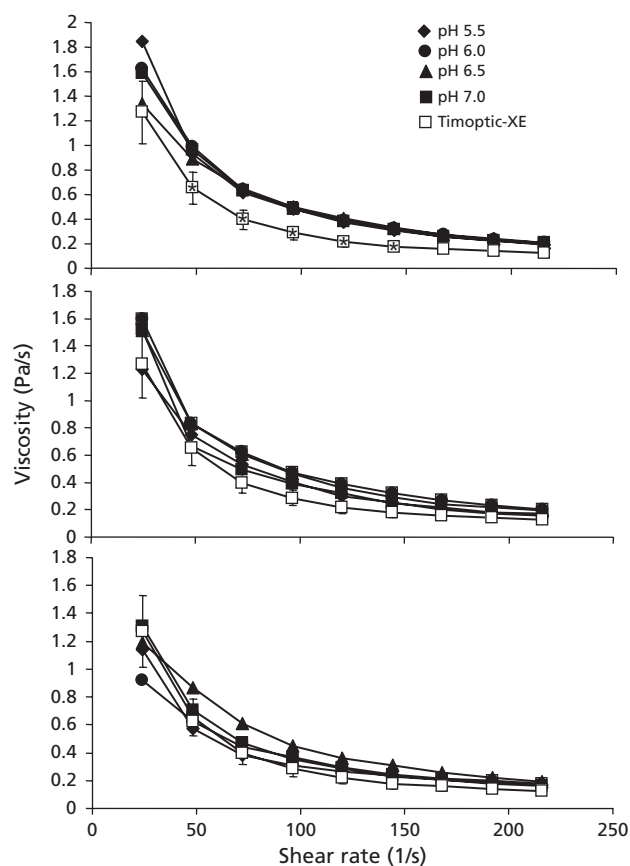

Figure 4 Viscosity profiles of L-carnosine-buffered gellan gum formulations. The formulations contained 0.06% (w/v) (bottom), 0.1% (middle) and 0.6% (top) gellan gum at pH 5.5, 6, 6.5 and 7 t 50–150/s, gellan gum (0.6% w/v) formulated with L-carnosine buffer had significantly higher viscosity than Timoptic-XE at all four pH values tested. Values are means from at least three determinations; error bars have been omitted for clarity (except Timoptic-XE controls).

as expected, given the same gelling agent and mechanism (Figure 4). However, L-carnosine buffered gellan gum formulations had better gelling capacity than Timoptic-XE (e.g. speed and extent of gel formation). The nature of the gel formed is known to depend on the polymer concentration,[8] which was evidently absent in the case of formulations buffered with 7.5 mM L-carnosine ranging from 0.06 to 0.6% w/v gellan gum content (Figure 4). Published examples of gellan gum preparations with active drugs (i.e. timolol) are reported at 0.6% w/v,[2,12] the only gellan gum concentration at which L-carnosine-buffered formulations retained significantly elevated viscosity values under higher shear rates of 50–150/s at all pH values tested (Figure 4). Furthermore, formulations that were hypotonic and hypertonic with respect to STF (169–187 and 470–561 mOsm/kg, respectively) at pH 5.5 and 7.0 (Table 1) displayed identical rheological characteristics to isotonic L-carnosine-buffered gellan gum as well as commercial Timoptic-XE systems (data not shown).

Ophthalmic tolerability

In general, topical dosing was well tolerated (Pfizer DSRD internal study number 05OPH047). Eye drops of the test formulation (50 μl) – 0.017% w/v (7.5 mM) L-carnosine-buffered vehicle containing 0.6% w/v gellan gum, 4.5% w/v D-mannitol at pH 6–6.5 (using trace HCl for pH adjustment) and ionic strength of approximately 300 mOsm/kg – were instilled three times daily in one eye, while the contralateral eye was used for control. Ophthalmic monitoring was performed for 3 days. Typical unremarkable observations associated with topical eye drops were recorded with administration of the L-carnosine-buffered gellan gum formulation, and there were no changes in basal IOP. Any conjunctival redness was transient (lasting 5–10 min after administration) and was generally localised to a minor area of the conjunctival surface. Conjunctival redness, chemosis and corneal opacity were assessed. Only conjunctival redness received a + score, while all other observations were absent.

Pharmacodynamic equivalency in IOP lowering

In normotensive dogs, topically administered 0.5% (w/v) timolol (equivalents) elicited detectable reductions in IOP from baseline. Reductions in IOP were achieved without any noteworthy side-effects known to occur in this animal model.[26] Similar lowering of IOP was recorded in eyes treated with Timoptic-XE and those treated with 0.017% w/v (7.5 mM) L-carnosine-buffered gellan gum formulations of timolol maleate and hemihydrate (0.5% w/v active equivalents) (Figure 5).

Discussion

The specific and general influences of excipients in topically applied ocular formulations on tolerance and efficiency towards delivery of resident active substances is significant. Tonicity, pH and topical anaesthetics affect tear production and drainage of instilled solutions, and therefore bioavailability of ocular drugs following topical dosing.[27,28] Studies investigating topical ocular drug delivery ascertained the important effect of vehicle on intraocular drug bioavailability in comparisons of solutions with suspensions, oleaginous
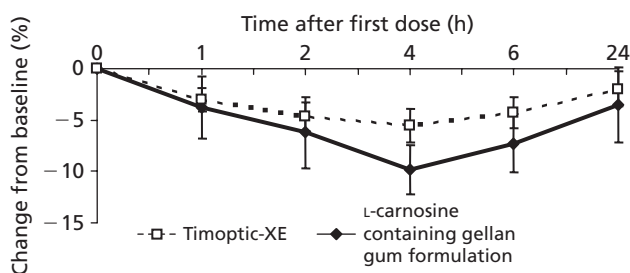

Figure 5 Pharmacodynamic equivalency in lowering of intraocular pressure. The graph shows the lowering of intraocular pressure (IOP) in normotensive dogs by 0.5% (w/v) timolol maleate administered in a 7.5 mM L-carnosine-buffered 0.6% w/v gellan gum solution (pH 6–6.5; ~300 mOsm/kg) and 0.5% timolol maleate (equivalent dose) as Timoptic-XE (label claim pH 7). Tonometry results are expressed as % changes from predose IOP values over the course of 24 h.

vehicles and ointments of the same active. From these studies, duration of aqueous humour levels and the net amount penetrating were found to depend on precorneal contact time and mixing efficiency with the resident tear film.[29,30] Flow properties, or viscosity, of topical ophthalmic formulations also affect intraocular bioavailability of applied ophthalmic actives.[31] In the current study, we have developed L-carnosine as a functionally synergistic buffer for topical ophthalmic use specifically compatible with pharmaceutical dosage forms containing gellan gum. Buffering capacity, chemical stability and rheology of prototype formulations were determined, as were in-vivo tolerance and topical pharmacodynamic equivalency to Timoptic-XE.

L-Carnosine has higher buffering capacity when compared with tromethamine at pH values of 6.5–7.6. When L-carnosine was evaluated as a potential buffer in a series of titrations, its buffer capacity, β, ranged from 0.002 to 0.01 at 7.5–44 mM of the dipeptide. Buffer capacity values up to 0.1 are acceptable in parenteral dosage forms.[32] The highest concentration of L-carnosine titrated was ~44 mM, selected on the basis of reports of this physiological dipeptide being safely administered to rabbits in preclinical evaluations as a 1% w/v solution.[33] The lowest concentration, at 7.5 mM L-carnosine, was selected for comparison with tromethamine (Figure 1) used as a commercial excipient for maintaining the pH of Timoptic-XE at 7–7.4.[2] Buffer capacity must be sufficient to maintain formulation pH for a reasonable duration. Changes in product pH may result from degradation or interaction of solution components with one another (or with the product package closures, etc.). On the other hand, the buffer capacity of ophthalmic and parenteral products must be low enough to allow rapid readjustment to physiological pH upon administration. Van Slyke predictions of β were marginally higher than the experimental values, which can be explained by the ion-interaction model, taking into account temperature- and solubility-dependent activity coefficients for the species evaluated (Figure 1).[34] Ion interaction also partially explains the time- and temperature-dependent deviations in pH of L-carnosine and L-histidine buffer solutions, which were most apparent at 5–15 mM concentrations, under 4°C storage (Figure 2).

L-Carnosine is chemically more stable than L-histidine from the point of view of formulation robustness. Accelerated studies indicated the chemical conversion of L-carnosine to L-histidine, followed by urocanic acid (Figure 1). Relative to L-histidine at equivalent concentrations, L-carnosine appeared to be 3–4 times more resistant to thermal acid/base-driven decomposition under most limiting conditions (Figure 3). Disappearance of L-carnosine buffer from prototype formulations may be through a complex high-order reaction, with L-histidine being an intermediate that rapidly converts to urocanic acid. The generation of L-histidine from L-carnosine appears as the slow step (estimated $t_{1/2}$ of 15 weeks at 40°C) in the buffer's stability profile, especially at high concentrations of L-carnosine, preceding conversion of L-histidine (estimated $t_{1/2}$ of 4 weeks at 40°C) to urocanic acid isomers (Figures 1 and 3). Trans-urocanic acid (Figure 1) is found in mammalian skin and can form upon deamination of L-histidine.[35] While it absorbs in the same spectrum as DNA, it may also act as an endogenous sunscreen, protecting epithelia from UV damage.[36] Although identified degradation byproducts originating from L-carnosine are of a physiological nature (L-histidine and urocanic acid, Figure 1), their safety and disposition in the context of long-term administration in topical ophthalmic products carrying L-carnosine as a synergistic buffer needs to be qualified in future studies. Furthermore, inventions describing stabilisation approaches in ophthalmic formulations of L-carnosine and related compounds have been reported.[37]

L-Carnosine-buffered formulations containing 0.06% (w/v) gellan gum exhibit solution–gel transition comparable to that of Timoptic-XE. The use of low polymer concentrations would be expected to decrease the likelihood of adverse effects due to chronic topical application of gelling agents. Gelling agents are one of many ingredients successfully used in topical ocular formulations to prolong drug contact time with the surface of the eye, thereby increasing the extent of penetration across the cornea and conjunctiva.[38–40] Gellan gum, which is a natural polysaccharide, forms a rigid and stable gel in the presence of mono/divalent cations. The cations in mammalian tears, especially Na^+, Ca^{2+} and Mg^{2+}, are particularly suited to initiate gelation of the polymer at ~37°C when instilled as a liquid solution into the inner eyelid. Once gelled, the formulation resists the natural drainage process from the precornea. Gel formation occurred following integration of STF, in theory after diffusion of inorganic cations (e.g. Na^+, K^+, Ca^{2+} and Mg^{2+}) present in STF into the formulation milieu (Figure 4). Solution–gel transitions for L-carnosine-buffered gellan gum test formulations at pH 5.5–7.0 were confirmed in parallel to performance of Timoptic-XE at 37°C, and formula-to-STF ratio of 3.6 : 1, demonstrating the biophysiological suitability of L-carnosine buffer. Findings with L-carnosine-buffered formulations containing 0.06 and 0.1% gellan gum showed comparable gelation to Timoptic-XE, a commercial example with unspecified Gelrite content, suggesting that gelation may also depend on the physicochemical characteristics of formulation components. Phenomenological observations best described as a lack of dependence of gelation and viscosity on polymer concentration and formulation osmolality (Table 1 and Figure 4), in contrast to known characteristics of gellan gum,[22,25] suggest that using L-carnosine as a buffer offers molecular synergism.

Earlier studies investigated the use of gellan gum to improve duration of ocular drugs from several pharmacological classes such as β-blockers, α-agonists, fluoroquinolones, corticosteroids, non-steroidal anti-inflammatory drugs and carbonic anhydrase inhibitors.[2,5–10,26,38,41–43] Having a (pre) clinically proven topical ocular drug delivery concept, gellan gum has been successfully commercialised in glaucoma products such as Timoptic-XE. However, because of the added requirements of precise pH, buffer capacity and control of ionic strength for topical ophthalmic formulations, the use of gellan gum is currently limited to drugs with a narrow range of suitable physicochemical properties. Findings in normotensive dogs showed similar lowering of IOP with Timoptic-XE and L-carnosine-buffered gellan gum formulations of 0.5% (w/v) equivalents timolol maleate and hemihydrate (Figure 5; data for hemihydrate were identical to maleate (not shown), suggesting insignificant contribution of ionic effect from weakly acidic salt counter ions in the presence of L-carnosine buffer). Pharmacological evaluation of two formulations delivering the same single active entity did not reveal significant differences in IOP at any time point, indicating that the formulations were pharmacodynamically equivalent. Lowering the pH of topical ophthalmic timolol formulations to 6–6.5 doesn't change ocular absorption but reduces systemic exposure (e.g. current case using L-carnosine-buffered gellan gum vehicle in IOP modulation, Figure 5), while alkaline pH values with preservatives incorporated into eye drops lead to increases in ocular absorption, as well as undesirable systemic absorption.[44] Correlation of well-established pharmacokinetic and dose–response relationships for topical ocular timolol supports these findings.[45–47] L-Carnosine-mediated lowering of IOP and reduction of prostaglandin-induced ocular hypertension in normotensive rabbits has been previously reported. These studies, however, used 0.2 ml intracameral injections of 10 mM L-carnosine to modulate IOP.[48] An equivalent injected dose of 2 μmol L-carnosine was delivered intraocularly,[48] an amount significantly larger than is achievable by topical absorption in our in-vivo safety and pharmacology studies (i.e. a 0.05 μl 7.5 mM L-carnosine eye drop topically delivers 0.375 μmol of the substance). High potency and capacity topical ocular formulations of L-carnosine may still lower IOP; however, this is unlikely at levels suggested herein for functional application as a buffering excipient (Figure 5).

Studies presented here provide support for further development of L-carnosine as a functionally synergistic buffer for topical ophthalmic use, attuned with pharmaceutical dosage forms containing gellan gum. A wider array of topical ophthalmic agents that require dosing more often than once daily because of disease aetiology or pharmacological mechanism can be accommodated using this approach. More specifically, a wide range of L-carnosine concentrations can be used to stabilise and/or solubilise (if ionisable) compounds in the pH range 6.5 ± 0.5. Enhanced gelation may be a further consequence of physiological active uptake of L-carnosine from formulation–tear mixtures by mammalian proton-coupled peptide transporters (e.g. PepT1[49,50])

following instillation into the conjunctival cul-de-sac. Since L-carnosine is a model substrate for PepT1, formation of a slightly acidic tear film in the microenvironment of conjunctival epithelial cells can facilitate epithelial absorption of the proposed dipeptide buffer and osmotic resorption of mono/divalent cations from tears into the formulation matrix. This theoretical occurrence is supported by identified transporter protein factor(s) endogenous to the mammalian conjunctiva,[51] with a two-fold topical ocular administration synergism: a more rapid and efficient gelation of gellan gum polymer units on a molecular level, combined with better ocular surface tolerance resulting from active uptake of L-carnosine in a proton-coupled mechanism catalysing tear fluid pH homeostasis.

Conclusions

We report here the application of a novel topical ophthalmic formulation of L-carnosine as a buffering excipient in specific combination with a carbohydrate polymer with in-situ gelling properties upon mixing with mammalian tear fluid. The buffer capacity of L-carnosine is superior to that of tromethamine (the only commercial example of a buffer used in combination with Gelrite) in the pH range 5.5–7.5. Longer term chemical stability and buffering activity of L-carnosine (versus L-histidine) under standard storage conditions offers the possibility of further development as a multifunctional excipient in ophthalmic solutions. Solution–gel transition efficiency of L-carnosine-buffered gellan gum formulations is marginally better than that of commercial Timoptic-XE when mixed with STF under experimental conditions, demonstrating physiological appropriateness and suitability. *In vivo*, acute topical ocular L-carnosine dosing is well tolerated and pharmacodynamically equivalent to Timoptic-XE when combined with an equimolar dose of timolol in the presence of 0.6% w/v gellan gum. Functional synergy between 0.06–0.6% w/v gellan gum formulations buffered with at least 7.5 mM L-carnosine enables the testing of topical ocular dosage forms with sustained precorneal residence for pharmacologically suitable and chemically diversified classes of molecules.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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