Evaluation of prolonged adherent with benzalkonium chloride on corneal protein secondary structure that assessed by Fourier transform infrared spectroscopy

Eman S. Mohamed* , Eman M. Aly

Biophysics and Laser Science Unit, Research Institute of Ophthalmology, Giza, Egypt. P.O. Box:90. 2 Al-Ahram Street, Giza, Egypt
Eman.saad@windowslive.com

Abstract: Benzalkonium chloride (BAC) is a commonly used preservative in eye drops and is known to induce complex inflammatory mechanisms on the ocular surface causing allergy and toxicity, as well as it may increase the rate of dry eye. The present study was conducted to evaluate and reveal the toxic effect(s) of different concentrations of BAC namely; 0.005%, 0.01% and 0.02% when applied twice a day for a maximum period of 16 days on corneal protein secondary structure that was assessed by deconvoluted Fourier transform infrared spectroscopy (FTIR). The results indicated that corneal protein secondary structures are tolerable for the 0.005 % BAC, while they were greatly affected by the other two concentrations of BAC. Moreover, the formed intra-molecular β-sheets that detected in the 0.005 % treated subgroup are involved in protein folding while; they are associated to protein aggregation in the other two BAC-treated subgroups; 0.01 % and 0.02 %.
[Eman S. Mohamed , Eman M. Aly. **Evaluation of prolonged adherent with benzalkonium chloride on corneal protein secondary structure that assessed by Fourier transform infrared spectroscopy.** Journal Journal of American Science 2011; 7(12):758-764]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: eye, corneal protein, FTIR, ophthalmic preservative, benzalkonium chloride.

1. Introduction

Benzalkonium chloride is a quaternary ammonium compound with a C_8 to C_{18} chain length of alkyl groups (Figure 1); it exerts toxic effects on microorganisms, and has been used as an effective germicide and preservative in cosmetic industry and medicine[1].

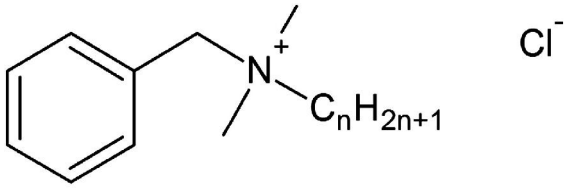

Figure 1. General structure of benzalkonium chloride.

BAC is the most common antimicrobial preservative used in topical ophthalmic preparations as well as in most intraocular pressure lowering agents. It has the potential to accumulate in ocular tissues, and to cause toxicity and/or adverse effects, particularly if there is chronic dosing[2,3], or longer medications[4,5].

Exaggerated use of BAC can also induce epithelial damage[2] and lead to an increased corneal epithelial permeability[6]. In vivo and ex vivo studies involving eye drops, attribute the induced ocular surface disease to the presence of the preservative BAC. In cell culture, BAC induced epithelial cell death[7], pro-inflammatory or pro-apoptotic mediators[8], and oxidative stress that including mitochondrial activity and glutathione injury[9]. For better understanding these ocular toxic effects, the chronic adherent with different concentrations of BAC; 0.005%, 0.01% and 0.02% for 4, 8, 12 and 16 days on the corneal protein secondary structure was evaluated by Fourier transform infrared spectroscopy.

2.Materials and Methods

Materials

Benzalkonium chloride was obtained from Acros organics (NJ, USA). Rats were randomly selected from the animal house facility at the Research Institute of Ophthalmology, Giza, Egypt. The experimental protocol was approved by the local ethical committee that applies by The Association for Research in Vision and Ophthalmology (ARVO) statements of using animals in ophthalmic and vision research.

Benzalkonium chloride treatment

Benzalkonium chloride was dissolved in deionized water in order to prepare the three concentrations 0.005, 0.01 and 0.02 % (w/v) that will be applied to the animal's eyes. These BAC solutions were freshly prepared each day. The rats were randomly classified into control (n=10, 20 eye balls) and three BAC-treated groups (each composed of 40 rats/4 subgroups) that received topical instillation of 10 L twice a day of 0.005, 0.01 or 0.02% BAC. The animal's of each BAC-treated subgroups (n=10) were monitored at 4, 8, 12 and 16 days (D).

Fourier transform infrared spectroscopy (FTIR)

Corneas were obtained from rats via cutting through the ora serata. The corneas from all animal's subgroups were weighed separately, and then crushed to powder by the aid of liquid nitrogen and mortar. The resulted corneal powder was freeze-dried for 24 h then mixed with potassium bromide (KBr) powder (95 mg KBr:5 mg cornea) in order to prepare the KBr disks that will be used for the FTIR measurement. FTIR spectra were recorded using Shimadzu FTIR spectrometer, where the instrument was operated under continuous flow of dry nitrogen gas to minimize the effect of water vapor and atmospheric CO_2. Hundred interferograms were recorded for each sample to enhance the signal to noise ratio, these interferograms were co-added, baseline corrected and smoothed by Savitzky-Golay method before Fourier transform. The spectra that belong to each BAC-treated subgroup were averaged using OriginPro7.5 software to obtain the final average subgroup spectrum. This final average subgroup spectrum was subjected to the curve enhancement procedure; a combination of Fourier deconvolution and non-linear curve fitting, to resolve the contour of amide I band (1750-1600 cm^{-1}) to its underlying peaks. The number of the resulted underlying peaks was confirmed by the second derivative of the subgroup spectrum.[10]

Statistical analysis

Data were presented as the mean±SD. In order to compare between multiple groups, analysis of variance (ANOVA) was employed using commercially available software package (SPSS-11 for windows, SPSS Inc., Chicago, IL, USA), where the significance level was set at $p<0.05$. All the spectral analyses were performed using OriginPro 7.5 software package (Origin Lab Corporation, Northampton, MA, USA).

3. Results

FTIR spectroscopy is a non-destructive technique, which provides quantitative biochemical information about biological samples. It is a valuable technique due to its high sensitivity in detecting changes in the molecular constituents of tissues. The amide I absorption is mainly associated with protein C=O stretching vibrations, and is sensitive to protein conformation. Therefore, it is suitable as a probe to determine the different secondary structures of proteins and polypeptides[10,11]. Since natural proteins fold in more than one conformation, the corresponding amide I is thus a superposition of several such structural components. The amide I band of the original infrared spectra, in general, is too complex and featureless to be of quantitative significance unless band narrowing procedure such as Fourier deconvolution and curve fitting was applied to enhance the potential of this band as a meaningful structural probe[12].

Figure (2) shows the FTIR spectra between 1750 and 1600 cm^{-1} that corresponds to amide I of protein. The original amide I band of the control corneas was centered at 1656±3 cm^{-1} while it was centered at an average frequency of 1656±2.4 cm^{-1}, 1657±1.5 cm^{-1} and 1656±2.0 cm^{-1} for those corneas of rats received 0.005, 0.01 or 0.02% BAC respectively. Decomposition of amide I band using the curve enhancement procedure resolved the contour of the control amide I into four structural components of protein secondary structure. These bands were discernible at 1689 cm^{-1} (β-turn structure, labeled as 1), 1658 cm^{-1} (α-helix structure, labeled as 2), 1637 cm^{-1} and 1622 cm^{-1} (β-sheet, labeled as 3) as shown in Figure 1 and given in Table 1. The contour of the amide I band was resolved to five different structural components due to the application of 0.005, 0.01 or 0.02% BAC, except the group received 0.005 % for 4-days, where the number of estimated structural components was mimicking the control ones.

Table 1 shows that the band position of the higher frequency β-turn component was affected (increased) by the different applied concentrations of BAC. This effect can be noticed at: 0.005 % BAC; at those subgroups followed up for 12-D and 16-D (the longer application periods), 0.01 % BAC; at the two subgroups monitored for 8-D and 12-D (the intermediate application periods) and 0.02 % BAC; for the two subgroups studied at 4-D and 8-D (the earlier application periods). In the same context, α-helix band position was greatly affected (reduced) after the topical application of BAC with concentration of 0.005 % and followed up for 4, 8, 12 and 16 days. There are three subgroups; 8-D (0.005% BAC), 4-D (0.01% BAC) and 4-D (0.02% BAC), that were characterized by the presence of β-sheet structure with average vibrational frequency of 1618 cm^{-1}.

The analysis of amide I band with the curve narrowing procedure that was shown in Figure 1 can be used to estimate the content of the different protein secondary structure components by expressing it as an area fraction (%) of the total area. It is notable from Figure (2) that the protein secondary structure of corneas received topical application of 0.005 % BAC was characterized by reduced α-helix content, that decreased from 52.1±3.4 % in the control, to an average group value of 32.3±4.9 % ($p<0.001$), but not for 4-D subgroup, associated with a reduction in the β-sheet content from 20.4±1.8 % in the control to an average group value of 10.4±0.8 % ($p<0.001$), and an elevated

content of β-turn structure that increased to an average group value of 52.8±9.4 relative to 27.5±3.7 for the control ($p<0.001$). As the concentration of the daily topically applied BAC increased to 0.01%; the α-helix content was reduced in all groups monitored at 4, 8, 12 and 16-D with an average group value of 37.9±4.3 % ($p<0.001$). On the other hand, the content of β-turn structure was increased in all groups (average group value =32.9±1.1 %, $p<0.01$). Meanwhile, the content of β-sheet structure showed paradox phenomenon, it reduced after 4 days of application of the 0.01% BAC solution (17.6±2.4 %, $p<0.01$), and increased at the rest of subgroups monitored at 8, 12 and 16 days of BAC application (average value =30.8±1.7 %, $p<0.001$).

For the highest daily applied concentration of BAC, 0.02%, there were fluctuated changes in the protein secondary structure components. The α-helix content temporarily increased after 4-days of application (65.2±1.8 %, $p<0.001$) associated with reduced β-sheet content (13.2±1.9 %, $p<0.001$) as well as the β-turn structure content (21.6±2.2 %, $p<0.001$). As the applied period increased to 8 days, the protein secondary structure components were affected in a different way to that noticed at 4-D subgroup, both α-helix and β-sheet contents were reduced to 38.9±2.1 % ($p<0.001$) and 16.3±1.1 % ($p<0.001$) respectively. This was concomitant with increased β-turn content (44.8±3.1 %, $p<0.001$). After 12 days of the topically applied 0.02 % BAC solution, β-turn structural component was unaffected and mimicking the normal value, while reduced α-helix content (35.7±3.6 %, $p<0.001$) and increased β-sheet content (36.9±1.7%, $p<0.001$) were noticed. For the longest application period, 16 days, there was no change in the α-helix content relative to the control. β-sheet content was reduced to 8.5±1.3% ($p<0.001$) and β-turn was increased to 38±2.4% ($p<0.001$).

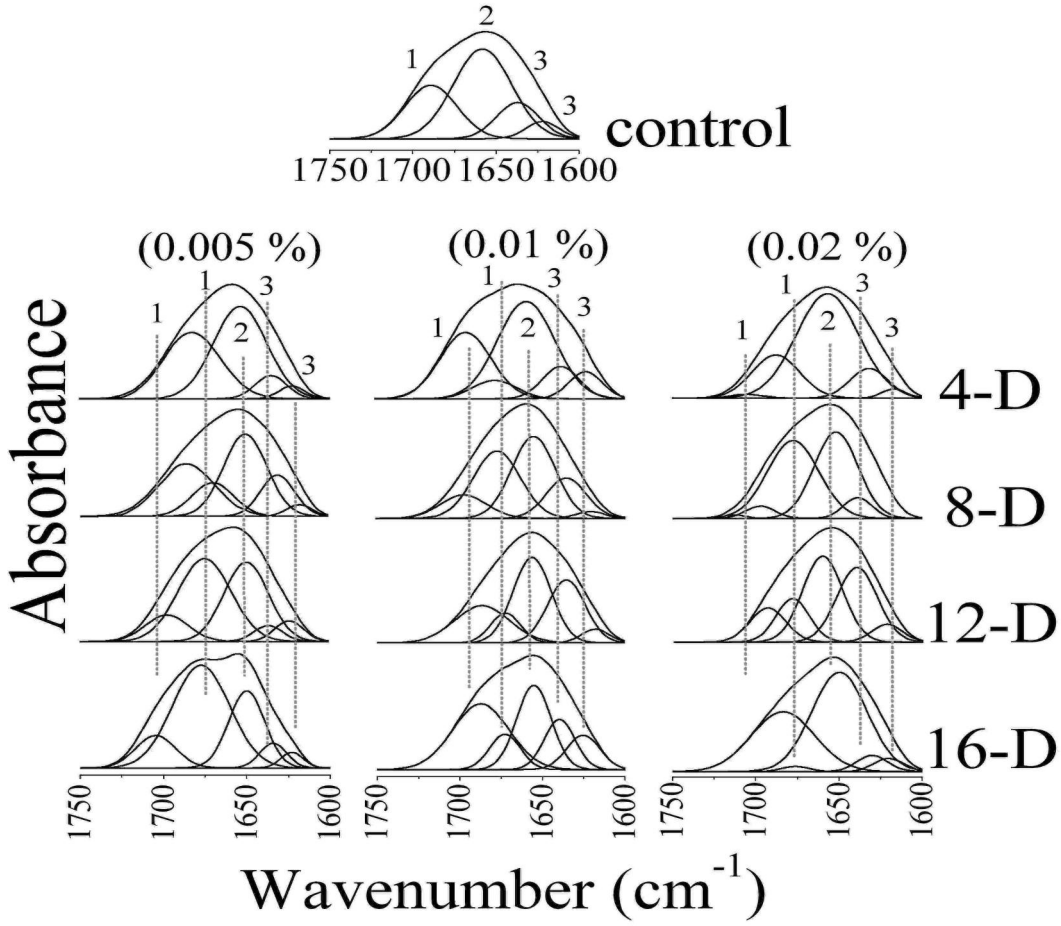

Figure 2. Amide I region (1750 – 1600 cm^{-1}) showing the underlying protein secondary structure components. The numbers above the peaks to facilitate their identification; (1) β-turn, (2) α-helix and (3) β-sheet structures.

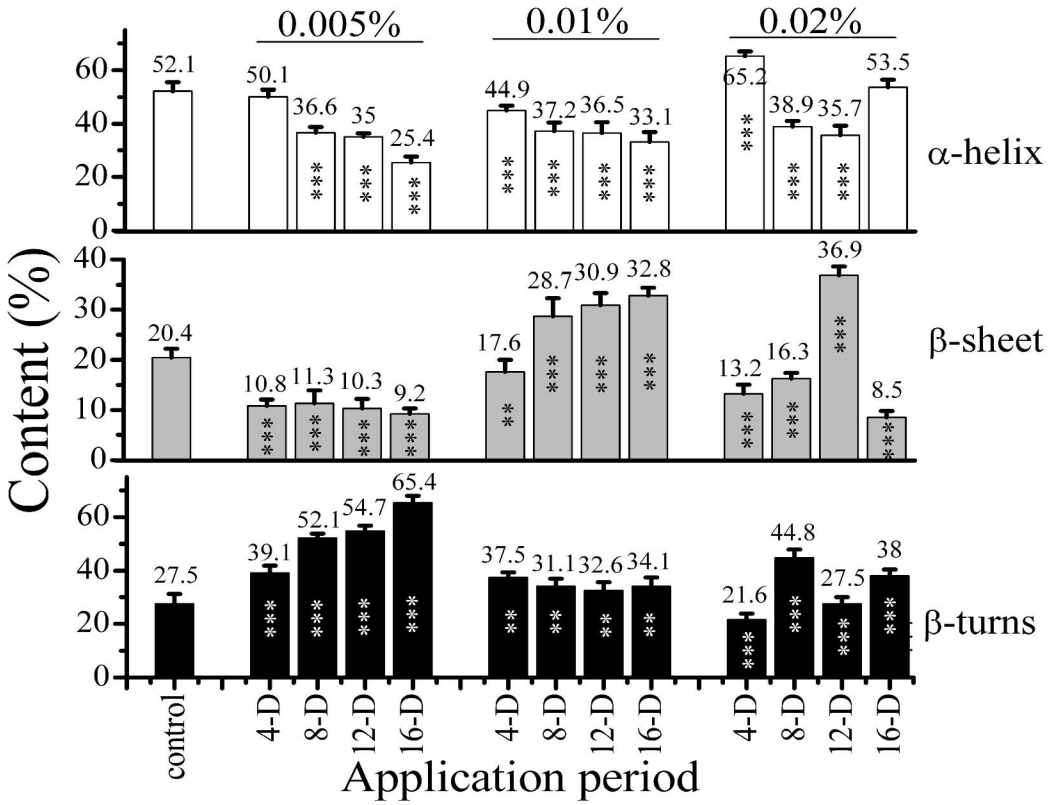

Figure 3. The content of different protein secondary structure components expressed as area percentage relative to the total area. The numbers above each column represents the mean value. **p<0.01 and ***p<0.001.

Table 1. The mean vibrational frequency ±SD (cm^{-1}) of the different estimated protein secondary structure components

		(1) β-turn	(1) β-turn	(2) α-helix	(3) β-sheet	(3) β-sheet
Control		1689±3	-	1658±2	1637±3	1622±3
0.005 %	4-D	1682±4	-	†1651±2	1635±2	1622±2
	8-D	1686±2	1670±1	†1650±2	1633±2	1618±3
	12-D	†1697±2	1675±3	†1650±1	1636±3	1624±2
	16-D	†1704±3	1675±2	†1650±3	1634±3	1623±1
0.01 %	4-D	1686±1	1672±4	1656±2	1635±1	1618±2
	8-D	†1697±3	1677±2	1655±2	1635±1	1621±2
	12-D	†1695±1	1678±3	1659±1	1638±1	1623±2
	16-D	1687±2	1673±2	1655±3	1640±2	1625±2
0.02 %	4-D	†1705±3 1688±1	-	1657±1	1632±3	1618±2
	8-D	†1697±2	1678±4	1654±3	1639±3	1626±2
	12-D	1693±3	1679±3	1659±3	1640±1	1622±3
	16-D	1684±3	1676±2	1655±2	1632±2	1620±2

†Statistically significant

4. Discussion

Eye drops are multiple dosage forms protected against microbial contamination by means of preservatives. Dry eye syndrome, associated with eye drops containing BAC, is a growing public health problem that can have a considerable impact on visual function and quality of life. As the most commonly used preservative[13], benzalkonium chloride (BAC), has shown its high level of toxicity in vitro and ex vivo by stimulating epithelial cell death[7,9], acting as pro-inflammatory or pro-apoptotic mediators[8], inducing oxidative stress[14-16], and significantly altering the precorneal mucins[17]. In vivo, these iatrogenic effects were most particularly found with the eye drops used for treating long-term pathologies such as glaucoma, with concentrations ranging from 0.005% to 0.01%. At a concentration of 0.005%, BAC was found to cause epithelial cell wrinkling and peeling with exposure of underlying cells. At 0.01%, it may induce strong epithelial alterations, and at higher concentrations (> 0.01%) it causes strong damage to corneal stroma and endothelium[18]. The threshold concentration of BAC was observed by Brasnu et. al. (2008)[19] and found to be 0.005 %. The results of this study are revealing some of the underlying mechanisms, related to corneal proteins, which can lead to the observed ocular toxicity associated with BAC. Protein in biological membranes can perturb lipid environment and, depending on their nature and concentration, influence membrane fluidity[20]. Moreover, proteins carry out most of the important tasks in living cells. For proteins to perform these important tasks, they must fold to their proper, unique three-dimensional structure[21]. Poor protein folding and insolubility lead to inefficient functional protein. There are three common secondary structures in proteins, namely α-helix, β-turns and β-sheets. β-turns are conformations that enable protein to adopt globular structures, and their formation is often rate limiting for folding[22]. They are the smallest type of the secondary structure, joining other elements of secondary structures as α-helix and β-sheets, and abruptly change the direction of the polypeptide chain[23]. The formed β-turn structure may serve as a nucleation site for folding/refolding of proteins[24]. β-sheets are another protein secondary structure conformation; although the importance of β-sheets in the folded structures of proteins has long been recognized, there is growing interest of molecular interactions among β-sheets. β -sheets have dual functions; in normal tissue, they are important for biological activities, and they are involved in many diseases such as cancer[25]. Inter-molecular β-sheet interactions (band < 1620 cm^{-1})[26] are associated with biomolecular recognition, protein quaternary structure, protein-protein interactions and peptide and protein aggregation. While, intra-molecular β-sheet interactions (band between 1630-1620 cm^{-1})[26] are associated with protein folding. Thus, vibrational spectra, as those of the FTIR spectra, were directly related to the structural features of molecules, hence; our results indicate that the inter-molecular β-sheets are transitionally exist with 0.005 % after 8 days; 0.01 % after 4 days, and 0.02 % after 4 days of BAC instillation. It has been suggested that protein insolubility depends on the content of β-sheet structure; the more β-sheet structure means the more insoluble protein[10], and that the decrease in α-helix may be due to the formation of an intramolecularly hydrogen-bonded β-sheet structure. Figure (3) showed that the corneal proteins were more folded and its solubility was increased as the β-sheet content reduced in the animal group received a daily dose of 0.005 % BAC. Protein stability is intimately connected with protein folding therefore, the daily application of BAC with 0.005 % concentration, up to 16 days, was found to be associated with increased corneal protein stability and solubility against BAC-induced stress. This finding supports that of Brasnu et. al. (2008)[19], where the threshold concentration of BAC was 0.005 %. This BAC concentration does not lead to the formation of intramolecular hydrogen-bonded β-sheet structure, the formation of such β-sheet structures resulted from the conversion of α-helix to β-sheet; but this is not the case where both α-helix and β-sheets contents were decreased as a result of the applied 0.005 % concentration of BAC. On the other hand, increasing concentration of BAC solution to 0.01 % resulted in insoluble and more folded corneal protein (Figure 3) which reflects that the corneal proteins become aggregated. This finding is also observed from the noticeable decreased in the α-helix content that was concomitant with the increase in β-sheet content due to formation of intramolecular hydrogen-bonded β-sheet structure.

The application of 0.02 % BAC was found to induce paradox effects on protein secondary structure components and accordingly on its physical characteristics (stability and solubility). The corneal protein solubility (reflected by the β-sheet content) was significantly increased after 4, 8 and 16 days of the topical application of 0.02 % BAC while, it greatly reduced after 12 days of the topical application. The variation in corneal protein solubility was associated with fluctuations in protein folding that reflected by the content of β-turns; more folded proteins can be noticed after 8 and 16 days while, less folded protein can be seen after 4 and 12 days. These variations in both protein solubility and folding are not functioning in the applied period. In ophthalmology, commonly administered

pharmacotherapeutic agents consist of 3 homologs of BAC with different N-alkyl chain lengths (C12, C14, and C16). These BAC homologs can result in different degrees of ocular toxicity. Uematsu et al., (2010)[27] found that the induced acute corneal epithelial toxicity is dependent upon the alkyl chain length of BAC homologs (C12 – C16), and that C12-BAC homolog exhibited the lowest corneal impairment whereas C14-BAC homolog induced the most severe impairment. Therefore, the contradictory results obtained in this study with 0.02 % BAC solution may be attributed to the difference in the alkyl chain length and that at higher concentration of BAC, the damaging effects of BAC on corneal protein is functioning in the alkyl chain length instead of the application period.

5. Conclusion

Altogether can lead to the conclusion that 0.005 % concentration of BAC may be used topically for a period of 16 days. While BAC concentration of 0.01 % seems to have a contradictory effects to that of 0.005 %. The use of BAC as ingredient of topical ocular formulations for long-term application with concentration range 0.01% - 0.02% should be considered with caution and, further investigations are required to reveal the paradox effects of 0.02 % BAC on corneal proteins; taking into account the possible role of different alkyl chain length of BAC homologs.

References

1. Radosław Ś, Tadeusz H , Wojciech W, Barbara K, Zofia G, & Wanda M (2008): Pulmonary irritation after inhalation exposure to benzalkonium chloride in rats. Int J Occup Med Environm Health 21, 157-163
2. Berdy G J, Abelson M B, Smith I M & George M A (1992): Preservative -free artificial tear preparation: assessment corneal epithelial toxic effects. Arch Ophthalmol 110, 528–532
3. Noecker R (2001): Effects of common ophthalmic preservatives on ocular health. Adv Ther 18, 205-215
4. Baudouin C (2008): Detrimental effect of preservatives in eye drops: implications for the treatment of glaucoma. Acta Ophthalmol 86, 716–726
5. Leung E W, Medeiros F A & Weinreb R N (2008): Prevalence of ocular surface disease in glaucoma patients. J. Glaucoma 17, 350–355
6. Gobbels M & Spitznas M (1992) : Corneal epithelial permeability of dry eyes before and after treatment with artificial tears. Ophthalmology 99, 873-878
7. Rolando M, Brezzo G ,Giordano P et. al. (1991): In The lacrimal system (Van Bijsterveld O P, Lemp M A & Spinelli D, eds) pp 89–91, Kugler, Amsterdam
8. De Saint J M , Brignole F, Bringuier F, et.al. (1999): Effects of benzalkonium chloride on growth and survival of Chang conjunctival cells. Invest Ophthalmol Vis Sci 40, 619–630
9. Debbasch C, Pisella P J, De Saint J M et.al. (2001): Mitochondrial Activity and Glutathione Injury in Apoptosis Induced by Unpreserved and Preserved β-Blockers on Chang Conjunctival Cells. Invest Ophthalmol Vis Sci 42,2525–2533
10. Lin S Y, Li M J, Liang R C & Lee S M (1998):Non-destructive analysis of the conformational changes in human lens lipid and protein structures of the immature cataracts associated with glaucoma. Spectrochemica Acta (B) 54,1509-1517
11. Haris P I & Chapman D (1994): Analysis of polypeptide and protein structures using Fourier transform infrared spectroscopy. Meth Mol Biol 22:183-202
12. Lamba O P, Borchman D, Sinha S K, Shah J, Renugopalakrishanan V & Yappert M C (1993):Estimation of the secondary structure and conformation of bovine lens crystallins by infrared spectroscopy: quantitative analysis and resolution by Fourier self-deconvolution and curve fit. Biochem Biophys Acta 1163, 113-123
13. Reck R A (1982): In Encyclopedia of Chemical Technology (Mark H F, Othmer D F, Overberger C G & Seaborg G T, eds) pp 521–531, John Wiley & Sons, Toronto
14. Debbasch C, Brignole F, Pisella P J, Warnet J M, Rat P & Baudouin C. (2001): Mitochondrial Activity and Glutathione Injury in Apoptosis Induced by Unpreserved and Preserved β-Blockers on Chang Conjunctival Cells: Invest Ophthalmol Vis Sci 42, 642–652
15. Guenoun J M, Baudouin C, Rat P, Pauly A, Warnet J M, Brignole- Baudouin F. (2005): In vitro study of inflammatory potential and toxicity profile of latanoprost, travoprost, and bimatoprost in conjunctiva- derived epithelial cells. Invest Ophthalmol Vis Sci 46:2444–2450
16. Guenoun J M, Baudouin C, Rat P, Pauly A, Warnet J M, Brignole- Baudouin F (2005): In vitro comparison of cytoprotective and antioxidative effects of latanoprost, travoprost, and bimatoprost on conjunctiva-derived epithelial cells. Invest Ophthalmol Vis Sci 46:4594–4599

17. Chung S H, Lee S K, Cristol S M, Lee E S, Lee D W, Seo K Y & Kim E K (2006): Impact of short-term exposure of commercial eyedrops preserved with benzalkonium chloride on precorneal mucin. Mol Vis 12, 415–421
18. Wollensak G, Iomdina E (2009): Biomechanical and histological changes after corneal crosslinking with and without epithelial debridement. J Cataract Refract Surg. 3: 540-54
19. Brasnu E, Brignole-Baudouin F, Riancho L, Guenoun J M, Warnet J M & Baudouin C (2008) :. In vitro effects of preservative-free tafluprost and preserved latanoprost, travoprost, and bimatoprost in a conjunctival epithelial cell line. Curr Eye Res 33, 303–312
20. Chapman D, Gomez-Fernhndez J C & Gofii F (1979) :Intrinsic protein-lipid interaction. Physical and biochemical evidence. FEBS Letters 98, 211–223
21. Trevino S R, Schaefer S, Scholtz J M & Pace C N (2007) : Increasing protein conformational stability by optimizing beta-turn sequence. J. Mol. Biol. 373(1):211-218
22. Fullera A A, Du D, Liu F, Davoren J E, Bhabha G, Kroon G et al (2009) : Evaluating beta-turn mimics as beta-sheet folding nucleators. PNAS 106, 11067-11072
23. Rose G D, Young W B & Gierasch L M (1983): Interior turns in globular proteins.. Nature 304, 654-657
24. Orpiszewski J, Schoromann N, Kluve-Beckerman M, Liepnieks J J & Benson M D (2000): Protein aging hypothesis of Alzheimer disease. FASEB J 14, 1255-1263
25. Khakshoor O & Nowick J S (2008): Artificial b-sheets: Chemical Models of b-Sheets . Curr Opin Chem Biol 12, 722–729
26. Szalontai B (2009) : Membrane protein dynamics: Limited lipid control. P M C Biophys 2, 1-17
27. Uematsu M, Kumagami T, Shimoda benzalkonium K, Kusano M, Teshima M, Sasaki H & Kitaoka T (2010): Influence of alkyl chain length of chloride on acute corneal epithelial toxicity. Cornea 29,1296–1301

12/12/2011