

Note d'application

## Validation of a UPLC Method for a Benzocaine, Butamben, and Tetracaine Hydrochloride Topical Solution

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## Abstract

This application note demonstrates the successful development and validation of an UltraPerformance LC (UPLC) method for the analysis of these compounds.

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## Introduction

Benzocaine (4-Aminobenzoic acid ethyl ester), Butamben (Butyl 4-aminobenzoate), and Tetracaine (4-(Butylamino)benzoic acid 2-(dimethylamino)ethyl ester) are topical anesthetics. The formulated mixture of these three active ingredients is indicated for the production of anesthesia of all accessible mucous membranes except the eyes. In this application note, we demonstrate the successful development and validation of an UltraPerformance LC (UPLC) method for the analysis of these compounds.

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## Experimental

### Materials

Benzocaine, butamben, tetracaine HCl, benzalkonium chloride, cetyl dimethyl ethyl ammonium bromide, ammonium bicarbonate, and ammonium hydroxide were purchased from Sigma-Aldrich Co. (St Louis, MO). Water was purified with a Milli-Q System (Millipore, Billerica, MA). Acetonitrile was purchased from Mallinckrodt Baker, Inc. (Phillipsburg NJ) and was of HPLC grade. The formulated topical solution was purchased from a local pharmaceutical supplier and was labeled to contain 14.0% benzocaine and 2.0% each of butamben and tetracaine HCl as well as the inactive ingredients benzalkonium chloride (0.5%) and cetyl dimethyl ethyl ammonium bromide (0.005%). All bottles of the topical solutions were of the same production lot.

### UPLC Apparatus and Conditions

The Waters ACQUITY UPLC System consisted of a Binary Solvent Manager (BSM), Sample Manager (SM), and an ACQUITY UPLC Tunable UV (TUV) Detector. System control, data collection, and data processing were accomplished using Waters Empower 2 Chromatography Data Software. Separations were performed

at 40 °C using an ACQUITY UPLC BEH C<sub>18</sub>, 1.7 µm, 2.1 mm x 50 mm Column. Elution was accomplished using a 10.0 mM ammonium bicarbonate mobile phase (adjusted to pH 10.0 with ammonium hydroxide) filtered through at 0.45 µm membrane, and acetonitrile (60:40) at 1.0 mL/min. 1 µL injections were used for both standards and samples. The sample manager was equipped with a 5 µL loop and used the Partial Loop Needle Overfill mode with 1 µL air gaps. To minimize carryover, 1200 µL of the weak wash (60:40 water/acetonitrile) and 400 µL of the strong wash (10:90 water/acetonitrile) were used. Detection wavelengths were optimized for each compound and collected on a timed event basis (Table 1). A sampling rate of 20 points per second and a filter constant of 0.10 seconds was used throughout. The total run time was 1.5 minutes (Figure 1).

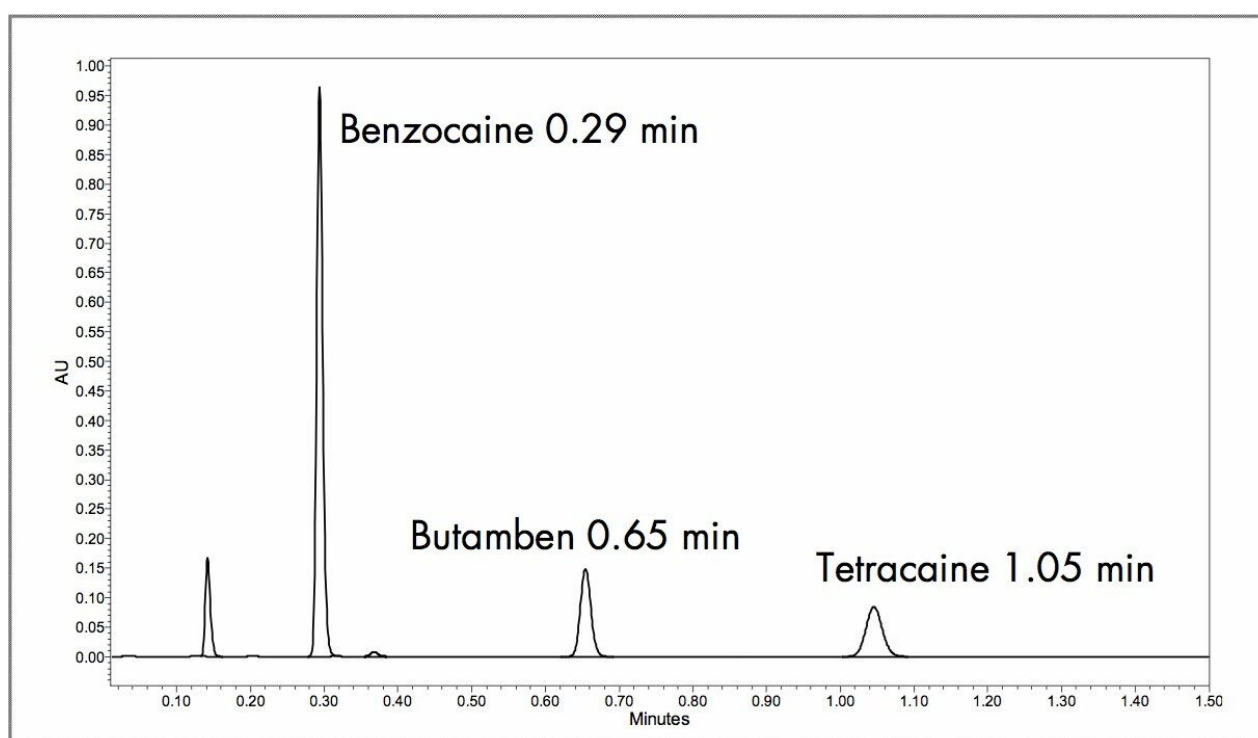


Table 1. Detector time table.

<i>Time range (min)</i>	<i>Wavelength (nm)</i>	<i>Compound</i>	<i>RT (min)</i>
0.00 - 0.50	220	Benzocaine	0.29
0.50 - 0.90	290	Butamben	0.65
0.90 - 1.50	307	Tetracaine	1.05

Figure 1. UPLC separation of Benzocaine, Butamben, and Tetracaine.

## Preparation of Standard Solutions

### Stock Solutions

A standard solution (200.0 mL) of benzocaine at 1.0 mg/mL and a standard solution (200.0 mL) containing 0.15 mg/mL each of butamben and tetracaine hydrochloride was prepared by dissolving appropriate amounts of each in the diluent (a mixture of 10.0 mM ammonium bicarbonate at pH 10.0 and acetonitrile (60:40)).

### Working Solutions

Working standard solutions (5 levels, 100.0 mL of each) were prepared by diluting appropriate amounts of stock solutions in the diluent to give concentrations ranging from 0.10–0.30 mg/mL for benzocaine and 0.015–0.045 mg/mL for butamben and tetracaine hydrochloride (Table 2).

Standard	Benzocaine Stock (mL)	Butamben Tetracaine HCl Stock (mL)	Approximate concentrations of working solutions (mg/mL)		
			Benzocaine	Butamben	Tetracaine HCl
Level 1	10.0	10.0	0.10	0.0150	0.0150
Level 2	15.0	15.0	0.15	0.0225	0.0225
Level 3	20.0	20.0	0.20	0.0300	0.0300
Level 4	25.0	25.0	0.25	0.0375	0.0375
Level 5	30.0	30.0	0.30	0.0450	0.0450

Table 2. Working standard solutions.

#### Topical Solution Sample Preparation

About 300 mg of Topical Solution was accurately weighed and transferred to a 200 mL volumetric flask. Approximately 180 mL of diluent was added and mixed on a wrist action shaker for 5 minutes. The sample was diluted to final volume and mixed thoroughly. An aliquot of the sample was transferred to a 2 mL vial and capped.

### Chromatographic Analysis Procedure

Equal volumes (1.0 µL) of the 5 standard preparations and the topical solution sample preparation were separately injected into the chromatograph, chromatograms recorded, peak areas were measured for the 3 major peaks. A calibration curve was then created based on the 5 standard preparations. The amounts were calculated, in mg/mL, for benzocaine, butamben and tetracaine hydrochloride in the portion of topical solution sample preparation by comparing the area of each peak to the linear regression line of the calibration curve, based on the five standard preparations.

The amount was calculated, by weight % of benzocaine, butamben and tetracaine hydrochloride in the topical solution using the formula:

$$\text{Concentration (mg/mL)} \times (200 \text{ mL} \div \text{Weight Topical Solution in Volumetric (mg)}) \times 100\%$$

The current monograph from the United States Pharmacopeia(USP) states that Benzocaine (14%), Butamben (2%), and Tetracaine Hydrochloride (2%) Topical Solution should contain not less than 90.0% and not more than 110.0% of the labeled amounts of benzocaine, butamben, and tetracaine hydrochloride.

## Suitability Criteria

Based on the chromatograms of standard preparations, the retention times (in minutes) are about 0.30 for benzocaine, 0.65 for butamben, and 1.05 for tetracaine; the resolution,  $R$ , between the benzocaine peak and the butamben peak and between the butamben peak and the tetracaine peak is not less than 8; and the relative standard deviation for retention time of replicate injections is not more than 1.0% for each of the 3 analyte peaks. The relative standard deviation for amount of replicate injections is not more than 2.0% for each of the 3 analyte peaks.

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## Results and Discussion

### Assay Validation And Results

#### Specificity

To demonstrate specificity, injections of individual reference standard, a diluent blank, and a solution that contains approximate concentration of labeled non-active ingredients were evaluated. The reference standards and diluent blanks were prepared using the same protocols as the assay preparation to ensure no contamination is added during the sample preparation steps. Samples were evaluated using photodiode array detection and MS detection and peak purity were assessed using Empower 2 Software.

#### Acceptance Criteria for Specificity

The diluent blanks must show no interference with the peaks of the active ingredients. Resolution between the main sample peaks should be greater than or equal to 2.0. The resolution factors between all sample peaks and any other peak should be greater than or equal to 2.0. Any co-elution of significant non-active ingredient, excipient or unknown peaks must be clearly noted in the final reporting of the results. To determine peak purity, the threshold must exceed the peak purity angle for the main sample peaks.

#### Specificity Results

Injections of a diluent blank showed no extraneous peaks. Injections of individual reference standards gave the following retention times: benzocaine 0.29 minutes, butamben 0.65 minutes, and tetracaine 1.05 minutes. No other peaks were noted in the chromatograms from the individual standards. A solution that contained the inactive ingredients benzalkonium chloride (0.5%) and cetyl dimethyl ethyl ammonium bromide (0.005%) in water was analyzed. No peaks were noted in the regions of the active ingredients. Photodiode array analysis and single quadrupole mass spectrometry detection were applied to a sample. Resolution requirements outlined in the acceptance criteria for specificity were easily met (Table 3).

Name	RT (min)	Resolution	Purity Angle	Purity Threshold
Unknown	0.14	—	$3.611 \pm 8.9\%$	$0.498 \pm 5.0\%$
Benzocaine	0.30	$11.3 \pm 1.8$	$0.225 \pm 11.5\%$	$0.604 \pm 2.1\%$
Unknown	0.37	$4.5 \pm 1.4$	$2.117 \pm 15.5\%$	$2.612 \pm 8.4\%$
Butamben	0.66	$12.9 \pm 0.9$	$0.252 \pm 9.0\%$	$0.419 \pm 7.7\%$
Tetracaine	1.05	$11.7 \pm 0.5$	$0.437 \pm 9.9\%$	$0.603 \pm 8.9\%$

Table 3. Specificity results.

Photodiode array peak purity results of the sample showed purity angles less than purity thresholds for the 3 peaks of interest, indicating each peak had a high degree of peak homogeneity. Evaluation of the mass spectral data confirmed spectral identity of the 3 peaks of interest (Figure 2). Based on these results, the method was determined to be specific for the analysis of benzocaine, butamben, and tetracaine.

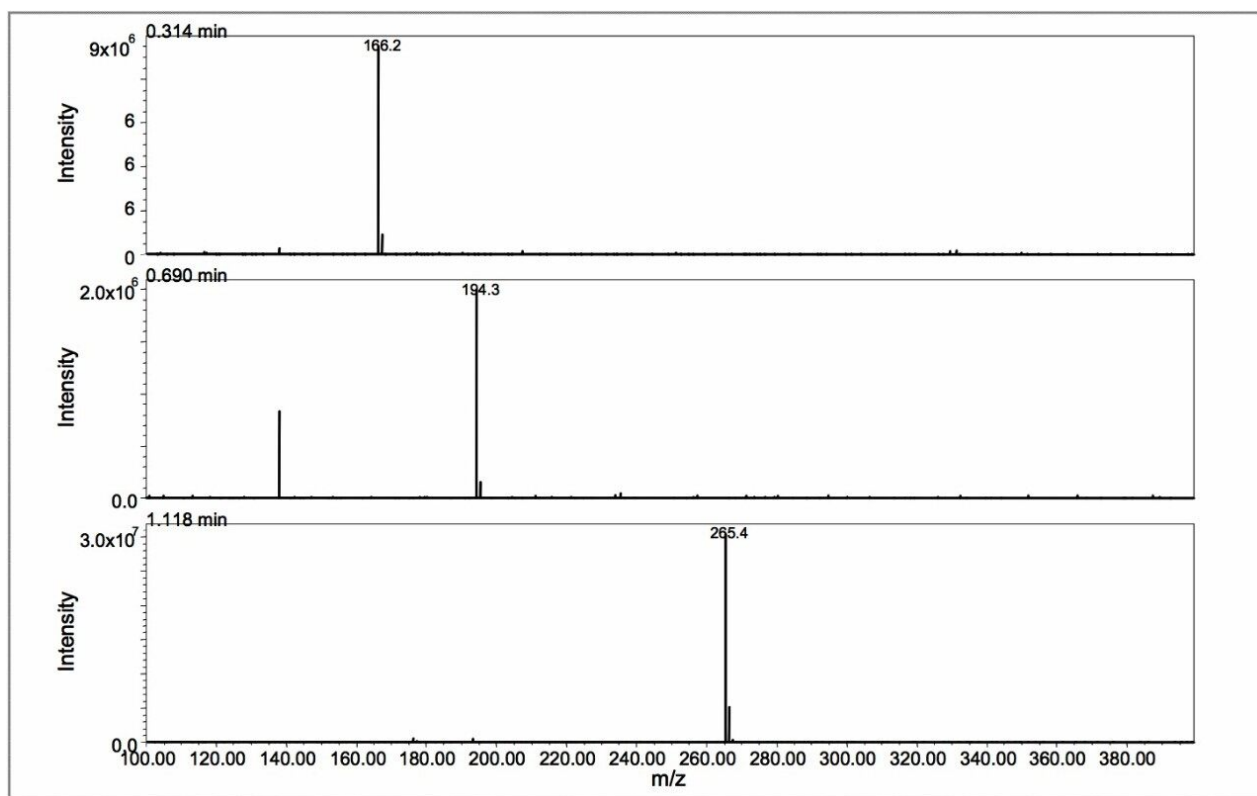


Figure 2. Mass spectra of benzocaine (top), butamben (middle) and tetracaine (bottom).

## Linearity

Serial dilutions from a stock standard solution were made to obtain 5 linearity solutions incorporating levels from 50% to 150% of the nominal analytical concentration of the sample components. Each solution was injected in duplicate. The linear regression of these injections provided the correlation coefficient, y-intercept, and the residual sum of squares.

### Acceptance Criteria

The correlation coefficient should not be less than 0.999 for all the sample components and the y-intercept should be within  $\pm 2.0\%$  when compared to the 100% level. The plot of the residuals should indicate linearity.

### Linearity Results

The linearity of all 3 compounds met the criteria described above (Table 4). Linearity plots are shown in Figures 3 and 4. Residuals at all 5 standard levels were all less than 2% and indicated linearity (Figure 5).



Name	R <sup>2</sup>	Y Intercept %Difference	Equation
Benzocaine	0.999855	1.08%	$Y = 2.49e+006 X + 5.47e+003$
Butamben	0.999897	0.37%	$Y = 4.71e+006 X + 5.39e+002$
Tetracaine	0.999883	0.16%	$Y = 4.24e+006 X - 2.09e+002$

Table 4. Linearity results.

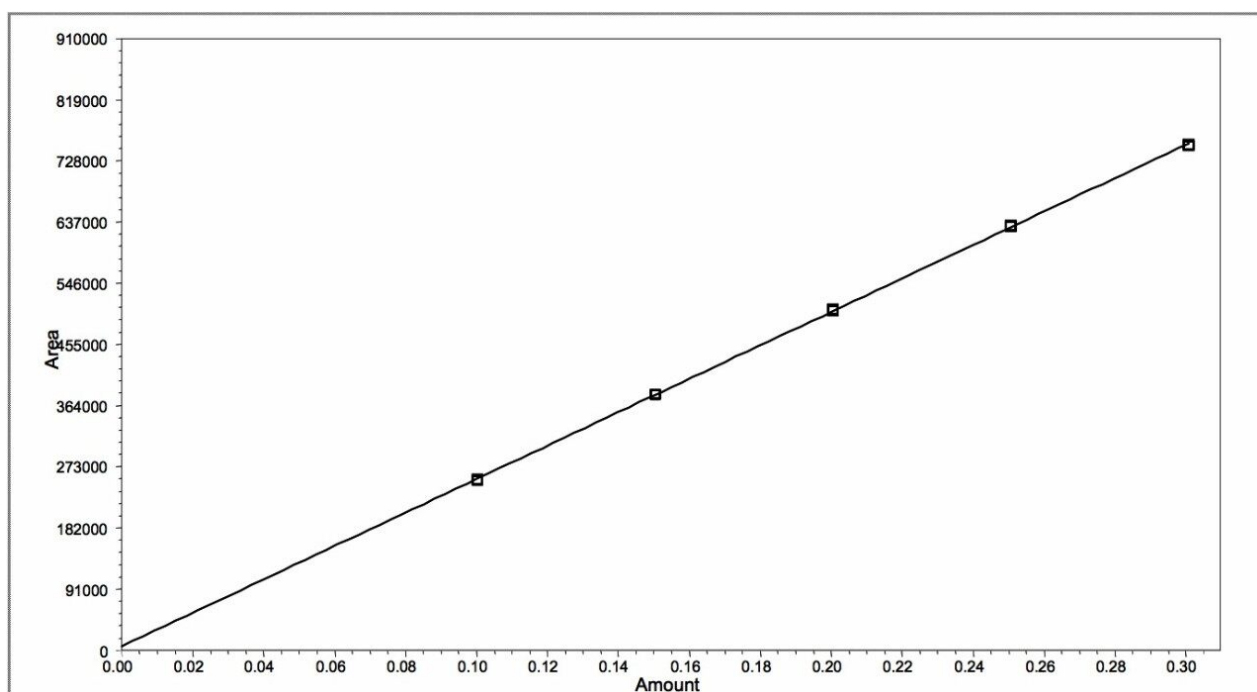


Figure 3. Calibration curve for benzocaine.

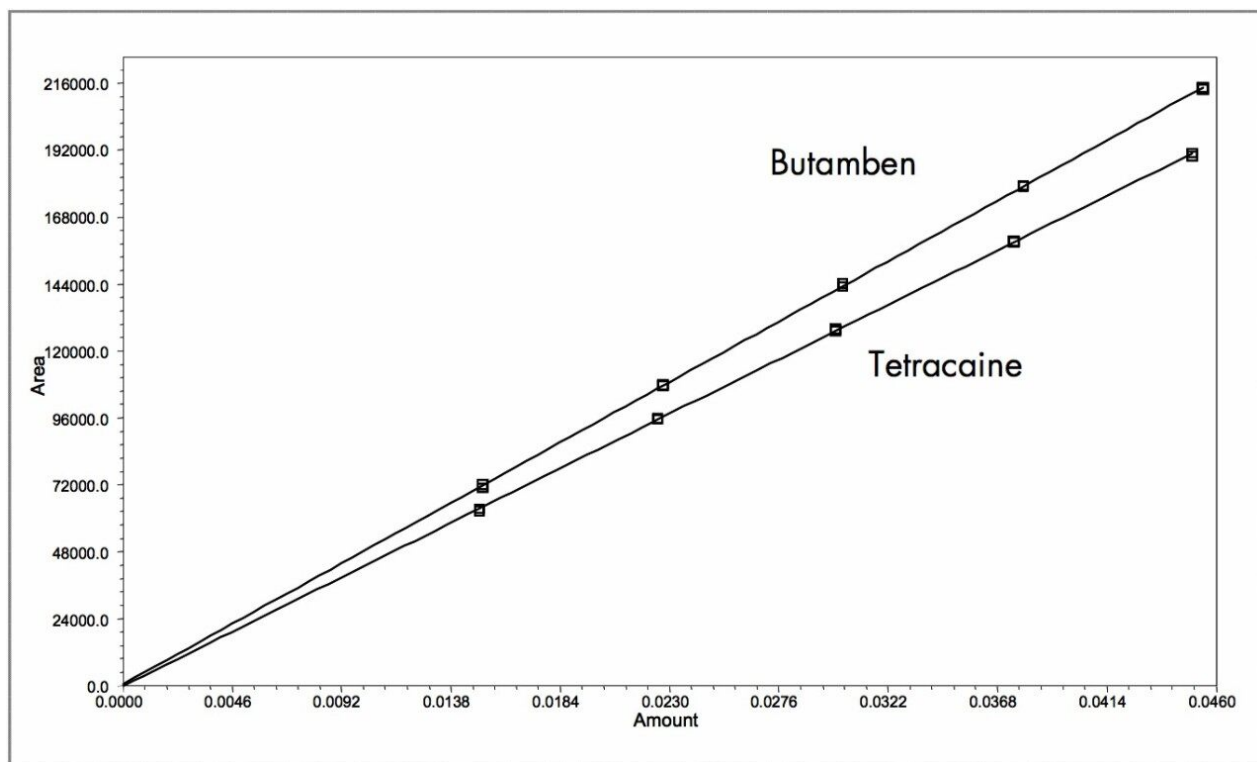


Figure 4. Calibration curves for butamben and tetracaine.

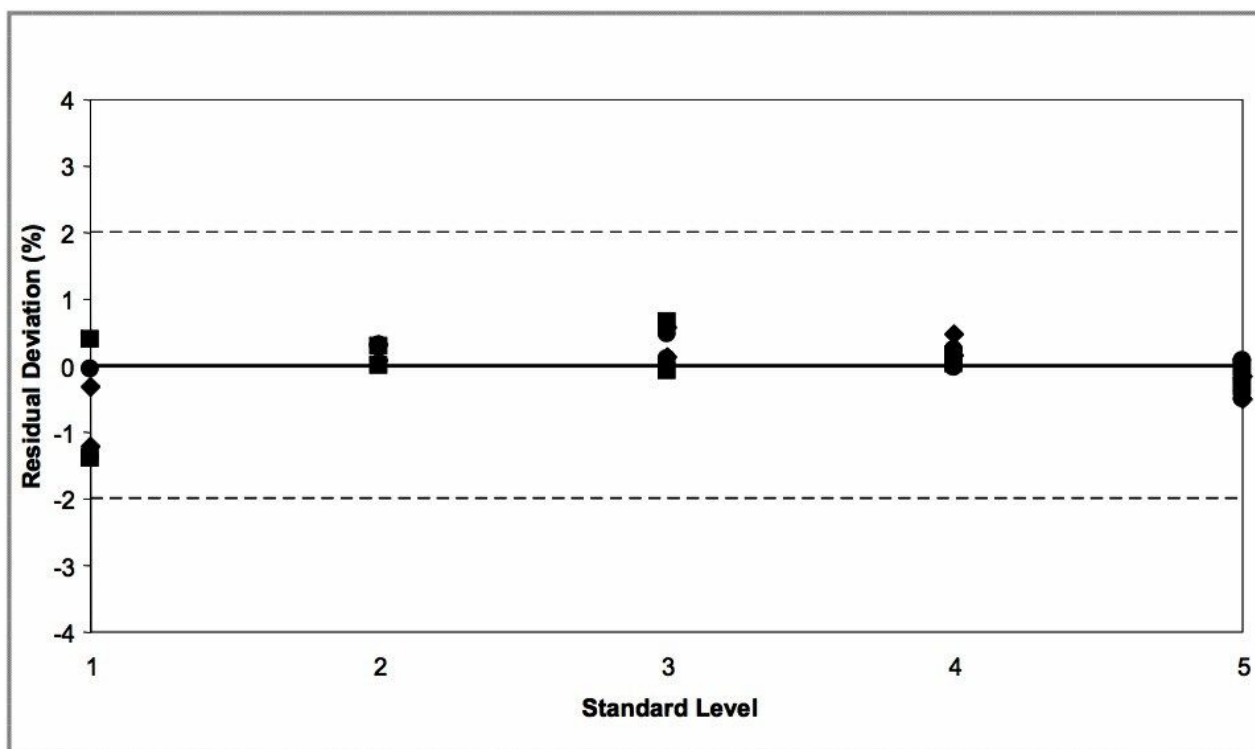


Figure 5. Residuals plot for standard curves of benzocaine, butamben, and tetracaine.

### Precision–Repeatability

The repeatability of the main sample peaks was measured by making 6 replicate injections of a single standard solution at each of 3 levels (50%, 100%, and 150%).

#### Acceptance Criteria for Precision–Repeatability

The %RSD for amount should not be more than 2.0% for each of the main sample components at each level. The %RSD for retention time should not be more than 1.0% for each of the main sample components at each level.

#### Precision–Repeatability Results

The repeatability for all three compounds met the acceptance criteria (Tables 5, 6, and 7).

	Benzocaine	Butamben	Tetracaine
RT (min)			
Mean	0.296	0.659	1.050
Std. Dev.	0.001	0.002	0.002
% RSD	0.46	0.30	0.22
Amount			
Mean	0.101	0.0152	0.0150
Std. Dev.	0.0003	0.0001	0.0001
% RSD	0.34	0.561	0.485

Table 5. Repeatability at the 50% level (n=6).

	Benzocaine	Butamben	Tetracaine
RT (min)			
Mean	0.295	0.658	1.051
Std. Dev.	0.001	0.002	0.003
% RSD	0.41	0.29	0.25
Amount			
Mean	0.204	0.0306	0.0305
Std. Dev.	0.001	0.0002	0.0002
% RSD	0.54	0.62	0.62

Table 6. Repeatability at the 100% level (n=6).

	Benzocaine	Butamben	Tetracaine
RT (min)			
Mean	0.295	0.657	1.050
Std. Dev.	0.001	0.001	0.002
% RSD	0.29	0.21	0.17
Amount			
Mean	0.302	0.0457	0.0452
Std. Dev.	0.001	0.0001	0.0001
% RSD	0.36	0.28	0.30

Table 7. Repeatability at the 150% level (n=6).

### Precision–Intermediate Precision

To establish the effects of random events on the precision of the analytical procedure, 2 analysts prepared and analyzed 6 sample assay preparations from 1 batch and 2 preparations each from 2 additional batches. Each analyst prepared their own standards and solutions, used a column from a different lot, and used different systems to evaluate the sample solutions.

#### Acceptance Criteria

The %RSD obtained for the 10 preparations for % active ingredient by both analysts should not be more than 2.0%. The mean analyst 1 result for each batch tested should not differ from the mean analyst 2 result for sample peaks by more than 2.0%.

#### Precision–Intermediate Precision Results

Intermediate precision results for both analysts met the criteria described above, demonstrating acceptable intermediate precision for the assay. Results are summarized in Table 8.

[% Active]	Benzocaine			Butamben			Tetracaine		
	Analyst 1	Analyst 2	%Diff.	Analyst 1	Analyst 2	%Diff.	Analyst 1	Analyst 2	%Diff.
Mean	13.9	14.0	0.70	1.99	1.98	0.50	1.96	1.97	0.50
Std. Dev.	0.05	0.03		0.007	0.004		0.005	0.004	
% RSD	0.33	0.23		0.36	0.22		0.26	0.22	

Table 8. Intermediate precision results.

## Precision–Reproducibility

Reproducibility is assessed by means of an inter-laboratory trial. Analysts from 2 different labs (different from the analysts involved in the intermediate precision) prepared and analyzed 6 sample assay preparations from one batch and 2 preparations each from 2 additional batches. Each analyst prepared their own standards, solutions, used a column from a different lot, and different systems to evaluate the sample solutions.

### Acceptance Criteria

The %RSD obtained for the 6 preparations by both analysts should not be more than 2.0% The mean lab 1 result for each batch tested should not differ from the mean lab 2 result for the sample peaks by more than 2.0%.

### Precision–Reproducibility Results

Precision reproducibility results from both laboratories met the criteria described above demonstrating acceptable reproducibility for the assay. Results are summarized in Table 9.

[% Active]	Benzocaine			Butamben			Tetracaine		
	Lab 1	Lab 2	% Diff.	Lab 1	Lab 2	% Diff.	Lab 1	Lab 2	% Diff.
Mean	14.0	13.8	1.43	1.98	1.95	1.51	2.02	2.00	1.00
Std. Dev.	0.07	0.14		0.012	0.021		0.026	0.027	
% RSD	0.51	1.04		0.59	1.08		1.30	1.36	

Table 9. Reproducibility results.

## Robustness

Robustness is the ability of a method to remain unaffected by small changes in conditions. If the changes are within the limits that produce acceptable chromatography, the method is considered robust. 6 replicate injections of a standard and a sample solution were performed under both the specified and modified conditions shown in Table 10.

<i>Parameter</i>	<i>Specified Conditions</i>	<i>Modified Conditions</i>
Wavelength (nm)	220, 290, 307	225, 295, 312 and 215,285, 302
Flow Rate (mL/min)	1.00	0.90, 0.95, 1.05, 1.10
Column Temperature (°C)	40	38, 39, 41, 42
Injection Volume (µL)	1.0	0.8, 0.9, 1.1, 1.2
Mobile Phase Composition	60/40	50/50, 55/45, 65/35, 70/30
Buffer Concentration mMol	10	8, 9, 11, 12
Buffer pH	10.0	9.0, 9.5, 10.5, 11.0
Sample Prep Shake time (min)	5	2, 5, 10

*Table 10. Tested robustness criteria.*

#### Acceptance Criteria

The average values obtained at the modified conditions should not differ by more than 2.0% for the sample components from those values obtained using the specified conditions.

#### Robustness Results

The results for the various method modifications are summarized in Table 11. For changes applied to the method, calculated amounts of the active ingredients did not vary by more than 2% (relative to the specified method conditions), except in the case of column temperature, where the results from the 38 °C column temperature fell outside of this range for all 3 active ingredients (Table 11). These results demonstrate that the method was robust, although care must be taken to ensure the specified column temperature.



<i>Method Condition</i>	<i>Benzocaine</i>	<i>Butamben</i>	<i>Tetracaine</i>
Wavelengths +5 nm	13.93±0.02	1.99±0.01	2.01±0.01
*Specified Wavelengths	13.90±0.02	1.98±0.01	1.98±0.01
Wavelengths -5 nm	13.91±0.03	1.98±0.01	2.01±0.01
Flow Rate 0.90 mL/min	13.97±0.02	1.99±0.01	2.00±0.02
Flow Rate 0.95 mL/min	13.94±0.04	1.99±0.01	1.99±0.01
*Flow Rate 1.00 mL/min	13.88±0.10	1.97±0.00	1.98±0.01
Flow Rate 1.05 mL/min	13.83±0.04	1.97±0.01	1.98±0.01
Flow Rate 1.10 mL/min	13.87±0.03	1.98±0.01	1.98±0.01
Column Temperature 38°C	<b>13.51±0.02<sup>1</sup></b>	<b>1.92±0.01<sup>1</sup></b>	<b>1.94±0.02<sup>1</sup></b>
Column Temperature 39°C	13.92±0.02	1.98±0.00	2.00±0.01
*Column Temperature 40°C	14.01±0.01	2.00±0.01	2.00±0.01
Column Temperature 41°C	13.98±0.03	1.99±0.01	1.99±0.01
Column Temperature 42°C	13.87±0.02	1.97±0.01	1.97±0.02
Injection Volume 0.8 µL	13.90±0.04	1.98±0.01	1.98±0.01
Injection Volume 0.9 µL	13.93±0.02	1.99±0.00	1.99±0.01
*Injection Volume 1.0 µL	13.84±0.03	1.96±0.01	1.96±0.01
Injection Volume 1.1 µL	13.91±0.03	1.98±0.01	1.98±0.02
Injection Volume 1.2 µL	13.89±0.02	1.98±0.01	1.98±0.01
Mobile Phase Composition 50/50	14.34±0.04	2.00±0.01	2.03±0.02
Mobile Phase Composition 55/45	14.19±0.04	2.02±0.01	2.02±0.01
*Mobile Phase Composition 60/40	14.19±0.03	2.03±0.01	2.03±0.02
Mobile Phase Composition 65/35	14.23±0.05	2.03±0.01	2.05±0.01
Mobile Phase Composition 70/30	14.10±0.07	2.03±0.01	2.17±0.05
Buffer Concentration 8 mMol	13.87±0.07	1.97±0.01	1.98±0.02
Buffer Concentration 9 mMol	13.87±0.04	1.97±0.01	1.98±0.01
*Buffer Concentration 10 mMol	13.85±0.04	1.97±0.01	1.98±0.01
Buffer Concentration 11 mMol	13.80±0.05	1.97±0.01	1.98±0.03
Buffer Concentration 12 mMol	13.90±0.04	1.98±0.02	1.98±0.01
Buffer pH 9.0	13.90±0.03	2.00±0.01	1.99±0.01
Buffer pH 9.5	14.00±0.03	1.99±0.01	2.01±0.01
*Buffer pH 10	13.91±0.03	1.99±0.01	2.00±0.02
Buffer pH 10.5	13.80±0.04	1.98±0.01	1.99±0.01
Buffer pH 11.0	13.90±0.04	1.98±0.01	2.00±0.01
Sample Prep Shake time 2 min	13.66±0.04	2.09±0.01	2.14±0.01
*Sample Prep Shake time 5 min	13.65±0.02	2.09±0.01	2.14±0.02
Sample Prep Shake time 10 min	13.62±0.03	2.07±0.01	2.13±0.02



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<sup>1</sup>Variation of >2.0% compared to the result obtained at the specified method conditions.

Table 11. Method robustness results.

## Accuracy/Recovery

The 3 analytes were spiked into a blank sample matrix at 3 levels: 80% of label, 100% of label, and 120% of label. These spiked samples were prepared according to topical solution sample preparation directions and analyzed according to the chromatographic analysis procedure. Amounts were determined using the same quantitation procedure as was used in the final method procedure. The percent recovery was then calculated.

### Acceptance Criteria

The measured value of the sample peaks in the spiked placebos should be within  $\pm 2.0\%$  of the spiked value.

### Accuracy/Recovery Results

The percent recovery values are summarized in Table 12. Recovery values for all 3 compounds at the 3 spike levels were within 2% of the expected value.

	<i>Benzocaine</i>	<i>Butamben</i>	<i>Tetracaine</i>
Spiked at 80% of label	100.4 $\pm$ 1.1	100.2 $\pm$ 1.2	98.8 $\pm$ 1.1
Spiked at 100% of label	100.5 $\pm$ 0.6	100.5 $\pm$ 0.5	98.8 $\pm$ 0.8
Spiked at 120% of label	101.5 $\pm$ 0.9	98.8 $\pm$ 0.8	100.3 $\pm$ 1.0

Table 12. % Recovery of spiked assay samples at 3 levels (n=6).

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## Conclusion

A linear, accurate, selective, robust, and reproducible UPLC method for the determination of Benzocaine, Butamben, and Tetracaine Hydrochloride Topical Solution was successfully validated. By taking advantage of ACQUITY UPLC System performance and BEH C<sub>18</sub> 1.7  $\mu$ m Columns, the entire method validation process was shortened from weeks to days, yielding a rugged method with superior resolution and speed.

## Acknowledgements

The technical assistance of Katherine Hynes and the participation of Mark Benvenuti and Michael D. Jones are gratefully acknowledged.

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