Effect of Different Concentrations of Benzalkonium Chloride on the Cornea

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Abstract: Aim of the work: The overall objective of this study is to evaluate the effect of benzalkonium chloride (BAK) on the conformational characteristics of the cornea. Materials and methods: New Zealand white rabbits were used in this study for application of different concentration of BAK (0.005%, 0.01% and 0.02%) for different periods (4, 8, 12 and 16 days). Results: The study reports the corneal structure alterations that may be induced as a result of BAK applications that were studied by Fourier transform infrared spectroscopy (FTIR). The resulting IR spectra were analyzed using the band enhancement procedure. The obtained data clearly indicate that there are different structural and conformational changes as the method of BAK applications.
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Key words: Benzalkonium chloride, Eye, Cornea, FTIR, Rabbits.

1. Introduction:

The most common preservative in topical ophthalmic preparations is benzalkonium chloride (BAK). This is a quaternary ammonium compound composed of a mixture of alkylbenzyldimethyammonium chloride homologues with n-C_{12} H_{25}, n-$C_{14}H_{29}$ and n-$C_{16}H_{33}$ comprising a major portion of the alkyl groups present (United States Pharmacopeia–National Formulary [USP–NF] 2005)[(1)]. It is commonly used at concentrations of 0.004−0.025%. Several investigations using animal models have suggested the existence of links between BAK and cytotoxic effects on several components of the eye.

Using a rabbit model, Furrer et al. (2001)[(2)] demonstrated that 28 days of treatment with beta-blockers preserved in BAK 0.01% or benzododecinium bromide 0.012% resulted in microlesions covering nearly 9% of the corneal surface. Administration of solutions containing 0.01% BAK has been linked to the infiltration of immunocompetent cells into the limbus and bulbar conjunctiva in rats[(3)]. The inflammatory reaction was associated with severe damage to the cornea and conjunctiva, including epithelial alterations and keratinization. Several other animal studies have shown that preservatives are linked to the onset of chronic fibrosis in the conjunctiva[(4,5)]. Noecker et al. (2004)[(6)] investigated the extent of epithelial and corneal damage associated with Purite® (Allergan, Inc, Irvine, California, USA), a stabilized oxychloro complex, and with topical antiglaucoma medications preserved with BAK. In addition to damaging the cornea and conjunctiva, preservatives may also cause severe lesions in the retina. In pigmented rabbits, subconjunctival injection of timolol (0.5%) or befunolol (1%) preserved with BAK caused retinal lesions, retinal detachment, loss of visual acuity and atrophy of the pigmented epithelium of the retina and choroids [(7)]. Jaenen et al. (2007)[(8)] showed that signs of damage to the conjunctiva, cornea and eyelids significantly decreased when patients were switched from preserved to preservative-free medication, or even when the number of eye drops containing BAK was decreased. This effect demonstrated the dose-dependency of BAK-induced manifestations. Masahiko and Atsuo (2010) [(9)] evaluated the cytotoxicity of prostaglandin analog eye drops preserved with BAK in multiple corneoconjunctival cell lines and they concluded that various dilutions and exposure times provided a unique evaluation of cytotoxicity among ophthalmic solutions. Zhirong et al. (2011) [(10)] found that topical administration of 0.2%BAK in mouse induces changes resembling that of dry eye syndrome in humans.

In the present work, the effect of different concentrations of BAK; 0.005%, 0.01% and 0.02% for 4, 8, 12 and 16 days on the conformational characteristics of the cornea was evaluated by Fourier transform infrared spectroscopy.

2. Materials and methods

Rats (*Rattus Norvigicus*) were randomly selected from the animal house facility at the Research Institute of Ophthalmology, Giza, Egypt. The research protocol was approved by the local ethical committee that applies the ARVO (the Association for Research in Vision and Ophthalmology) statements for using animals in ophthalmic and vision research. Benzalkonium chloride was obtained from Acros organics (NJ, USA) then was dissolved in de-ionized 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 BAK solutions were freshly prepared each day. The rats were randomly classified

into control (n=10, 20 eye balls) and three BAK-treated groups (each composed of 40 rats/4 subgroups) that received topical instillation of 10µL twice a day of 0.005, 0.01 or 0.02% BAK. The animals of each BAK-treated subgroup (n=10) were monitored at 4, 8, 12 and 16 days (D). Corneas were obtained from rats via cutting through the ora serrata. The corneas from all animals subgroup 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 before Fourier transform. The spectra that belong to each BAK-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 NH group (3900-3000 cm^{-1}), CH group (3000-2800 cm^{-1}) and the finger print region (1400-1000 cm^{-1}) to its underlying peaks. The number of the resulted underlying peaks was confirmed by the second derivative of the subgroup spectrum.

Statistical analysis

Data were expressed as the mean±SD. Comparison between multiple groups was performed using analysis of variance (ANOVA), commercially available statistical software package (SPSS-11, for windows) was used where the significance level was set at $p<0.05$. All the spectral analysis was performed with OriginPro 7.5 software (Origin Lab Corporation, Northampton, MA, USA).

3. Results

Figure (1) shows the infrared frequency range 3900-3000 cm^{-1} that corresponds to the stretching of NH-OH groups. The curve enhancement procedure; a combination of Fourier deconvolution and non-linear curve fitting, revealed the mean peak of the normal pattern into two components centered at 3598±3 cm^{-1} and 3492±2 cm^{-1} due to OH stretching ($_{str}OH$), a peak centered at 3319±2 cm^{-1} due to NH asymmetric (NH_{asym}) and CH ring centered at 3073±3 cm^{-1}. As shown in table 1, due to treatment with BAK $_{str}OH$ was splitted into three components in 0.01 % treated group regardless the follow up period. On the other hand, treatment with the lowest BAK concentration was associated by fluctuated changes in the number of estimated components as well as in their band position and band width. The last observation regarding this band can be seen in the highest BAK treated group, where the number of the estimated bands was mimicking the control ones but for 8-D group. In addition, the two modes of vibrations; OH_{asym} and OH_{sym}, was detected (appeared) in the BAK treated groups in a manner that do not directly relate to the applied periods and the concentration as well. In the same context, the NH_{sym} mode of vibrations was also detected (appeared), as compared to the normal, in many of the BAK treated groups; this mode is sensitive to the highest concentration; 0.02 %. Meanwhile, the NH_{asym} mode of vibration was also found to be sensitive to the BAK treatment; it was restricted at 8-D group (0.05%), 4-D and 16-D groups (0.01%) and 8-D and 12-D groups treated with 0.02%. Finally, for the CH_{ring} mode of vibration, there were two common observations; there was no change in the band frequency that was associated with significantly increased band width.

The CH stretching region (3000-2800 cm^{-1}) shown in fig (2) indicates the presence of four bands in the control samples centered at 2962 ±3, 2926±3, 2874±4 and 2854±3 cm^{-1} that can be assigned to $_{asym}CH_3$, $_{asym}CH_2$, $_{sym}CH_3$ and $_{sym}CH_2$ stretching vibration, respectively. In the data of the CH stretching region given in table (2), the $_{asym}CH_3$ vibrational mode was transitionally affected after treatment with 0.02% BAK solution for 12 days where its band position and bandwidth were significantly increased; meanwhile they remained unchanged to the rest of BAK treated groups. On the other hand, the $_{asym}CH_2$ mode of vibration shows different characteristics where its bandwidth was reduced as a result of BAK treatment with different concentrations, while its band frequency was unaffected and remained in the range of the control one. The characteristics (band position and bandwidth) of the symmetric modes of vibration (CH_3 and CH_2) were also unchanged in all BAK treated groups for all the followed periods involved in the study.

The third region of the FTIR shown in fig.3 is the finger print region (1500-900 cm^{-1}). The normal sample indicates the presence of eight bands: (1) CH_2 bending at 1457±3 cm^{-1}, (2) COO_{sym} at 1398±2, (3) CH_3 bending at range 1335±2 to 1284±3 cm^{-1}, (4) $_{asym}PO_2$ at 1238±2 cm^{-1}, (5) CH deformation at 1200±3 cm^{-1}, (6) $COOC_{asym}$ at 1167±2 cm^{-1}, (7) NH_3 rocking at 1127±1 cm^{-1} and (8) $_{sym}PO_2$ at 1069±3 cm^{-1} [11]. As a result of the application of BAK some observations can be concluded from Table (3):

CH_2 bending: No change in band position. The bandwidth decreased in all groups but 0.02 %, 4-D group.

COO_{sym}: No change in band position and bandwidth after application of BAK

CH_3 bending:

- No change in the frequency of the lower frequency component (≈1280 cm^{-1})
- No change in frequency of the higher frequency component (≈ 1335cm^{-1})
- At 0.01 % concentration applied for 8-D, only one component was detected with different frequency (1323 cm^{-1}) relative to the control ones
- The band detected at ≈1312 cm^{-1} disappeared in 8-D group (0.005 %).
- The lowest frequency component (≈1280 cm^{-1}) was absent in the following groups 4-D (0.01%), 4-D and 12-D (0.02%).
- The bandwidth was reduced for the detected higher frequency (≈1335 cm^{-1}). Meanwhile, the bandwidth of the detected lower frequency component fluctuated without any trend that can be related to application of BAK.

$_{asym}PO_2$: Although this band was restricted at 4-D group as a result of 0.005% BAK treatment, there were no changes in its band position or bandwidth from the other different BAK concentration treatment.

CH deformation: The band detected in different BAK treatment groups was characterized by unchanged band position associated with increased bandwidth in two groups only; 4-D (0.005) and 4-D (0.01%). The most interesting observation is the absence of this mode of vibration at the longer period (16-D) of different BAK concentrations used in this study.

$COOC_{asym}$: As a result of application of BAK with concentration of 0.005% for 4 days and 8 days; this mode of vibration was restricted. With the increase in the applied period as well as by increasing the BAK concentration, this mode was detected with lower frequency as compared with the control one. This was concomitant with variation in its bandwidth that cannot be related to application of BAK.

NH_3 rocking: The vibrational frequency of NH_3 rocking increased for the 4-days group with the lowest concentration of BAK (0.005%) then decreased for all other studied groups. The band width showed the same phenomena.

$_{sym}PO_2$: There is an increase in the vibrational frequency for all studied groups compared to the normal. Also there is a splitting of the peak for the 8-days group (0.01 %). Also fluctuations of the band width were observed.

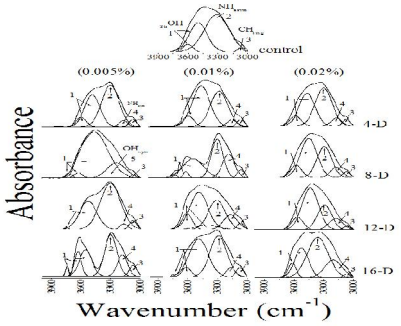

Figure 1: Representative FTIR spectra of the normal and BAK treatment groups in the NH-OH region and the estimated component.

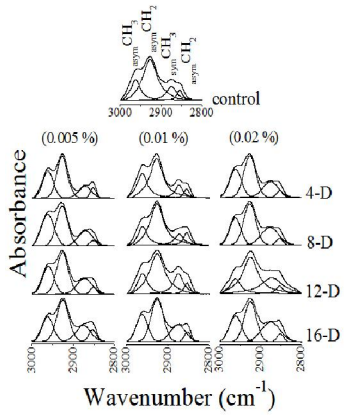

Figure 2: FTIR spectra for the wavenumber ranging from 3000 to 2800 cm^{-1} of the normal corneas and BAK treatment groups.

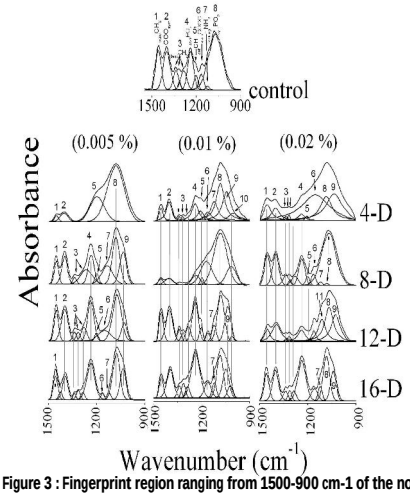

Figure 3 : Fingerprint region ranging from 1500-900 cm-1 of the normal corneas and BAK treatment groups; showing the corresponding estimated components.

Table 1. Band assignment, wavenumber (cm^{-1}), bandwidth (cm^{-1}) and estimated components of corneal tissue in the NH-OH region for normal and all groups receive benzalkonium chloride treatment

		$_{Str}$OH			OH_{asym}	NH_{asym}	OH_{sym}	NH_{sym}	CH_{ring}
	Control	3598±3	3492±2			3319±2			3073±3
		92±5	156±8			254±6			51±3
0.005%	4-D	3600±3	3490±3			†3303±2		3172±2	3071±4
		93±5	154±8			†173±8		75±1	†64±4
	8-D	3684±2	3627±2	3476±2			3244±2		3070±3
		195±12	85±6	287±10			52±1		†187±2
	12-D	3740±3	3526±2			†3303±3		3168±3	3076±3
		27±4	171±3			†201±10		71±1	†66±4
	16-D	3743±3	3631±3	3553±21124±7		†3300±3		3182±3	3073±3
		28±4	68±4			†122±9		99±1	†73±3
0.01%	4-D	3601±3	3471±3				†3291±2	3162±3	3075±4
		115±4	177±4				†174±1	89±1	†66±3
	8-D	3733±4	3624±3	3552±3		†3310±3		3196±3	3073±4
		37±5	47±4	205±5		†114±7		118±2	†63±3
	12-D	3740±4	3611±3	3491±31178±6		†3310±3		3173±4	3072±3
		216±9	99±6			†166±7		136±2	†61±4
	16-D	3738±4	3614±3	3491±3190±5			†3292±4		3075±4
		20±3	86±7				†167±2		†67±4
0.02%	4-D	3586±2	3469±1			†3302±3		3172±2	3073±3
		114±2	158±9			†168±6		98±2	†63±4
	8-D	3576±2			3452±2		†3290±3	3175±3	3071±2
		115±4			162±1		†144±2	107±1	†66±5
	12-D	3565±3	3437±2		3437±2		†3290±2	3180±2	3070±3
		117±3	162±8		162±2		†138±1	129±2	†63±4
	16-D	3621±2	3526±2			†3362±2		3203±3	3070±3
		76±6	137±8			†209±9		173±2	†59±2

† Statistically significant; The second line represent the bandwidth± SD.

Table 2. Frequency values and bandwidth (cm-1) of the CH stretching region of normal and all groups received BAK.

		$_{asym}CH_3$	$_{asym}CH_2$	$_{Sym}CH_3$	$_{Sym}CH_2$
	Control	2962±3	2926±3	2874±4	2854±3
		26±6	47±3	29±6	16±5
0.005%	**4-D**	2962±3	2926±2	2872±2	2852±3
		27±5	†26 ±5	32±4	13±2
	8-D	2963±3	2927±2	2870±2	2852±3
		31±5	†28±4	36±2	16±3
	12-D	2962±4	2926±4	2873±3	2852±3
		28±4	†29±2	34±3	15±4
	16-D	2962±3	2925±3	2875±3	2853±3
		28±4	†30±2	35±4	18±5
0.01%	**4-D**	2962±3	2928±2	2873±2	2855±3
		28±3	†36±1	22±6	16±3
	8-D	2961±3	2926±4	2872±2	2854±4
		25±5	†37±2	24±2	16±2
	12-D	2962±3	2927±339±2	2857±4	2854±2
		27±3		27±3	15±4
	16-D	2962±3	2926±4	2875±3	2852±3
		27±1	†30±2	36±4	14±3
0.02%	**4-D**	2963±4	2926±4	2876±3	2853±3
		30±5	†30±2	39±6	15±1
	8-D	2962±3	2927±3	2877±3	2853±2
		†28±3	†30±2	40±6	15±1
	12-D	†2873±4	2853±3	2975±2	2859±4
		†61±2	†18±1	39±7	20±6
	16-D	2960±3	2925±2	2875±4	2850±3
		30±4	†29±2	40±7	15±2

†Statistically significant;
The first line in each cell reflects the band position, while second line reflects the band width

Table 3. General band assignment of the fingerprint region for normal and all groups received Benzalkonium chloride.

		CH_2 bending	COO_{sym}	CH_3 bending	$_{asym}PO_2$	CH deformation	$COOC_{asym}$	NH_3 rocking	$_{sym}PO_2$	COC	CH bending
	Control	1457±3 †(45±4)	1398±2 (45±3)	1335±2 (40±3) 1312±3 (15±6) 1284±3 (37±6)	1238±2 (43±7)	1200±3 (24±7)	1167±2 (26±6)	1127±1 (62±5)	1069±3 (98±5)		
0.005%	**4-D**	1456±2 †(25±6)	1403±3 (50±4)			1200±2 †(91±8)			†1077±1 (110±9)		
	8-D	1454±2 †(25±3)	1400±1 (38±6)	1338±2 (20±4)† 1268±5 (77±8)†	1235±2 (35±4)	1199±3 (21±6)		†1136±2 (69±4)	†1081±1 †(44±8)	1035±2 (43±1)	
	12-D	1455±2 †(26±5)	1399±3 (42±4)	1339±3 (17±2)† 1316±2 (22±1) 1281±3 (33±4)	1237±2 (36±3)	1202±3 (17±5)	†1153±2 †(80±2)		†1076±1 †(52±7)	1028±2 (34±1)	
	16-D	1457±1 †(24±4) 1425±2 †(17±5)	1395±2 (38±5)	1338±2 (12±5)† 1314±4 (20±1) 1286±2 (20±6)†	1236±1 (49±5)		†1163±1 (17±1)	†1120±2 †(16±6)	†1079±2 †(43±6)	1035±2 (37±1)	
0.01%	**4-D**	1454±2 †(23±2)	1402±2 (36±7)	1338±2 (8±3)† 1317±3 (14±2)	1239±2 (44±3)	1204±3 †(43±8)	†1160±1 †(46±3)	†1120±1 †(50±3)	†1082±2 †(57±4)	1042±2 (60±4)	1001±6 (58±5)
	8-D	1455±3 †(26±7)	1403±3 (47±2)	1323±3 (53±3)	1234±4 (50±6)	1201±2 (16±5)	†1160±2 †(73±4)		†1081±2 †(80±6)		1013±4 (62±2)
	12-D	1454±1 †(26±2)	1400±2 (41±2)	1338±2 (18±4)† 1316±2 (19±2) 1282±3 (29±6)†	1239±2 (36±6)	1202±1 (17±5)	†1160±1 (21±1)	†1120±1 †(25±3)	†1079±2 †(41±5)	1035±1 (36±2)	1008±2 (15±3)
	16-D	1454±2 †(24±6)	1396±1 (43±1)	1338±2 (22±3)† 1313±2 (21±1) 1287±3 (23±5)†	1237±1 (54±7)		†1160±1 † (40±3)	†1120±2 †(23±4)	†1084±4 †(40±4)	1047±5 (37±1)	1021±5 (28±5)
0.02%	**4-D**	1456±1 (56±8)	1400±1 (50±4)	1339±3 (22±5)† 1316±3 (12±1)	1238±2 (43±4)	1203±1 (21±4)	†1151±2 †(198±3)		†1083±1 (83±2)	1033±1 (117±2)	
	8-D	1454±1 †(35±3)	1398±1 (39±5)	1336±3 (31±3)† 1312±2 (13±3) 1280±4 (40±5)	1237±2 (37±4)	1201±1 (23±3)	†1164±1 (26±1)	†1120±1 †(19±6)	†1081±1 (14±3)† 1067±1 (95±1)		
	12-D	1454±1 †(33±4)	1397±2 (56±9)	1337±4 (16±4)† 1316±3 (25±1)	1239±2 (48±4)	1207±4 (15±4)	†1162±1 (25±3)		†1076±1 †(59±4)	1027±2 (60±4)	
	16-D	1457±1 †(30±5)	1400±3 (42±3)	1338±2 (26±3)† 1311±3 (20±2) 1285±2 (24±3)†	1236±1 (53±8)		†1158±1 (36±4)	†1122±1 †(22±7)	†1084±1 †(40±6)	1040±2 (39±1)	

†Statistically significant, the numbers between brackets represent the bandwidth ±SD

4. Discussion

Ophthalmic solutions are formulated to achieve long shelf-life, effective antimicrobial action, comfort to the patient, penetration and action of the active agent(s), and minimal side effects. Tissue reactions from these preparations are often tolerated to gain one or more specific benefits. However, there are frequent situations when specific drug components may induce serious iatrogenic diseases, possibly vitiating any beneficial effects on the primary disease process. The cationic surfactant benzalkonium chloride is a common disinfectant in topical ophthalmic formulations [12] and its side effect on the cornea has been frequently investigated [13,14,15,16,17,18]. So in the present work the effect of different concentrations of BAK; 0.005%, 0.01% and 0.02% for 4, 8, 12 and 16 days on the conformational characteristics of the cornea was evaluated by FTIR.

The NH-OH region (table, 1) was very sensitive to the BAK treatment whether the concentration was 0.05% (lowest concentration) or 0.02 % (the highest concentration). These bands are found in membrane constituents as the lipid, protein and the genetic material as well. Thus BAK treatment induces functional changes in the membrane constituents that may be related to the previously documented biohazard

effects of BAK when used as an ophthalmic preservative. Several studies [19,20,21] found that benzalkonium chloride can denature corneal protein and cause irreversible damage to the eye.

The CH vibrational region (table, 2) is used generally to characterize the lipid molecules. The band width of the asym CH_2 vibrational frequency was decreased after application of BAK also $_{asym}CH_3$ vibrational frequency was affected indicating an environmental change. The cooperative effect between the changes in the NH-OH region and the CH vibrational region may also be seen in the fingerprint region. The PO_2 stretching modes are characterized by some changes in their environment. This may reflect interaction/binding mechanism(s). The CH_2 bending is of special interest, because it can be used to monitor the tissue disorder. As shown in table (3), there is no change in the vibrational frequency but the bandwidth was decreased after application of BAK. Also the changes in the vibrational frequency and the bandwidth of $COOC_{asym}$ or NH_3 rocking confirm the changes in the environment.

The greatest biocidal activity is associated with the BAK. The mechanism of bactericidal/ microbicidal action is thought to be due to disruption of intermolecular interactions. This can cause dissociation of cellular membrane lipid bilayers, which compromises cellular permeability controls and induces leakage of cellular contents [22,23,24,25].

5. **Conclusion**:

From this study, FTIR spectroscopy is a technique, which provides quantitative biochemical information about biological samples. BAK induces structural and conformational changes of the cornea. Also the extensive use of BAK in ophthalmic preparations must be critically reviewed and its use, if indicated, curtailed. The studies of the induced changes effects of BAK should be done to the different ocular structure.

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