

Determination of "Bioavailable" Cetylpyridinium Chloride in Mouthrinses by an *In Vitro* Disk Retention Assay

References:

SOP HCA 101 – *Guidelines for Analytical Method Validation*

USP 24 – *Validation of Compendial Methods*

ICH Guideline Q2B – *Validation of Analytical Procedures: Methodology*

Scope : This method is designed as a performance assay to analyze solutions containing 0.03% to 0.1% cetylpyridinium chloride (CPC) in aqueous mouthrinses for "bioavailable" CPC.

Principle: "Bioavailable" CPC is the amount of CPC adsorbed to cellulose disks after one minute exposure. The amount of "bioavailable" CPC is thought to correlate with antimicrobial efficacy. The current method is performed by pipetting the mouthrinse on a stack of 2 prewetted cellulose disks, allowing it to equilibrate for 1 minute, centrifuging the stack of disks, and measuring the CPC level in the centrifugate by HPLC with UV detection at 265 nm. This is the amount of non-bioavailable CPC. The "bioavailable" CPC is the difference between the amount of total CPC in the mouthrinse and non-bioavailable CPC.

<u>Reagents</u>	<u>Suggested Type or Source</u> (Equivalent Items May be used)
Ammonium Formate	J.T. Baker (Cat. No. A10699)
Methanol	HPLC grade
Ethanol (absolute)	Aaper
Polysorbate 80 NF	J. T. Baker
Cetylpyridinium Chloride	USP Reference Standard
Water	Distilled, Deionized

<u>Equipment</u>	<u>Suggested Type or Source</u> (Equivalent Items May be used)
Metal Syringe Filter Holder	13 mm Millipore Swinney (Cat. No. XX3001200)
13 mm Cellulose Disks	Whatman AA disks Cat. no. 2017-013 (no substitutions)

<u>Equipment</u>	<u>Suggested Type or Source</u> (Equivalent Items May be used)
Balances	Capable of reading ± 0.01 g and 0.0001 g
Centrifuge Filter	Whatman Vectaspin 3 Centrifuge Filter (Cat. no. 68380005)
Autosampler Vials	Waters low volume HPLC vials
UV Detector	Waters 486
HPLC pump	Waters 625
Autosampler	Waters 717 plus
Column	250 mm X 4.6 mm, 5 micron Whatman Partisil 10 SCX (Cat. no. WC4227-001)
Data Collection	Waters Empower Client/ Server
Timer	Capable of timing minutes and seconds
Parafilm	VWR

Mobile Phase – Dissolve 7.56 g of ammonium formate in 200 mL of water. Dilute to 1000 mL with Methanol and mix with stir bar.

Check Standard – Add (400 mg \pm 20 mg) Polysorbate 80 N.F., 50 mg (\pm 2.5 mg) CPC (adjusted for moisture) and 18 mL of ethanol to a 100 mL volumetric flask. Dilute to volume with water and mix. This solution contains an expected value of 68.5% bioavailable CPC.

Stock Standard – Weigh accurately, approximately 100 mg of CPC, USP reference standard into a 50 mL volumetric flask. Dilute to volume with water. The concentration of CPC is calculated using equation 1.

Calibration Standards – Pipet the appropriate volumes (aliquot of stock standard into volumetric flasks and dilute to the volumes shown below with mobile phase.

<u>Standard</u>	<u>Aliquot (mL)</u>	<u>Final Volume</u> (mL)	<u>Final Conc. (ppm)</u>
Cal 10	0.05	10	10
Cal 25	0.125	10	25
Cal 50	0.25	10	50
Cal 100	0.5	10	100
Cal 200	1	10	200
Cal 400	2	10	400
Cal 600	3	10	600
Cal 800	4	10	800
Cal 1200	6	10	1200

Chromatographic Conditions

Wavelength 265 nm
Flow Rate 1.0 mL/ min
Injection Volume 30 μ L

Sample Preparation and Analysis

Total CPC

The mouthrinse samples are directly injected into HPLC and need no sample preparation

Non-bioavailable CPC

1. Prewet disks Prewet disks - Pipet 200 μ L of water onto stack of 2 cellulose disks that are placed into the bottom half of a metal syringe filter holder (see attachment 1). Cover with parafilm and place in a Vectaspin 3 centrifuge filter with the filter membrane and cap removed (see attachment 2). Exactly 1 minute after the pipetting step, centrifuge for 5 minutes @ 5445 RCF(relative centrifugal force). Discard the centrifugate and place a low volume HPLC vial in bottom of the receiving tube that is the bottom part of the centrifuge filter assembly(see Attachment 1).
2. Sample Preparation of Check Standard and Mouthrinse Sample – Pipet 200 μ L of mouthrinse sample or check standard onto the stack of cellulose disks and immediately cover with parafilm. Exactly 1 minute after the pipetting step, centrifuge for 5 minutes @ 5445 RCF. The centrifugate is placed in an autosampler and is ready for analysis of non-bioavailable CPC. Each sample should be prepared 6 times.

The HPLC system is calibrated for CPC by injecting the calibration standard solutions from lowest to highest concentration. A linear calibration curve is generated from calibration standard concentrations (ppm) and associated peak areas. CPC concentrations in the samples are calculated using the appropriate calibration curve (equation 3). Non- bioavailable CPC concentrations are subtracted from Total CPC concentrations to calculate “Bioavailable CPC” levels (equation 4). “% Bioavailable CPC” is calculated using equation 5. The average “Bioavailable CPC” or “% Bioavailable CPC” values of the 6 replicates are calculated for each sample and reported as one value.

System Suitability

Parameter	Limits
Retention time Precision	%RSD \leq 5% for 5 replicate check standard injections
Peak Area Precision	%RSD \leq 5% for 5 replicate check standard injections
Tailing Factor	T < 2.5
Linearity	The calibration curve looks linear and R \geq 0.990
% Recovery	Average %bioavailable CPC of check standard is 90 – 110% of expected value of 68.5% bioavailable CPC (Equation 6)
Check Standard and Sample Precision	%RSD \leq 20% for 6 replicate sample and check standard preparations

Equations

Equation 1. Concentration of Stock Standard

$$\text{Conc. } (\mu\text{g/mL or ppm}) = W_i (\text{mg}) / 100 \text{ mL} \times 1000 \mu\text{g/mg} \times \text{purity } (\%) / 100\%$$

Equation 2. Concentration of Calibration Standards

$$\text{Conc. Cal Std (ppm)} = \text{Conc. Stock (ppm)} \times \text{Volume Stock (mL)} / \text{Final Volume (mL)}$$

Equation 3. CPC Concentration (Total and Non-Bioavailable)

$$\text{CPC (ppm)} = \frac{(\text{Peak Area}) - \text{Intercept}}{\text{Slope}}$$

Equation 4. Bioavailable CPC Concentration

$$\text{Bioavailable CPC Conc. (ppm)} = \text{Total CPC Conc. (ppm)} - \text{Non-Bioavailable CPC Conc. (ppm)}$$

Equation 5. % Bioavailable CPC

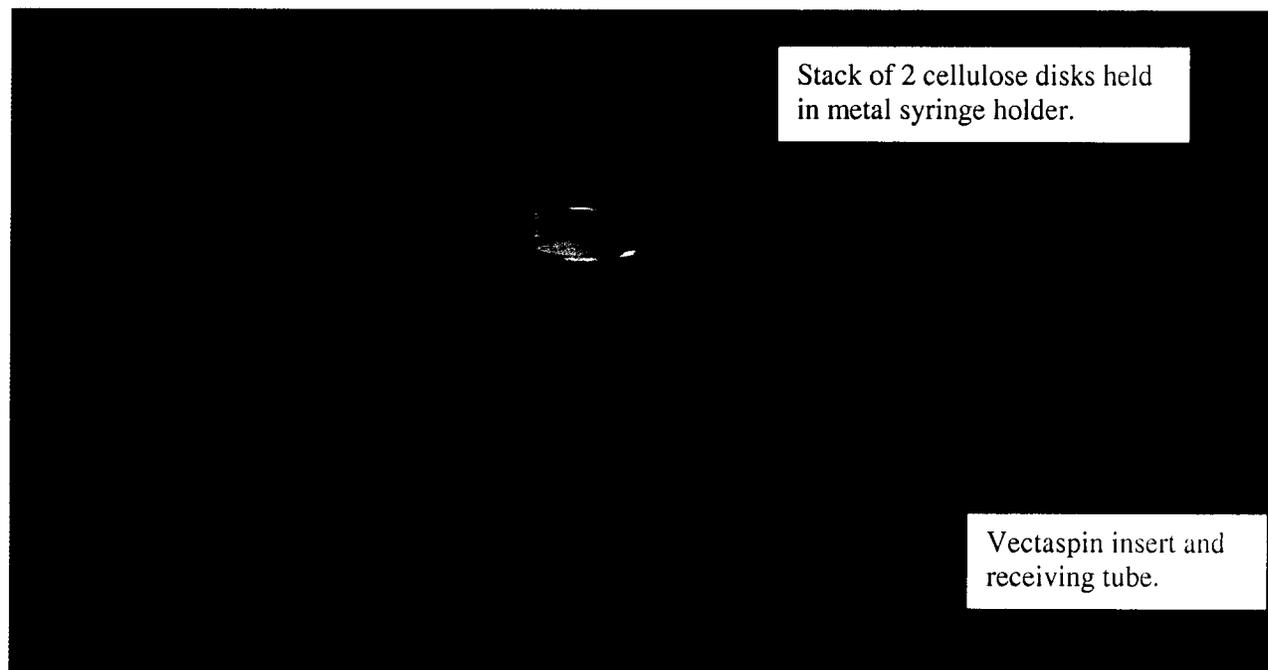
$$\% \text{Bioavailable CPC Conc. (ppm)} = \frac{100 \times \text{Bioavailable CPC Conc. (ppm)}}{\text{Total CPC Conc. (ppm)}}$$

Equation 6. % Recovery of Check

$$\% \text{Recovery} = 100 \times \frac{(\text{Average Concentration (ppm) Bioavailable CPC Found})}{(68.5\% \text{ Bioavailable CPC Expected})}$$

Attachment 1

Filter Holder Assembly during prewetting step.



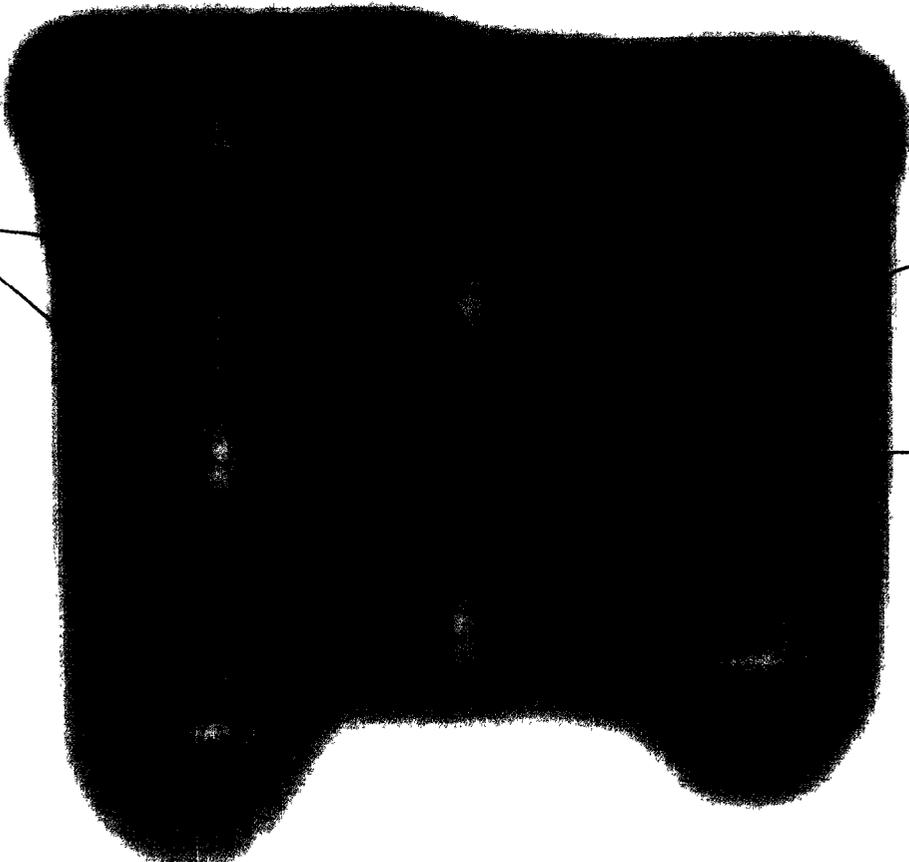
Filter Holder Assembly containing HPLC autosampler vial inside Vectaspin receiving tube.



Attachment 2

Vectaspin 3 Assembly

Cap and Filter Membrane must be removed on insert.



Vectaspin 3 receiving Tube

Vectaspin 3 insert with cap