



## Microbiology Final Report

### MINIMUM INHIBITORY DILUTION (MID) AND MINIMUM BACTERICIDAL DILUTION (MBD) ANALYSIS OF HIGH BIOAVAILABLE CPC RINSE FORMULATION AGAINST ORGANISMS IMPLICATED IN GINGIVITIS.

**Study Number:** Addendum 2 to OC 0011 2002

**Study Director:** Nivedita Ramji

**Technical Analyst:** John Barnes

**Documentation:** Laboratory Notebook HCS3400 p.18

**Addendum written on:** 10/31/03

**Work Done On:** October 16<sup>th</sup> 2002 to December 20<sup>th</sup> 2002

**Test Facility:** Health Care Research Center: Microbiology Facility  
8700 Mason-Montgomery Road  
Mason, Ohio 45040-9462

**Test Products:** Crest Gum Care (Batch # HCS 2879-021)  
0.05% High Bioavailable CPC Rinse Formulation  
0.036% CPC Solution prepared in house  
Sterile Water (to be used as negative control)

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#### **PURPOSE:**

The purpose of this experiment was to evaluate the *in vitro* microbial hostility of High Bioavailable Cetylpyridinium Chloride (HBA-CPC), a standard CPC rinse in water and Crest Gum Care™ dentifrice against a battery of organisms well accepted as the representative organisms responsible for or associated with gingivitis. The battery included *Actinomyces viscosus*, *Campylobacter rectus*, *Candida albicans*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Haemophilus actinomycetemcomitans*, *Lactobacillus casei*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Streptococcus sanguinis*, and pooled saliva sample. A minimum inhibitory dilution (MID) and minimum bactericidal dilution (MBD) study was planned for this purpose. The MID / MBD assays have historically provided an appropriate measure of the efficacy of actives / products. The assay is based on the concentration of the test product required to inhibit or kill the representative microorganisms. A lower inhibitory and kill concentration indicates greater efficacy of the test product(s).

#### **BACKGROUND:**

CPC, a quaternary ammonium chloride, is a cationic surface-active agent and has a long heritage as a broad-spectrum antimicrobial against oral bacteria. The CPC molecule has both a positively charged hydrophilic region and a hydrophobic region. The surfaces of bacteria under normal physiological conditions have a net negative charge, which associates with the positively charged CPC region. Subsequently the hydrophobic portion of CPC then inserts into the cell membrane, which causes leakage of cellular components, disruption of bacterial metabolism, inhibition of cell growth, and finally cell death. Common excipients added to commercial oral care formulations such as surfactants can diminish or even completely neutralize the antimicrobial activity of CPC<sup>1,2</sup>. Evaluation of CPC-containing mouthrinses using the *In Vitro* Disk Retention Assay and *Ex Vivo* Plaque Glycolysis methods have been published by P&G in the *J. Clin. Dent.* 8: 107-113, 1997. This publication established that CPC in the experimental mouthrinse formulations prepared in-house were sufficiently available and biologically active to deliver antimicrobial benefits. The formulations studied had 0.025%, 0.05%, 0.075% and 0.1% CPC and showed percentage availability in the range of 72-77%. The activity of these High Bioavailable CPC rinse formulations were compared to commercial rinses including ACT® Fluoride Anti-Cavity Treatment (Johnson and Johnson Products Inc., Skillman, NJ), Cepacol® Mouthwash / Gargle (J. B. Williams Co., GlenRock, NJ), Scope® Mouthwash / Gargle (Procter and Gamble Co., Cincinnati, OH) and a placebo rinse (i.e experimental mouthwash

formulation minus CPC). The three commercial rinses showed percentage availability for CPC of 4%, 54%, and 38%, respectively. From the published analyses, it was apparent that formulations with higher available CPC are associated with greater biological activity as measured by plaque glycolysis, and suggest that these formulations would have a higher probability of showing clinical efficacy.

The FDA Plaque Subcommittee, after a series of meetings (from 1993 to 1998) in which they reviewed 40 actives, provided their report to the FDA with recommendations that only three Class I (Safe and Efficacious) actives: cetylpyridinium chloride, a fixed combination of essential oils, and stannous fluoride were entitled to make anti-gingivitis and anti-plaque claims under the monograph rule-making process. Based on this it was thought to be a reasonable approach to generate microbiocidal data for both CPC and stannous fluoride<sup>3</sup>. The current investigation was designed to dimensionalize the antimicrobial activity of a 0.05% HBA-CPC rinse formulation and a stannous dentifrice against organisms causing gingivitis in support of the claim "Fights Germs that Cause Gingivitis."

## **REQUIREMENTS:**

### **Test products**

#### **0.05% High Bioavailable CPC Rinse Formulation**

##### Preparation:

0.053g CPC was dissolved per 100 mL of water (this accounts for the 95% purity of CPC used in the making of this formulation). Product was prepared in the PRLO and dispensed in approximately 500 mL volumes in amber colored bottles (Lot# HCS 723-067).

#### **0.036% CPC Solution prepared in house**

##### Preparation:

This solution was prepared fresh before use in each experiment. Each experiment used 50 mL volume of the standard CPC solution; 0.0189g of CPC (M# 10045571, batch #12) was dissolved in 50 mL sterile water (this accounts for the 95% purity of CPC used in the making of this solution).

#### **sterile water (to be used as negative control)**

#### **Crest Gum Care™**

##### Preparation:

The slurry was prepared fresh before use in each experiment. 5g of the dentifrice was weighed out in a sample cup. 45 mL sterile diluent was added to the weighed dentifrice and the contents were vortexed at high speed until no solid pieces of the dentifrice remained (vortexing done typically for 3-5 minutes). The slurry was subsequently centrifuged at 1,500 rpm for 15 minutes and the supernatant was used in the MID / MBD assays.

(Product Batch # for Crest Gum Care: HCS2879-021; Master formula # HCS887-155; this dentifrice formulation contains 0.454% SnF<sub>2</sub>).

### **Test organisms:**

#### ***Actinomyces viscosus* ATCC 19246**

Propagation: This organism was rehydrated and grown in Brain Heart Infusion Broth (BHI, Anaerobe Systems Catalog # AS-8725) at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) for 24 hrs before exposure to the MID / MBD assays.

Assay: The assay medium for MID was BHI. After inoculation of the test organism in the assay medium containing dilutions of the product(s), the tubes were incubated at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) for 24 hrs before being read visually for turbidity. 10 µL of the tubes showing no turbidity were plated onto BBA and further incubated at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) prior to being read for growth (MBD).

#### ***Campylobacter rectus* ATCC 33238**

Propagation: This organism was rehydrated and grown in Peptone Yeast Extract with Glucose, (PYG), with Formate and Fumarate (Anaerobe Systems Catalog # AS-8585) anaerobically at 36°C for 24-48 hrs before exposure to the MID / MBD assays.

Assay: The assay medium for MID was PYG. After inoculation of the test organism in the assay medium containing dilutions of the product(s), the tubes were incubated at 36°C under anaerobic conditions for 24-48 hrs before being read

visually for turbidity. 10  $\mu$ L of the tubes showing no turbidity were plated onto BBA and further incubated at 36°C under anaerobic conditions prior to being read for growth (MBD).

#### ***Candida albicans* ATCC 10231**

Propagation: This organism was rehydrated and grown in Yeast and Mold Broth (YM, Difco 271120; 0711-17; prepared in house) at 23°C under aerobic conditions for 24 hrs before exposure to the MID / MBD assays.

Assay: The assay medium for MID was YM. After inoculation of the test organism in the assay medium containing dilutions of the product(s), the tubes were incubated at 23°C under aerobic conditions for 24 hrs before being read visually for turbidity. 10  $\mu$ L of the tubes showing no turbidity were plated onto PDA and further incubated at 23°C under aerobic conditions prior to being read for growth (MBD).

#### ***Eikenella corrodens* ATCC 23834**

Propagation: This organism was rehydrated and grown in Brain Heart Infusion Broth (BHI, Anaerobe Systems Catalog # AS- 8725) supplemented with 16.7% filter sterilized Fetal Bovine Serum at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) for 48 hrs before exposure to the MID / MBD assays.

Assay: The assay medium for MID was BHI-Serum. After inoculation of the test organism in the assay medium containing dilutions of the product(s), the tubes were incubated at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) for 48 hrs before being read visually for turbidity. 10  $\mu$ L of the tubes showing no turbidity were plated onto BBA and GC Agar (BBL 211275 prepared in-house) and further incubated at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) prior to being read for growth (MBD).

#### ***Fusobacterium nucleatum* ATCC 10953**

Propagation: This organism was rehydrated and grown in Brain Heart Infusion Broth (BHI, Anaerobe Systems Catalog # AS-8725) at 36°C under anaerobic conditions for 24 hrs before exposure to the MID / MBD assays.

Assay: The assay medium for MID was BHI. After inoculation of the test organism in the assay medium containing dilutions of the product(s), the tubes were incubated at 36°C under anaerobic conditions for 24 hrs before being read visually for turbidity. 10  $\mu$ L of the tubes showing no turbidity were plated onto BBA and further incubated at 36°C under anaerobic conditions prior to being read for growth (MBD).

#### ***Haemophilus actinomycetemcomitans* ATCC 29522**

Propagation: This organism was rehydrated and grown in Brain Heart Infusion Broth (BHI, Anaerobe Systems Catalog # AS-8725) at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) for 24-48 hrs before exposure to the MID / MBD assays.

Assay: The assay medium for MID was BHI. After inoculation of the test organism in the assay medium containing dilutions of the product(s), the tubes were incubated at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) for 24-48 hrs before being read visually for turbidity. 10  $\mu$ L of the tubes showing no turbidity were plated onto BHI Agar and further incubated at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) prior to being read for growth (MBD).

#### ***Lactobacillus casei* ATCC 393**

Propagation: This organism was rehydrated and grown in Lactobacilli MRS Broth (Difco 0881; prepared in-house) at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) for 48 hrs before exposure to the MID / MBD assays.

Assay: The assay medium for MID was MRS Broth. After inoculation of the test organism in the assay medium containing dilutions of the product(s), the tubes were incubated at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) for 48 hrs before being read visually for turbidity. 10  $\mu$ L of the tubes showing no turbidity were plated onto MRS Agar and further incubated at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) prior to being read for growth (MBD).

#### ***Porphyromonas gingivalis* ATCC 33277**

Propagation: This organism was rehydrated and grown in Brain Heart Infusion Broth (BHI, Anaerobe Systems Catalog # AS-8725) anaerobically at 36°C for 48 hrs before exposure to the MID/MBD assays.

Assay: The assay medium for MID was BHI. After inoculation of the test organism in the assay medium containing dilutions of the product(s), the tubes were incubated at 36°C under anaerobic conditions for 48 hrs before being read visually for turbidity. 10  $\mu$ L of the tubes showing no turbidity were plated onto BBA and further incubated at 36°C under anaerobic conditions prior to being read for growth (MBD).

#### ***Prevotella intermedia* ATCC 25611**

Propagation: This organism was rehydrated and grown in 1:1 Chopped Meat Carbohydrate Medium (CMCM, Anaerobe Systems Catalog # AS-823) and Chopped Meat Medium (CMM Anaerobe Systems Catalog # AS-811) at 36°C under anaerobic conditions for 24 hrs before exposure to the MID / MBD assays.

**Assay:** The assay medium for MID was the same as the propagation medium. After inoculation of the test organism in the assay medium containing dilutions of the product(s), the tubes were incubated at 36°C under anaerobic conditions for 24 hrs before being read visually for turbidity. 10 µL of the tubes showing no turbidity were plated onto BBA and further incubated at 36°C under anaerobic conditions before being read for growth (MBD).

#### ***Pseudomonas aeruginosa* ATCC 27853**

**Propagation:** This organism was rehydrated and grown in Nutrient Broth (Difco 0003-17-8; prepared in house) aerobically at 33°C for 24 hrs before exposure to the MID / MBD assays.

**Assay:** The assay medium for MID was Nutrient Broth. After inoculation of the test organism in the assay medium containing dilutions of the product(s), the tubes were incubated at 33°C under aerobic conditions for 24 hrs before being read visually for turbidity. 10 µL of the tubes showing no turbidity were plated onto TSA and further incubated at 33°C under aerobic conditions prior to being read for growth (MBD).

#### ***Streptococcus mutans* ATCC 35668**

**Propagation:** This organism was rehydrated and grown in Brain Heart Infusion Broth (BHI, Anaerobe Systems Catalog # AS-872) at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) for 24 hrs before exposure to the MID / MBD assays.

**Assay:** The assay medium for MID was BHI. After inoculation of the test organism in the assay medium containing dilutions of the product(s), the tubes were incubated at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) for 24 hrs before being read visually for turbidity. 10 µL of the tubes showing no turbidity were plated onto TSA and further incubated at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) for 24 hrs prior to being read for growth (MBD).

#### ***Streptococcus sanguinis* ATCC 10556**

**Propagation:** This organism was rehydrated and grown in Brain Heart Infusion Broth (BHI, Anaerobe Systems Catalog # AS-8725) at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) for 24 hrs before exposure to the MID / MBD assays.

**Assay:** The assay medium for MID was BHI. After inoculation of the test organism in the assay medium containing dilutions of the product(s), the tubes were incubated at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) for 24 hrs before being read visually for turbidity. 10 µL of the tubes showing no turbidity were plated onto TSA and further incubated at 36°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) prior to being read for growth (MBD).

#### **Pooled saliva sample**

Stimulated saliva was collected from six subjects (15 mL per subject). Equal volumes from each were combined to produce pooled stimulated whole saliva. Pooled saliva was spun down at 1500 rpm for 15 minutes and subjected to the MID / MBD assays using Tryptic Soy Broth and Tryptic Soy Agar for MID and MBD respectively.

**Assay:** The assay medium for MID was TSB. After inoculation of the test organism in the assay medium containing dilutions of the product(s), the tubes were incubated at 33°C under aerobic conditions for 24 hrs before being read visually for turbidity. 10 µL of the tubes showing no turbidity were plated onto TSA and further incubated at 33°C under aerobic conditions prior to being read for growth (MBD).

#### **Equipment:**

1. Analytical Balance, Mettler, Serial # F45925 (HC 2211), located in DV1 404
2. Vortex Genie 2, #1, VWR Scientific Model G-560, Serial # 2-220005, located in DV1 614
3. Vortex Genie 2, #2, Fischer Scientific Model G-560, Serial # 2-138021, located in DV1 614
4. Walk-In 33°C Incubator, 60% RH, located in DV1-407
5. Walk- In 23°C Incubator, 60% RH, located in DV1-409
6. Incubator, VWR Scientific CO<sub>2</sub> Incubator, Model # 2300, HC 00003150
7. VWR Multi-Tube Vortexer, Serial # 0375, Catalog # 58816-115
8. Anaerobic Chamber, Coy Model AALC-3-DOOR, Serial # AC023450, located in DV1-404

#### **Lab Ware:**

1. Sample cups (120mL volume)
2. Calibrated Eppendorf pipettes
3. Eppendorf pipette tips, Catalog # 2249006-1 (for 1 and 2.5 mL)
4. Sterile glass tubes for preparing dilutions
5. 50 mL serological pipettes (VWR 53283-712)
6. 10 mL serological pipettes (VWR 53283-708)
7. 5 mL serological pipettes (VWR 53283-706)

**Design:**

1. MID media was used to make dilutions of all the products tested in this study. The dilutions were prepared in sterile glass tubes and the dilution chart is included in the subsequent section.
2. Each tube contained 5 mL of the product dilution in broth medium. Appropriate negative and positive controls were maintained in the experiment. The negative control contained plain 5 mL sterile broth while the positive control had the test culture inoculated into 5 mL broth medium without any test product.
3. Test Cultures: 12 representative organisms implicated in gingivitis and pooled saliva were used as the inoculum in this study. For pooled saliva, equal volumes of stimulated saliva from 6 subjects were combined and centrifuged at 1500 rpm for 15 minutes. The supernatant was used to inoculate all the tubes except the negative control. In case of the representative organisms a 24 to 48 hr old broth culture was used to inoculate the MID tubes. Each 5 ml dilution of the test product and the positive control was inoculated with 100  $\mu$ L of the test culture and the tubes incubated under desired growth conditions.
4. After appropriate incubation time all the tubes were visually examined for growth based on the turbidity they exhibit. The lowest concentration (the most dilute tube) of the test product which showed absence of growth / lack of turbidity was considered as the Minimum Inhibitory Dilution (MID) for the test product.
5. A 10  $\mu$ l-aliquot of each dilution showing no growth (no turbidity) was spread-plated onto an agar medium appropriate for the test culture, incubated under desired growth conditions, and examined for bacterial growth (viability). Additionally, 10  $\mu$ l aliquots of some of the dilutions that indicated growth (turbidity) were spread-plated onto an appropriate agar medium to serve as positive controls. The least concentrated (most dilute) sample of test product exhibiting absence of growth on the agar medium was considered to be the Minimum Bactericidal Dilution (MBD) or the Minimum Killing Dilution (MKD) for that test product. Comparisons of the MID / MBD values are made between different products to determine their microbial hostility.

**Methodology:**

**The dilution chart is as follows:**

Dilution	Use	Total Volume in mL	Vol. Stock in mL	Vol. broth diluent in mL	Discard Vol. in mL
1:10	1:10	5.0	5.0	0.0	0
1:20	1:10	5.0	2.5	2.5	0
1:50	1:10	5.0	1.0	4.0	0
1:100	1:10	5.0	0.5	4.5	0
1:150	1:100	6.0	4.0	2.0	1.0
1:200	1:100	5.0	2.5	2.5	0
1:250	1:100	5.0	2.0	3.0	0
1:300	1:100	6.0	2.0	4.0	1.0
1:350	1:100	7.0	2.0	5.0	2.0
1:400	1:100	8.0	2.0	6.0	3.0
1:450	1:100	9.0	2.0	7.0	4.0
1:500	1:100	5.0	1.0	4.0	0
1:550	1:100	5.5	1.0	4.5	0.5
1:600	1:100	6.0	1.0	5.0	1.0
1:800	1:100	8.0	1.0	7.0	3.0
1:1000	1:100	5.0	0.5	4.5	0

1:10 (45 mL of the broth diluent + 5 mL / grams of the product made 50 mL of stock of the 1:10 dilution of the product) and 1:100 (63 mL of the broth diluent + 7 mL of the 1:10 dilution made 70 mL of stock of the 1:100 dilution of the product) dilutions were prepared in larger volumes in sample cups since they were used as master / stock concentrations for subsequent dilutions as is mentioned in the dilution chart above.

The detailed methodology is as below:

- a. The above mentioned dilutions were prepared using MID broth as the diluent and dispensed as 5.0 ml of the various dilutions of MID broth containing test product into sterile test tubes.
- b. The tubes of MID broth-test product were inoculated with 100 µl of the test culture.
- c. As a positive control, a 5.0 ml tube of sterile MID broth containing no product was inoculated with the test organism.
- d. All the tubes were incubated under desired growth conditions (specified in the previous section on test organisms) and examined for growth. Turbidity (+/-) for each dilution was recorded. For each product, the highest dilution (lowest concentration) of product showing no turbidity was deemed to be the Minimum Inhibitory Dilution (MID) for the product towards the tested culture.
- e. As a measure of the cidal potential of each test product, 10 µl of post-incubation sample from MID medium / Product / Bacteria mix showing no turbidity was plated onto MBD medium and incubated under desired growth conditions.
- f. The agar plates were examined for microbial growth after an appropriate incubation period. No growth on MBD medium was interpreted as meaning that no organisms remained viable in the MID medium / Product / Bacteria mix, but had been killed by exposure to the test product. For each test product the highest dilution (lowest concentration) of the product showing no growth on MBD medium was deemed to be the Minimum Bactericidal Dilution for the dentifrice towards the tested culture (MBD).
- g. Each test was run in triplicate. In most cases the consistent value out of three runs was reported as the MID/MBDs. However in few cases where the three values were different the median was reported since the MID/MBDs obtained were within one dilution factor of each other.

#### Observations / Measurements :

Tubes of the MID medium were visually examined for turbidity indicating bacterial growth. Plates of MBD medium were visually examined for bacterial colonies indicating the presence / absence of viable bacteria.

#### **OBSERVATIONS / TEST RESULTS**

Refer to HCL8273 for the data. Tabulated on the following page is the summary of the MID / MBD data after exposure of the representative organism to the test product. The data presented here is an average of three sets.

## FOR 13 MICROORGANISMS

### MID / MBD DATA POST-PRODUCT EXPOSURE FOR REPRESENTATIVE ORGANISMS

Representative Organisms	0.05% HBA-CPC		0.036% CPC solution		Crest Gum Care		Listerine	
	MID	MBD	MID	MBD	MID	MBD	MID	MBD
<i>A. viscosus</i> ATCC19246	1:450	1:100	1:300	1:100	***	1:20	<1:10	<1:10
<i>C. albicans</i> ATCC 10231	1:100	1:50	1:50	1:30	***	1:50	<1:10	<1:10
<i>C. rectus</i> ATCC 33238	1:800	1:300	1:500	1:150	***	1:200	1:20	<1:10
<i>E. corrodens</i> ATCC 23834	<1:10	1:20	<1:10	1:10	***	>1:50	<1:10	1:10
<i>F. nucleatum</i> ATCC 10953	1:550	1:200	>1:1000	1:20	***	1:10	<1:10	<1:10
<i>H. actinomycetem-comitans</i> ATCC 29522	1:800	1:100	1:500	1:100	***	1:20	<1:10	<1:10
<i>L. casei</i> ATCC 393	1:150	<1:10	1:100	<1:10	***	<1:10	<1:10	<1:10
<i>P. intermedia</i> ATCC 25611	1:150	NR	1:100	NR	***	NR	<1:10	NR
<i>P. aeruginosa</i> ATCC 27853	<1:10	<1:10	<1:10	<1:10	***	1:10	<1:10	<1:10
<i>P. gingivalis</i> ATCC 33277	1:450	1:450	1:300	1:350	***	1:350	1:10	1:10
<i>S. sanguinis</i> ATCC 10556	1:450	1:200	1:300	1:150	***	1:450	<1:10	<1:10
<i>S. mutans</i> ATCC 35668	1:300	1:100	1:200	1:100	***	1:150	<1:10	<1:10
Stimulated Whole Saliva	1:300	1:200	1:150	1:100	***	1:50	<1:10	<1:10

\*\*\* The MID values for Crest Gum Care™ are not reported here since it was difficult to distinguish between the product turbidity and the turbidity due to growth of the organism. In this case therefore we would rely on the MBD values to evaluate the antimicrobial efficacy of Crest Gum Care™.

NR Not reported; the MBD values for *Prevotella intermedia* are not reported here because of inconsistencies in the MBD results, i.e. absence of growth on BBA plates for MID dilutions which were distinctly positive, render the MBD's unreliable.

<1:10 means the product did not inhibit the growth of the microorganism even at 1:10 dilution. Lower dilutions have not been performed here.

>1:1000 means the product inhibited the growth of the test organism at the highest dilution tested in this experiment  
The **water control** did not show any inhibition of the test organisms.

## Microbiology Final Report

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**Study Number:** Addendum 3 to OC 0011 2002

**Study Director:** Nivedita Ramji

**Technical Manager:** John Barnes

**Documentation:** HCL8723 p.153

**Work Done On:** July 17, 2003

**Addendum written on:** 10/31/03

**Test Facility:** Health Care Research Center: Microbiology Facility  
8700 Mason-Montgomery Road  
Mason, Ohio 45040-9462

**Test Products:** Crest Gum Care  
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Sterile Water (to be used as negative control)

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#### **PURPOSE:**

The purpose of this experiment is to evaluate the *in vitro* antimicrobial hostility of High Bioavailable CetylPyridinium Chloride (HBA-CPC), a standard CPC rinse in water and Crest Gum Care™ dentifrice against *Salmonella typhimurium*. A minimum inhibitory dilution (MID) and minimum bactericidal dilution (MBD) study was planned for this purpose. The MID / MBD assays have historically provided an appropriate measure of the efficacy of actives / products. The assay is based on the concentration of the test product required to inhibit or kill the representative microorganisms. A lower inhibitory and kill concentration indicates greater efficacy of the test product(s).

#### **BACKGROUND:**

CPC, a quaternary ammonium chloride, is a cationic surface-active agent and has a long heritage as a broad-spectrum antimicrobial against oral bacteria. The CPC molecule has both a positively charged hydrophilic region and a hydrophobic region. The surfaces of bacteria under normal physiological conditions have a net negative charge, which associates with the positively charged CPC region. Subsequently the hydrophobic portion of CPC then inserts into the cell membrane, which causes leakage of cellular components, disruption of bacterial metabolism, inhibition of cell growth, and finally cell death. Common excipients added to commercial oral care formulations such as surfactants can diminish or even completely neutralize the antimicrobial activity of CPC<sup>1,2</sup>. Evaluation of CPC-containing mouthrinses using the *In Vitro* Disk Retention Assay and *Ex Vivo* Plaque Glycolysis methods have been published by P&G in the *J. Clin. Dent.* **8: 107-113, 1997**. This publication established that CPC in the experimental mouthrinse formulations prepared in-house were sufficiently available and biologically active to deliver antimicrobial benefits. The formulations studied had 0.025%, 0.05%, 0.075% and 0.1% CPC and showed percentage availability in the range of 72-77%. The activity of these High Bioavailable CPC rinse formulations were compared to commercial rinses including ACT® Fluoride Anti-Cavity Treatment (Johnson and Johnson Products Inc., Skillman, NJ), Cepacol® Mouthwash / Gargle (J. B. Williams Co., GlenRock, NJ), Scope® Mouthwash / Gargle (Procter and Gamble Co., Cincinnati, OH) and a placebo rinse (i.e experimental mouthwash formulation minus CPC). The three commercial rinses showed percentage availability for CPC of 4%, 54%, and 38%, respectively. From the published analyses, it was apparent that formulations with higher available CPC are associated with greater biological activity as measured by plaque glycolysis, and suggest that these formulations would have a higher probability of showing clinical efficacy.

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approach to generate microbiocidal data for both CPC and stannous fluoride<sup>3</sup>. The current investigation was designed to dimensionalize the antimicrobial activity of a 0.05% HBA-CPC rinse formulation and a stannous dentifrice against organisms causing gingivitis in support of the claim " Fights Germs that Cause Gingivitis."

## **REQUIREMENTS:**

### **Test products**

#### **0.05% High Bioavailable CPC Rinse Formulation**

##### **Preparation:**

0.053g CPC was dissolved per 100 mL of water (this accounts for the 95% purity of the CPC used in the making of this formulation). Product was prepared in the PRLO and dispensed in approximately 500 mL volumes in amber colored bottles (Lot# HCS 723-067).

#### **0.036% CPC Solution prepared in house**

##### **Preparation:**

This solution was prepared fresh before use in each experiment. Each experiment used 50 mL volume of the standard CPC solution; 0.0189g of CPC (M# 10045571, batch #12) was dissolved in 50 mL sterile water (this accounts for the 95% purity of CPC used in the making of this solution).

#### **Sterile water (to be used as negative control)**

#### **Crest Gum Care™**

##### **Preparation:**

The slurry was prepared fresh before use in each experiment. 5g of the dentifrice was weighed out in a sample cup. 45 mL sterile diluent was added to the weighed dentifrice and the contents were vortexed at high speed until no solid pieces of the dentifrice remained (vortexing done typically for 3-5 minutes). The slurry was subsequently centrifuged at 1,500 rpm for 15 minutes and the supernatant was used in the MID / MBD assays.

(Product Batch # for Crest Gum Care: HCS2879-021; Master formula # HCS887-155; this dentifrice formulation contains .454% SnF<sub>2</sub>).

### **Test organisms:**

#### ***Salmonella typhimurium* ATCC13311**

**Propagation:** This organism will be rehydrated and grown in Nutrient Broth (Difco 0003-17-8; prepared in-house) aerobically at 33°C for 24 to 48 hours before being used for the MID/MBD assays.

**Assay:** The assay medium is Nutrient Agar (Difco 213000-001-17; prepared in house). After plating the plates are incubated aerobically at 33°C for 24 to 48 hours before being read in an automated colony counter.

### **Equipment:**

1. Analytical Balance, Mettler, Serial # F45925 (HC 2211), located in DV1 404
2. Vortex Genie 2, #1, VWR Scientific Model G-560, Serial # 2-220005, located in DV1 614
3. Vortex Genie 2, #2, Fischer Scientific Model G-560, Serial # 2-138021, located in DV1 614
4. Walk-In 33°C Incubator, 60% RH, located in DV1-407
5. Walk- In 23°C Incubator, 60% RH, located in DV1-409
6. Incubator, VWR Scientific CO<sub>2</sub> Incubator, Model # 2300, HC 00003150
7. VWR Multi-Tube Vortexer, Serial # 0375, Catalog # 58816-115
8. Anaerobic Chamber, Coy Model AALC-3-DOOR, Serial # AC023450, located in DV1-404

### **Lab Ware:**

1. Sample cups (120mL volume)
2. Calibrated Eppendorf pipettes
3. Eppendorf pipette tips, Catalog # 2249006-1 (for 1 and 2.5 mL)
4. Sterile glass tubes for preparing dilutions
5. 50 mL serological pipettes (VWR 53283-712)
6. 10 mL serological pipettes (VWR 53283-708)
7. 5 mL serological pipettes (VWR 53283-706)

**Design:**

1. MID media was used to make dilutions of all the products tested in this study. The dilutions were prepared in sterile glass tubes and the dilution chart is included in the subsequent section.
2. Each tube contained 5 mL of the product dilution in broth medium. Appropriate negative and positive controls were maintained in the experiment. The negative control contained plain 5 mL sterile broth while the positive control had the test culture inoculated into 5 mL broth medium without any test product.
3. A 24 to 48 hr old broth culture of the test organism was used to inoculate the MID tubes. Each 5 ml dilution of the test product and the positive control was inoculated with 100  $\mu$ L of the test culture and the tubes incubated under desired growth conditions.
4. After appropriate incubation time all the tubes were visually examined for growth based on the turbidity they exhibit. The highest dilution (the lowest concentration) of the test product which showed absence of growth / lack of turbidity was considered as the Minimum Inhibitory Dilution (MID) for the test product.
5. A 10  $\mu$ l-aliquot of each dilution showing no growth (no turbidity) was spread-plated onto an agar medium appropriate for the test culture, incubated under desired growth conditions, and examined for bacterial growth (viability). Additionally, 10  $\mu$ l aliquots of some of the dilutions that indicated growth (turbidity) were spread-plated onto an appropriate agar medium to serve as positive controls. The highest dilution (least concentration) of test product exhibiting absence of growth on the agar medium was considered to be the Minimum Bactericidal Dilution (MBD) or the Minimum Killing Dilution (MKD) for that test product. Comparisons of the MID / MBD values were made between different products to determine their microbial hostility.

**Methodology:****The dilution chart is as follows:**

Dilution	Use	Total Volume in mL	Vol. Stock in mL	Vol. broth diluent in mL	Discard Vol. in mL
1:10	1:10	5.0	5.0	0.0	0
1:20	1:10	5.0	2.5	2.5	0
1:50	1:10	5.0	1.0	4.0	0
1:100	1:10	5.0	0.5	4.5	0
1:150	1:100	6.0	4.0	2.0	1.0
1:200	1:100	5.0	2.5	2.5	0
1:250	1:100	5.0	2.0	3.0	0
1:300	1:100	6.0	2.0	4.0	1.0
1:350	1:100	7.0	2.0	5.0	2.0
1:400	1:100	8.0	2.0	6.0	3.0
1:450	1:100	9.0	2.0	7.0	4.0
1:500	1:100	5.0	1.0	4.0	0
1:550	1:100	5.5	1.0	4.5	0.5
1:600	1:100	6.0	1.0	5.0	1.0
1:800	1:100	8.0	1.0	7.0	3.0
1:1000	1:100	5.0	0.5	4.5	0

1:10 (45 mL of the broth diluent + 5 mL / grams of the product made 50 mL of stock of the 1:10 dilution of the product) and 1:100 (63 mL of the broth diluent + 7 mL of the 1:10 dilution made 70 mL of stock of the 1:100 dilution of the product) dilutions were prepared in larger volumes in sample cups since they were used as master / stock concentrations for subsequent dilutions as is mentioned in the dilution chart above.

The detailed methodology is as below:

- a. The above mentioned dilutions were prepared using MID broth as the diluent and dispensed as 5.0 ml of the various dilutions of MID broth containing test product into sterile test tubes.
- b. The tubes of MID broth-test product were inoculated with 100 µl of the test culture.
- c. As a positive control, a 5.0 ml tube of sterile MID broth containing no product was inoculated with the test organism.
- d. All the tubes were incubated under desired growth conditions (specified in the previous section on test organisms) and examined for growth. Turbidity (+/-) for each dilution was recorded. For each product, the highest dilution (lowest concentration) of product showing no turbidity was deemed to be the Minimum Inhibitory Dilution (MID) for the product towards the tested culture.
- e. As a measure of the cidal potential of each test product, 10 µl of post-incubation sample from MID medium / Product / Bacteria mix showing no turbidity was plated onto MBD medium and incubated under desired growth conditions.
- f. The agar plates were examined for microbial growth after an appropriate incubation period. No growth on MBD medium was interpreted as meaning that no organisms remained viable in the MID medium / Product / Bacteria mix, but had been killed by exposure to the test product. For each test product the highest dilution (lowest concentration) of the product showing no growth on MBD medium was deemed to be the Minimum Bactericidal Dilution for the dentifrice towards the tested culture (MBD).
- g. Each test was run in triplicate. In most cases the consistent value out of three runs was reported as the MID/MBDs. However in few cases where the three values were different the median was reported since the MID/MBDs obtained were within one dilution factor of each other.

#### Observations / Measurements :

Tubes of the MID medium were visually examined for turbidity indicating bacterial growth. Plates of MBD medium were visually examined for bacterial colonies indicating the presence / absence of viable bacteria.

#### **OBSERVATIONS / TEST RESULTS**

Refer to HCL8273 for the data. Tabulated on the following page is the summary of the MID / MBD data after exposure of the representative organism to the test product. The data presented here is an average of three sets.

#### **MID / MBD DATA POST-PRODUCT EXPOSURE FOR REPRESENTATIVE ORGANISMS**

Representative Organisms	0.05% HBA-CPC		0.036% CPC solution		Crest Gum Care		Listerine		Water	
	MID	MBD	MID	MBD	MID	MBD	MID	MBD	MID	MBD
<i>Salmonella typhimurium</i> ATCC13311	1:100	1:20	1:50	1:20	1:20	1:20	1:10	<1:10	<1:10	<1:10