In-vitro permeation studies of Benzalkonium chloride from FiteBac®

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Introduction

The in vitro permeation testing was performed on fitebac gel containing 0.1% benzalkonium chloride. The analytical method LC-MS/MS method was quantitation of Benzalkonium chloride was validated. In one protocol, the gel was loaded one time and the dose was equivalent to the dose applied through the maximum use trials. A custom made poloxamer gel containing same strength of Benzalkonium chloride from the same source was used as a control. Permeation studies were also performed using aqueous solution of banzalkonium chloride in the donor compartment. The second protocol, the gel was applied every 5 min for 12 hours in divided doses (called Maximum use trials) to assess the potential impact of frequent application of hand sanitizer product on the extent of absorption of Benzalkonium chloride. In vitro permeation testing would be a great tool to screen antibacterial products intended for frequent applications to assess the risk of systemic exposure to the actives incorporated in formulations.

Abbreviations

BAC-12 : Benzalkonium chloride (C-12)

BAC-14 : Benzalkonium chloride (C-14)

BAC-16 : Benzalkonium chloride (C-16)

IS : Internal Standard

ESI : Electrospray ionization

UPLC : Ultra Performance Liquid Chromatography

LC : Liquid Chromatography

MS : Mass spectrometry

DDAB : Didecyldimethylammonium bromide

BLQ : Below the limit of quantitation

1. LC-MS/MS Method Development and Validation of Benzalkonium chloride (C-12, C-14 and C-16)

1.1 INSTRUMENTATION AND METHOD

i. Liquid Chromatography

The chromatographic analysis was carried out using Acquity™ Ultra Performance LC equipped with binary solvent and sample manager (Waters, Milford, MA, USA). The 5 µl aliquots of processed samples was injected into a Acquity UPLC® HSS T3 (1.8µM, 2.1 X 50 mm; Milford, MA, USA). The isocratic mobile phase, a mixture of 0.1 % formic acid and acetonitrile (25:75, v/v) and was run on a flow gradient program for analysis (Table 1).

Table 1: UPLC Flow gradient program for analysis.

Time (min)	Flow (ml/min)	% A	% B
0.0	0.15	25	75
1.0	0.15	25	75
2.5	0.25	25	75
3.5	0.35	25	75

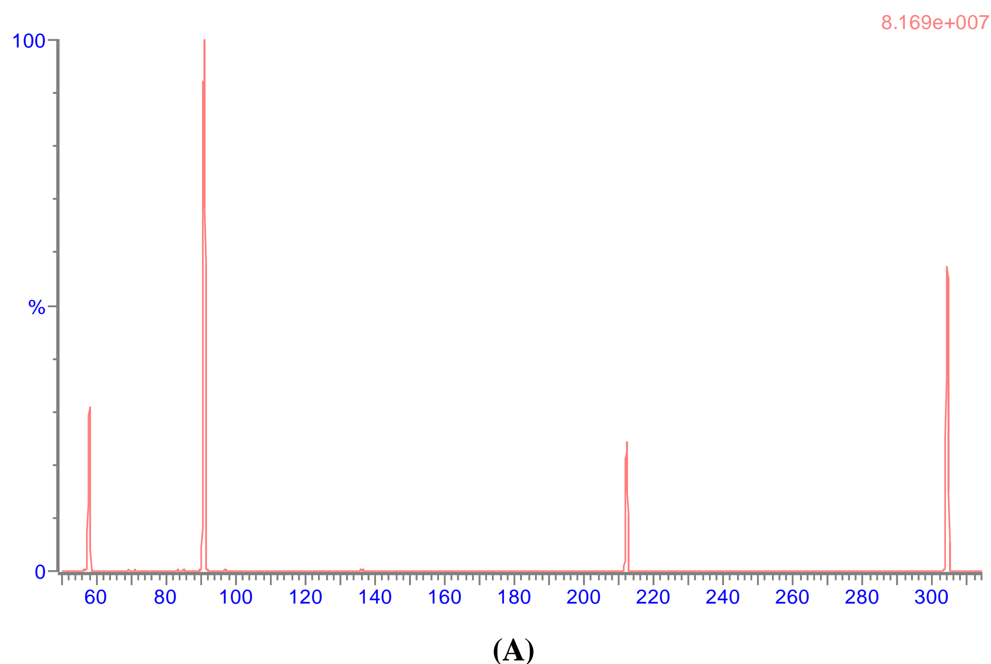
ii. Mass Spectrometry

Quantitation was achieved by MS/MS detection in positive ion mode for analyte and IS using a Waters Xevo® TQD (Waters, Milford, MA, USA) mass spectrometer, equipped with standard electrospray ionization (ESI). The source parameters viz., source temperature, desolvation temperature, cone gas flow, and desolvation gas, were 150 °C, 500 °C, 25 L/h, and 600 L/h, respectively. Nitrogen was used as the nebulizer and cone gas, while argon was employed as collision gas. The analytical data were processed by Masslynx. The ESI conditions were optimized for BAC-12, BAC-14, BAC-16 and IS, by performing quadrupole full scan in positive ion detection mode. Analytes were infused directly, the mass spectra for BAC-12, BAC-14, BAC-16 and DDAB (IS) revealed peaks at m/z 304.2, 332.3, 360.1 and 228.2, respectively, as protonated molecular ions, [M+H]+. Followed by thorough optimization of mass spectrometry conditions, the compound parameters (Table 2) obtained was used for

quantification purposes. The fragmentation patterns of BAC-12, BAC-14 and BAC-16 are shown in Figure 1, 2 & 3.

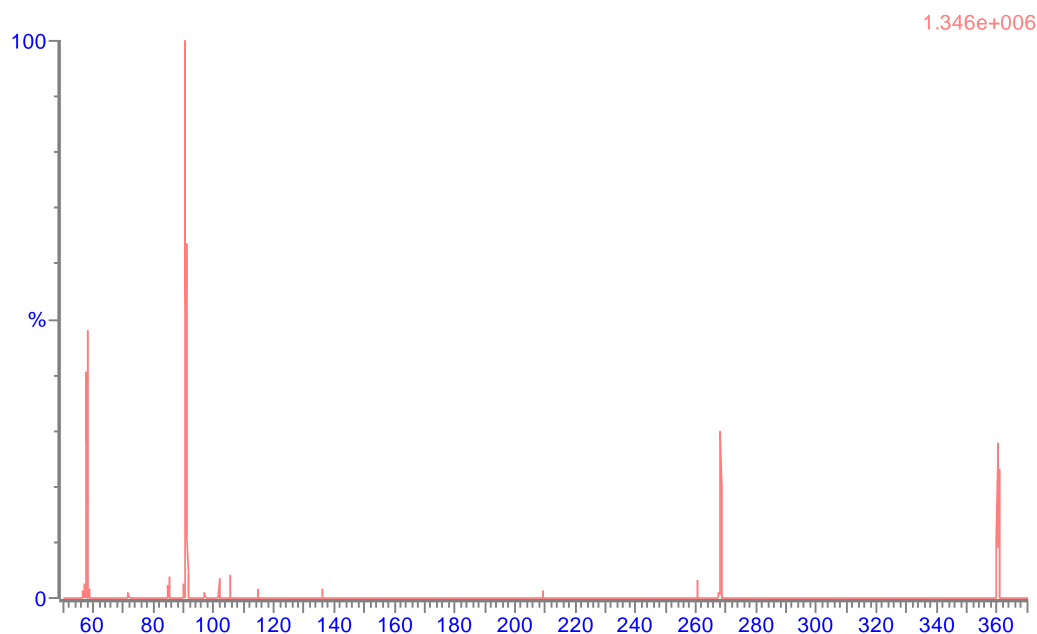
Table 2: The compound parameters for analysis of analytes.

Compound	Parent (m/z)	Daughter (m/z)	Dwell (s)	Cone (v)	Collision (v)
BAC-12	304.202	212.155	0.025	46	22
BAC-14	332.329	240.214	0.025	52	24
BAC-16	360.128	268.235	0.025	42	24



(A)

(B)



(C)

Figure 1: MS/MS Fragmentation pattern of (A) BAC-12, (B) BAC-14 and (C) BAC-16

Preparation of stock and standard solutions

The calibration standard and quality control (QC) primary stock solutions of BAC-12, BAC-14 and BAC-16 were prepared from separate weighing. The BAC-12, BAC-14, BAC-16 and IS

primary stocks (200 µg/ml) were made in methanol and stored at 4°C. The primary stock solutions of BAC-12, BAC-14 and BAC-16 were diluted with 80% methanol, which were subsequently used in the preparation of calibration curve (CC) and QC sample stock solutions. Appropriate dilutions of BAC-12, BAC-14 and BAC-16 stock solutions were made in 80% methanol to produce working stock solutions and QC stock solutions.

Table 3: The Concentrations of calibration standards and quality control samples

Standards and Quality control	**Benzalkonium chloride (ng/mL)**		
	C-12	**C-14**	**C-16**
Lower limit of quantitation (LLOQ)	5.07	5.07	5.12
Lower quality control (LQC)	15.2	15.2	15.4
Medium Quality control (MQC)	507	507	512
High Quality control (HQC)	761	761	768
Higher limit of quantitation	1015	1015	1024

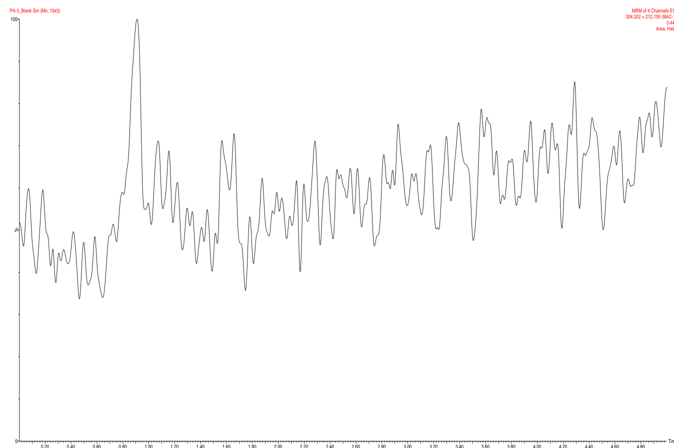
Working standard and QC stocks were used to prepare calibration standards and quality control samples. A working IS solution (100 ng/ml) was prepared in 80% methanol. To an aliquot of 1995 µl of water 5 µl of BAC-12, BAC-14, and BAC-16 working solution and 20 µl of IS were spiked to prepare the calibration standard and quality control samples. The 2 ml mixture was vortex-mixed in a cyclo-mixer for 3 min and dried under a gentle stream of nitrogen at 50℃ using automated evaporation system (Turbovap® LV, Biotage, MA, USA). The dried residue was reconstituted in 200 µl of acetonitrile, vortex-mixed and centrifuged at 14,000 rpm for 5 min (Centrifuge 5430 R, Eppendorff, Germany). The supernatant was transferred into vials and injected into LC-MS/MS for analysis.

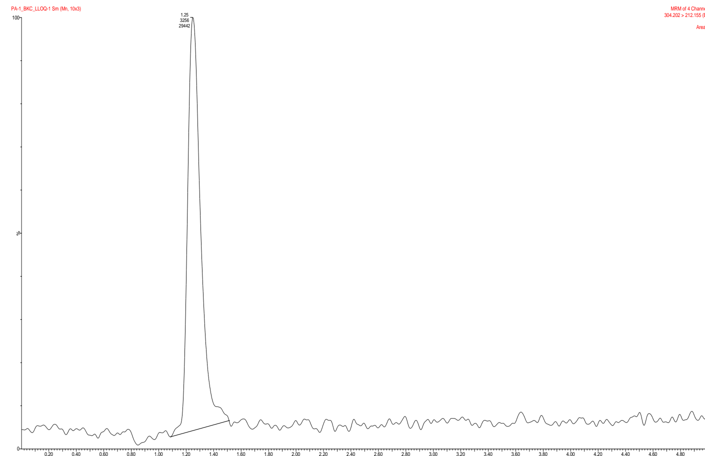
1.2 METHOD VALIDATION

Method validation for BAC-12, BAC-14 and BAC-16 in water was performed as per US FDA guidelines.

i. Specificity & selectivity

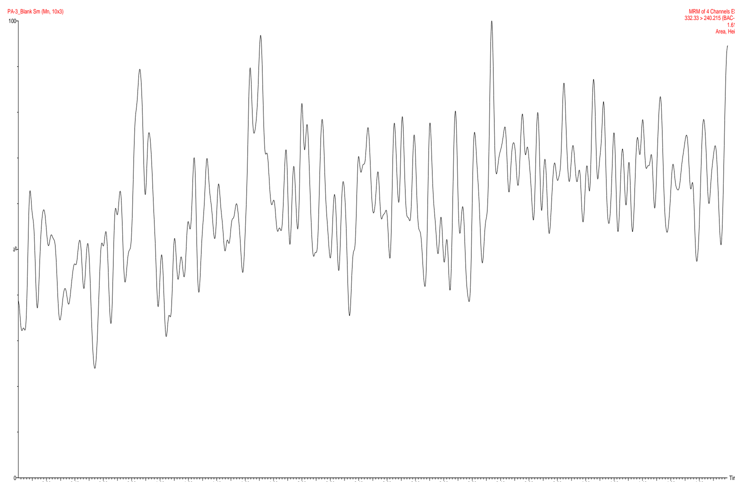
The specificity of the method was evaluated by analyzing six blank (water) samples processed as mentioned in the solution preparation to investigate the potential interferences at the LC peak region for analyte and IS. The acceptance criterion for the experiment was that, at least four out of six lots should have response less than five-times of the LLOQ level response in the same matrix. Figure 2, 3 and 4 shows a representative chromatogram of blank (water free of analyte and IS), and BAC-12, BAC-14 and BAC-16 spiked to water with IS. No interfering peaks were observed at the retention times of analytes and IS. The retention times of BAC-12, BAC-14, BAC-16 and IS was ~1.25, 1.68, 2.46 and 0.99 min, respectively.

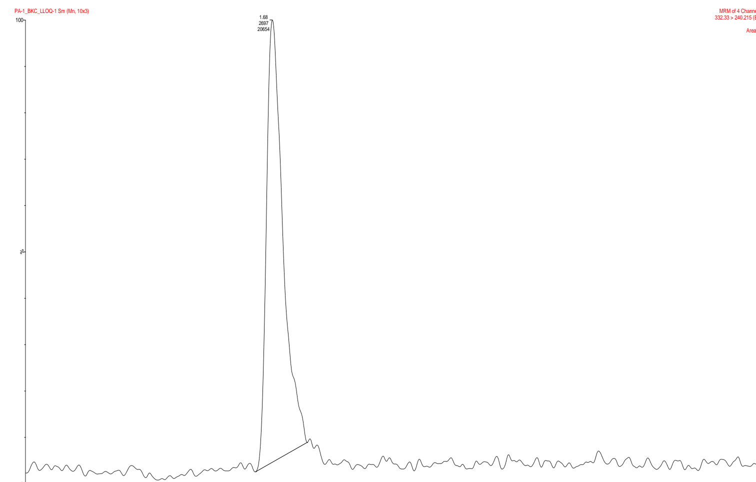




(A) **(B)**

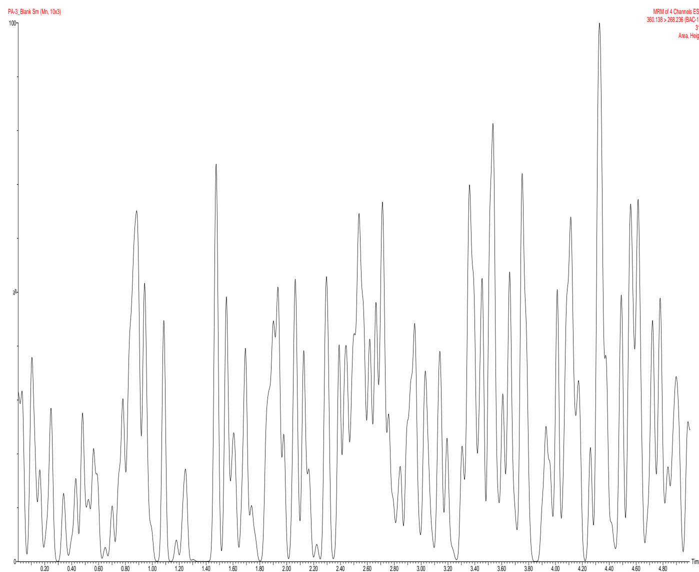
Figure 2: Representative Chromatogram of (a) Blank and (b) Benzalkonium chloride C-12 (5.07 ng/mL) at 304.202 > 212.155 MRM



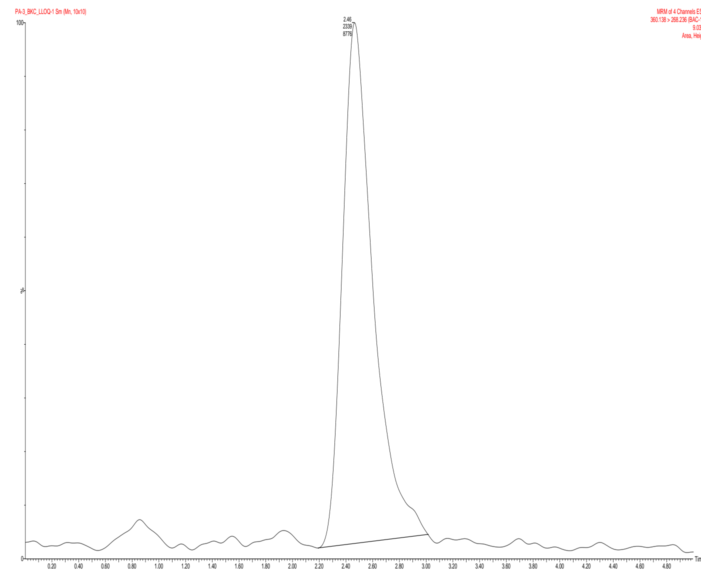


(A) **(B)**

Figure 3: Representative Chromatogram of (a) Blank and (b) Benzalkonium chloride C-14 (5.073 ng/mL) at 332.33 > 240.215 MRM



(A)



(B)

Figure 4: Representative Chromatogram of (a) Blank and (b) Benzalkonium chloride C-16 (5.122 ng/mL) at 360.138 > 268.236 MRM

ii. Extraction Recovery

Recovery of BAC and IS from water was determined by comparing the response of analytes and IS extracted from replicate QC samples (n = 6) with the response of analytes and IS from neat standards at the same concentration. Recovery of BAC was determined at LQC, MQC and HQC concentrations. The water evaporated using Turbovap® LV and reconstituted with acetonitrile was adequate to achieve recovery and cleaner samples. The results of the comparison of neat standards versus samples evaporated and reconstituted standards were estimated for BAC-12, BAC-14 and BAC-16.

Table 4: Mean recovery of BAC-12, BAC-14 and BAC-16 from the water

Sample ID	Percent Recovery ± SD		
	C-12	C-14	C-16
LQC	100 ± 12.2	109 ± 6.30	101 ± 7.24
MQC	100 ± 2.40	97.9 ± 2.91	98.6 ± 3.11
HQC	100 ± 1.63	99.0 ± 1.80	101 ± 1.82

iii. Calibration curve

The eight-point calibration curve was constructed by plotting the peak area ratio of Analyte: IS against the nominal concentration of calibration standards in water. Following the evaluation of different weighing factors, the results were fitted to linear regression analysis with the use of 1/X2 (X = concentration) weighing factor. The calibration curve with a correlation coefficient (r) of 0.99 or better was considered. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20%. The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. CC was prepared by determining the best fit of peak area ratios (peak area analyte/peak area IS) versus concentration, and fitted to the y = mx + c using weighing factor (1/X2). The average regression (n = 4) of Calibration curve was found to be >0.993, >0.996 and >0.998 for BAC-12, BAC-14 and BAC-16, respectively. The % accuracy observed for the mean of back-calculated concentrations for four CCs for BAC-12, BAC-14 and BAC-16 was within 88.2 to 113, 90.5 to 112 and 92.3 to 106, respectively. The BAC-12, BAC-14 and BAC-16 precision (%CV) values ranged from 0.79 to 7.28, 1.25 to 6.79 and 1.03 to 7.37, respectively.

iv. Precision & accuracy

The intra-day assay precision and accuracy were estimated by analyzing six replicates containing BAC-12, BAC-14 and BAC-16 at four different QC levels. The inter-day assay precision was determined by analyzing the four QC level samples on four different runs. The criteria for acceptability of the data included accuracy within 85–115% from the nominal values and a precision of within ±15% relative standard deviation (RSD) except for LLOQ, where it should be within 80–120% for accuracy and less than 20% of RSD. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

Table 5: Intra-day precision of Analytes in water.

Nominal Concentration (ng/ml)	Day	BAC-12		BAC-14		BAC-16	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
LLOQ	1	7.20	104	6.69	107	3.60	103
	2	12.7	101	18.5	100	14.1	88.4
	3	2.72	107	10.3	98.6	12.1	100
	4	6.63	88.3	6.58	104	5.42	112
LQC	1	7.51	100	1.69	105	4.27	101
	2	3.33	105	4.68	105	4.68	105
	3	5.00	96.7	6.62	104	2.85	100
	4	5.54	93.5	3.75	100	2.31	101
MQC	1	4.92	100	5.54	99.5	5.75	100
	2	4.18	107	4.99	104	4.99	104
	3	5.13	104	5.76	103	5.99	102
	4	4.51	103	4.53	102	4.42	101
HQC	1	8.06	99.2	7.82	102	5.40	102
	2	4.10	103	4.39	105	4.39	105
	3	3.59	100	3.30	102	4.41	105
	4	5.68	102	6.24	105	5.94	105

Table 6: Inter-day precision of Analytes in water.

Nominal Concentration (ng/ml)	Run	BAC-12		BAC-14		BAC-16	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
LLOQ	1	10.7	99.1	10.8	103	10.7	102
LQC	2	6.73	99.0	4.50	103	3.76	101
MQC	3	5.05	104	5.16	102	5.21	102
HQC	4	5.42	101	5.51	104	4.75	104

v. Stability experiments

The stability of analyte in the injection solvent was determined periodically by injecting replicate preparations of processed samples for up to 24 h (in the autosampler at 10°C) after the initial injection. The peak areas of the analyte and IS obtained at initial cycle were used as reference to determine the stability at subsequent points. Stability of BAC-12, BAC-14 and BAC-16 in water over 8 h (benchtop) was determined at ambient temperature (25 ± 2°C) at LQC and HQC concentrations in six replicates. The samples were processed using the same procedure as described in the sample preparation section. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e., 85–115% of nominal value) and precision (i.e., ±15% RSD) as compared with freshly spiked (0 h) samples. The predicted concentrations for BAC-12, BAC-14 and BAC-16 samples deviated within ±15% of the nominal concentrations in an in-injector (24 h) and benchtop (8 h). The results (Table 7, 8 & 9) were found to be within the assay variability limits during the entire process.

Table 7: BAC-12 Stability.

Nominal Concentration (ng/ml)	In-Injector Stability (24 h) ng/mL			Benchtop Stability (8 h) ng/ml		
	Mean ± SD	Accuracy (%)	CV (%)	Mean ± SD	Accuracy (%)	CV (%)
LQC	15.2 ± 0.83	91.1	5.47	14.9 ± 1.38	105	9.24
HQC	689 ± 12.0	88.1	1.74	744 ± 31.4	95.8	4.23

Table 8: BAC-14 Stability.

Nominal Concentration (ng/ml)	In-Injector Stability (24 h) ng/ml			Benchtop Stability (8 h) ng/ml		
	Mean ± SD	**Accuracy (%)**	**CV (%)**	**Mean ± SD**	**Accuracy (%)**	**CV (%)**
LQC	15.8 ± 1.91	100	12.1	15.9 ± 0.55	101	3.45
HQC	691 ± 16.3	86.3	2.36	760 ± 29.1	95.4	3.83

Table 9: BAC-16 Stability.

Nominal Concentration (ng/ml)	In-Injector Stability (24 h) ng/ml			Benchtop Stability (8 h) ng/ml		
	Mean ± SD	**Accuracy (%)**	**CV (%)**	**Mean ± SD**	**Accuracy (%)**	**CV (%)**
LQC	13.9 ± 0.56	86.8	4.04	15.6 ± 0.25	88.4	1.61
HQC	710 ± 16.1	87.7	2.27	785 ± 30.2	96.0	3.84

2. IN VITRO SKIN PERMEATION STUDIES

The experiments were performed using Franz diffusion cells across human cadaver skin model.

2.1 In vitro permeation protocol

i. Franz-diffusion cells

Franz diffusion cells used for the permeation studies consisted of two borosilicate glass components namely; the donor compartment and the jacketed receiver compartment. Human cadaver skin placed between the rubber washers was fixed in between the donor and the receiver compartments. The pinch clamp tightly seals the two chambers with the help of rubber washers. The sample for which permeation was to be tested is applied uniformly on the human cadaver skin. The skin surface was maintained at 32±1°C with the help of heat regulator and circulator which circulates water in the jacket present around the receiver compartment. Magnetic stir bar was used to mix the solution in the receiver compartment.

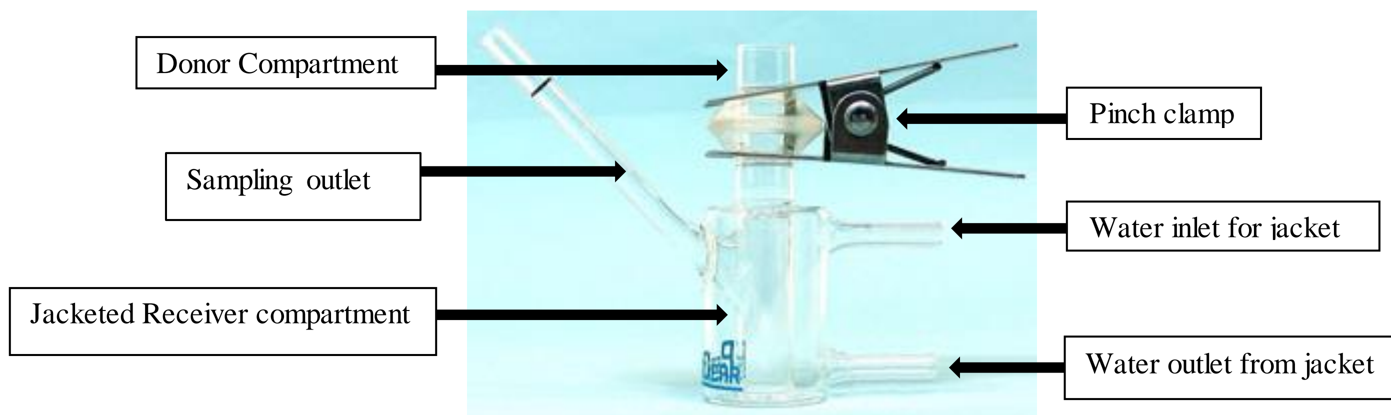


Figure 5: Typical Franz Diffusion Cell

ii. Washing protocol of Franz diffusion cells

Prior to the use of Franz diffusion cells for IVPT studies of FiteBac and poloxomer gel; the magnetic stir bars, rubber washers, donor and receiver compartments were washed alternatively with 50% methanol and de-ionized water. Later, they were sonicated with de-ionized water for 90 min and which was replaced with fresh de-ionized water for every 30 min. All the components were rinsed after sonication and any water remaining in the receiver compartment was completely drained out. The samples from final washings were subjected to LC-MS/MS analysis for confirmation of scrupulous washing.

iii. Skin mounting and resistance measurement

The experimental protocol involved the usage of a set of vertical Franz diffusion cells consisting of donor and receiver chambers. The human cadaver dermatomed skin was used as permeation membrane. Cryo-preserved human cadaver dermatomed skin devoid of any disease was obtained from Firefighter's skin bank and was stored in water impermeable plastic bags at below -70°C until the time of use. Prior to use, the skin was thawed at room temperature for 5 min and was cut in sections large enough to fit 2 cm^{2} active permeation area. The skin barrier integrity was checked before starting the experiment by measuring the resistance at a frequency of 10 Hz and low voltage of 100 mV. While checking integrity, phosphate buffer was used in the donor compartment and skin resistance was measured by placing electrodes in the donor and the sampling compartment. The skin having resistance value greater than 10 KΩ/cm2 was used for permeation studies.

2.2 Characterization of BAC coponents present in the Benzalkonium chloride used in the product (McKinley Resources inc)

The Benzalkonium chloride (McKinley Resources inc) was weighed and dissolved in methanol to prepare a stock solution of 1 mg/ml. The percentage of BAC-12, BAC-14 and BAC-16 was estimated by spiking 5 µl of stock solution and 20 µl of IS to an aliquot of 1995 µl of water. This mixture was vortex-mixed on a cyclo-mixer for 3 min and dried under a gentle stream of nitrogen at 50℃ using automated evaporation system. The dried residue was reconstituted in 200 µl of acetonitrile, vortex-mixed and centrifuged at 13,000 rpm for 5 min. The supernatant was

transferred into vials and injected into LC-MS/MS for analysis. The calibration curve (CC) for the estimation of percentage consisted of at least 6 non-zero calibration standards along with a blank and blank with internal standard samples, Study samples were analyzed along with interspersed quality control samples (low, medium and high QC samples).

Results

The benzalkonium chloride (McKinley Resources inc) consists of 52.6 %, 19.1 % and 0.26 % of BAC-12, BAC-14 and BAC-16, respectively. There was not BAC10 or BAC18 detected.

2.3 Preparation of Control product (0.1% Benzalkonium chloride-Poloxamer Gel)

10 mg of benzalkonium chloride was dissolved in 7 ml of deionized water present in a glass vial. 1.7 gm of poloxamer 407 was added into this solution and the total content was made up to 10 gm with addition of deionized water. The glass vial was placed in refrigerator overnight at 5°C to facilitate poloxamer dissolution in the solution. The clear solution was stirred with the help of spatula and kept in a water bath maintained at 32 ^{o}C for formation of gel.

2.4 Single loading of multiple doses

288 ± 2 mg of either FiteBac or Poloxomer gel was weighed and applied on the skin with the help of spatula and the dose was spread uniformly across the skin surface. Later, the drug loaded skin was placed on to the Franz cell and the donor chamber was placed on it and fixed tightly with a clamp.

2.5 In vitro Maximum Usage Trials

2 ± 0.2 mg equivalent of either FiteBac or Poloxomer gel was pipetted on to the skin surface and was spread uniformly with the help of the HPLC glass vial for every 5 min up to 12 hr.

2.6 Validation of dosing method

Eppendorf Research Plus pipette was used to dispense the dose on the skin. The pipette tip was cut to facilitate the ease dispensing. The dose pipetted was validated by optimizing the weight that was dispensed on the HPLC vial. Initially, an approximate dial reading was set in the pipette and the weight of the gel dispensed was measured. Based on the weight dispensed, the dial value was adjusted so as to dispense 2 ± 0.2 mg of gel on each discharge. The dose dispensed with set reading was checked for 5times for both the control and the test products.

Table 10: **Validation of dosing method**

S.no	**Quantity dispensed (mg)**	
	FiteBac	**Poloxomar Control**
1.	2.2	1.9
2.	1.9	2.1
3.	2.2	2.0
4.	2.1	1.8
5.	2.2	2.0
Avg	2.08	1.96
% C.V.	6.3	5.8

2.7 Sampling

The receptor solution was completely sampled and replaced with fresh de-ionized water at 0 h, 4 h, 8 h, 12 h, 16 h, 20 h and 24 h.

2.8 Sample preparation for analysis

The sample from receiver compartment were vortex-mixed on a cyclo-mixer for 3 min and to 2 mL of this sample, 20 µl of IS was spiked. The 2 mL of sample containing IS was dried under a gentle stream of nitrogen at 50℃ using automated evaporation system. The dried residue was reconstituted in 200 µl of acetonitrile, vortex-mixed and centrifuged at 13,000 rpm for 5 min (Centrifuge 5430 R, Eppendorf, Germany). The supernatant was transferred into vials and injected into LC-MS/MS for analysis. The calibration curve (CC) for the sample analysis consisted of at least 6 non-zero calibration standards along with a blank and blank with internal standard samples, Study samples were analyzed along with interspersed quality control samples (low, medium and high QC samples).

3. RESULTS

Single loading of multiple doses

Table 11: In Vitro permeation of BAC-12 (ng/7mL/2cm^2/4 h) from FiteBac gel across cryo-preserved human cadaver dermatomed skin

Cell no.	0 h	4 h	8 h	12 h	Cumulative
1	BLQ	BLQ	BLQ	BLQ	--
2	LQ	BLQ	BLQ	BLQ	--
3	BLQ	BLQ	BLQ	18.3	18.3
4	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	BLQ	BLQ	21.0	21.0
6	BLQ	BLQ	BLQ	BLQ	--

Table 12: In Vitro permeation of BAC-12 (ng/7mL/2cm^2/4 h) from poloxamer gel across cryo-preserved human cadaver dermatomed skin

Cell no.	0 h	4 h	8 h	12 h	Cumulative
1	BLQ	BLQ	BLQ	22.1	22.1
2	BLQ	BLQ	38.4	55.7	94.1
3	BLQ	BLQ	26.3	19.3	45.6
4	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	BLQ	BLQ	BLQ	BLQ
6	BLQ	BLQ	49.8	71.2	121.0

Table 13: In Vitro permeation of BAC-12 (ng/7mL/2cm^2/4 h) from aqueous solution of BAC (0.1%w/v) across cryo-preserved human cadaver dermatomed skin

Cell no.	0 h	4 h	8 h	12 h	Cumulative
1	BLQ	BLQ	11.5	22.1	33.6
2	BLQ	BLQ	28.4	65.9	94.3
3	BLQ	BLQ	36.5	49.3	85.8
4	BLQ	BLQ	38.8	42.3	81.1
5	BLQ	BLQ	46.7	112.8	159.5
6	BLQ	BLQ	62.1	28.9	91.0

BAC14 and BAC 16 were not detectable in the receiver compartment samples. Either they were BLQ or did not transport across the skin.

Maximum Usage Protocol

Table 14: In Vitro permeation of BAC-14 (ng/7mL/2cm^2/4 h) from FiteBac gel across cryo-preserved human cadaver dermatomed skin

Cell no.	0 h	4 h	8 h	12 h	Cumulative
1	BLQ	BLQ	BLQ	BLQ	--
2	BLQ	BLQ	BLQ	BLQ	--
3	BLQ	BLQ	BLQ	BLQ	--
4	BLQ	BLQ	BLQ	BLQ	--
5	BLQ	BLQ	BLQ	BLQ	--
6	BLQ	BLQ	BLQ	BLQ	--

Table 15: In Vitro permeation of BAC-14 (ng/7mL/2cm^2/4 h) from poloxamer gel across cryo-preserved human cadaver dermatomed skin

Cell no.	0 h	4 h	8 h	12 h	Cumulative
1	BLQ	BLQ	BLQ	BLQ	--
2	BLQ	BLQ	BLQ	BLQ	--
3	BLQ	BLQ	BLQ	BLQ	--
4	BLQ	BLQ	BLQ	BLQ	--
5	BLQ	BLQ	BLQ	BLQ	--
6	BLQ	BLQ	BLQ	BLQ	--

Conclusions

The formulation contained C12, C14 and C16 benzalkonium chloride. Only C12 was detected in the receiver compartment. BAC14 and BAC 16 were not detectable in the receiver compartment samples. Either they were BLQ or did not transport across the skin.

In case of Fitebac®, the amount of BAC14 transported was below the limit of detection in most cases except two cells in which some BAC14 was detected only in 12 hour samples in the single application protocol. Whereas in the maximum usage protocol, there was absolutely no BAC14 or any other components of BAC detected even after 12 hours.

The amount of BAC14 transported across the skin in case of custom made gel was relatively higher in single application protocol. However, in case of Maximum usage trials, there was no detectable levels of BAC in the receiver compartment fluid in any of the samples.

These studies clearly indicate that the extent of systemic uptake BAC from the topical products tested in this study would likely be negligible. In vivo studies in animal model or human subjects would give relatively better clarity about the extent of systemic uptake of BAC following topical application following single or frequent administration of the product.

Note: *The above mentioned study was not GLP compliant, however standard procedures and validated methods were used.*

In-vitro permeation studies of Benzalkonium chloride from Aqueous solution (0.13% w/v)

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Report of the Permeation of Benzalkonium chloride from 0.13% aqueous solution

3.1 Preparation of 0.13% Benzalkonium chloride Solution

13 mg of benzalkonium chloride was taken in a glass vial and made up to 10 gm using deionized water. The glass vial was vortexed so as to dissolve benzalkonium chloride completely till a clear solution is formed.

3.2 Single loading study

200 µl was pipetted into the donor compartments of the assembled Franz diffusion apparatus using Eppendorf Research Plus pipette and the solution was spread uniformly by slightly tilting the assembly.

3.3 Multiple application study

2 µl was pipetted into the donor compartments for every 5 min up to the first 20 min and every 10 minutes later till the end of 8 hours. (A total of 50 applications)

3.4 Sampling

The receptor solution was completely sampled and replaced with fresh de-ionized water at 0 h, 4 h and 8 h.

Results

The results of the single loading study is in the table 1 and table 2

Table 1: In Vitro permeation of BAC-12 (ng/7mL/2cm^2/4 h) from aqueous solution of BAC (0.13%w/v) across cryo-preserved human cadaver dermatomed skin.

Trial	BAC-12			
	0h	4h	8h	Cumulative Amount
1	BLQ	BLQ	56.89	56.9
2	BLQ	BLQ	BLQ	-
3	BLQ	BLQ	BLQ	-
4	BLQ	BLQ	37.98	38.0
5	BLQ	BLQ	34.90	34.9
6	BLQ	BLQ	48.20	48.2

Table 2: In Vitro permeation of BAC-14 (ng/7mL/2cm^2/4 h) from aqueous solution of BAC (0.13%w/v) across cryo-preserved human cadaver dermatomed skin

Trial	BAC-14			
	0h	4h	8h	Cumulative Amount
7	BLQ	BLQ	BLQ	-
8	BLQ	BLQ	BLQ	-
9	BLQ	BLQ	BLQ	-
10	BLQ	BLQ	32.36	32.4
11	BLQ	BLQ	43.78	43.8

12	BLQ	BLQ	36.00	36.0

The results clearly indicate the significant amount of BAC 12 permeated across the skin in single load studies in four out of six cells. BAC14 was detectable in three out of six cells.

The results of multiple application study are shown in table 4 and 5.

Table 4: In Vitro permeation of BAC-12 (ng/7mL/2cm^2/4 h) from aqueous solution of BAC (0.13%w/v) across cryo-preserved human cadaver dermatomed skin (Multiple application protocol)

Trial	BAC-12			
	0h	4h	8h	Cumulative Amount
1	BLQ	BLQ	34.90	34.9
2	BLQ	BLQ	38.90	38.9
3	BLQ	28.87	BLQ	28.9
4	BLQ	BLQ	46.77	46.8
5	BLQ	32.11	BLQ	32.1
6	BLQ	BLQ	44.12	44.1

Table 5: In Vitro permeation of BAC-14 (ng/7mL/2cm^2/4 h) from aqueous solution of BAC (0.13%w/v) across cryo-preserved human cadaver dermatomed skin (Multiple application protocol)

Trial	BAC-14 (ng/cm2)			
	0h	4h	8h	Cumulative Amount
7	BLQ	BLQ	BLQ	-
8	BLQ	BLQ	BLQ	-
9	BLQ	BLQ	BLQ	-
10	BLQ	BLQ	48.90	48.9
11	BLQ	BLQ	BLQ	-
12	BLQ	BLQ	42.34	42.3

In the multiple application study, the BAC12 was permeated in all the cells while BAC 14 was found in two out of six cells.