Method Development and Validation of an HPLC Assay for Choline in Carbachol Formulations Using a Post Column Reactor and a Chemically Modified Glassy Carbon Electrode

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Background

Carbachol (carbamylcholine chloride) is the active ingredient in some ophthalmic formulations used to induce miosis (pupil contraction) during eye surgeries. The miotic reduces the intraocular pressure in the eye by increasing the amount of fluid the eye drains. A diminished concentration of carbachol in the formulation may prevent the effective reduction of intraocular pressure, which may have several deleterious effects such as iris prolapse.

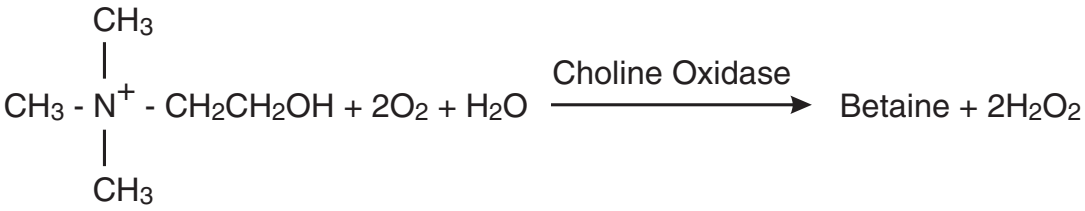
To ascertain whether the concentration of carbachol remains at a therapeutically active level under different storage conditions in a stability program, a method to selectively detect and quantitate choline, a hydrolytic degradation product of carbachol, was developed and validated at low levels of degradant (<1 µg/mL). Choline is neither UV-absorbing nor electroactive under reasonable conditions. However, via enzyme catalyzed reaction, it is converted to hydrogen peroxide, which can be readily oxidized or reduced.

Chromatography

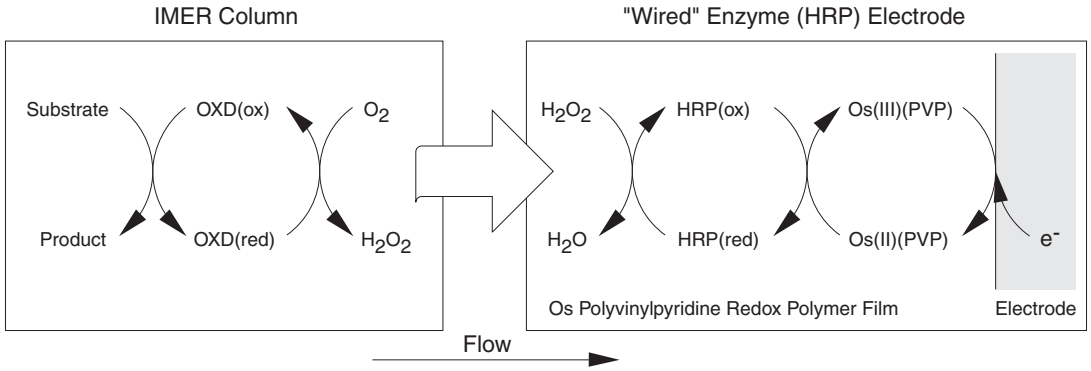
Choline is separated from other components in the matrix by ion exchange using a BAS Acetylcholine/Choline analytical column (#MF-6150). The isocratic separation is accomplished by using:

- 50 mM sodium phosphate (pH 8.0) mobile phase
- column/cell temperature: 30 °C
- flow rate: 1 mL/min
- injection volume: 20 µL

A BAS immobilized enzyme reactor (IMER, #MF-6149) containing choline oxidase covalently attached to a polymeric substrate is placed after the analytical column and is used to convert choline to hydrogen peroxide by the following enzyme catalyzed reaction:



The resultant H_2O_2 is reduced at the surface of a glassy carbon electrode, modified with a film of osmium poly(vinylpyridine) redox polymer sandwiched between horseradish peroxidase and the glassy carbon surface. The redox polymer effectively "wires" the horseradish peroxidase to the glassy carbon surface. The electrode is operated at only +100 mV (vrs Ag/AgCl) with a gain of 500 nA full scale.

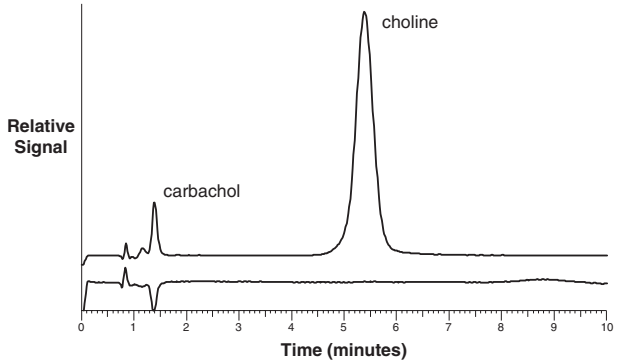


Redox cycles for oxidase IMER and wired peroxidase electrode.

The combination of an enzyme catalzyed reaction and a "wired" electrode operated at low potential creates a highly sensitive and selective detector.

Validation Results

Specificity



Overlay of chromatograms from acetic acid diluent blank and formulation sample. Carbachol peak elutes near void. No interfering peaks in the vicinity of the main band were observed in sample chromatograms.

System Suitability

Suitability Parameter	Criteria	Typical Value
Column Efficiency	≥1000 plates	1100 plates
Tailing Factor	$0.8<T_f<2.0$	1.1
Precision (Peak Area, n=5)	≤2%	0.5%
Precision (Retention Time, n=5)	≤2%	0.1%
Standard Check	≤2%	0.5%

Linearity

Response is linear from LOQ to 1% of nominal analytical concentration of carbachol.

Choline Concentration (µg/mL)	Number of Injections	Average Peak Area	%RSD
0.001	3	11271	5.8
0.002	3	17517	9.3
0.005	3	40522	3.0
0.01	3	77706	1.5
0.05	3	383497	0.7
0.1	3	774572	0.7
0.5	3	3842514	0.2
1.0	3	7685749	0.6
		Correlation coef.	1.00000
		Slope	7708942
		intercept	2350
		%intercept bias at nominal	0.6%
		Standard Error	2246
		LOQ	0.003 µg/mL
		LOD	0.0009 µg/mL

Criteria: correlation coefficient: ≥0.995; %intercept bias at nominal: ≤5.0%

Recovery/Accuracy

Method yields acceptable results for recovery and accuracy for sample preparations at 80, 100, and 120 percent of nominal levels of choline degradant.

% Nominal Concentration*	Number of Sample Preparations	Average Recovery	%RSD
120	3	102	1.1
100	3	102	0.6
80	3	102	1.5

Criteria: %Recovery: 90.0-110.0%; %RSD: ≤10%
*Nominal concentration: 0.5 µg/mL choline.

Solution Stability

With the addition of preservative (1% ProClin®), standard solutions are stable for 7 days at room temperature or under refrigeration.

Time Point (Day)	Conditions	% Recovery	% Initial
Initial	Room Temp	99	N/A
1	Room Temp	101	102
	Refrigerated	101	102
2	Room Temp	99	100
	Refrigerated	99	100
3	Room Temp	98	99
	Refrigerated	98	99
7	Room Temp	98	99
	Refrigerated	98	99

Criteria: %Recovery 98-102%; %Initial: 95-105%

Robustness

For reproducible peak area and retention time, flow rate and buffer concentration are critical parameters.

Parameters		Choline chloride			
		Average RT	% Difference	Average Peak Area	% Difference
Flow Rate (mL/min)	0.9	6.06	+11.7	2432138	+8.3
	1.0	5.39		2238396	
	1.1	4.99	-7.8	2117406	-5.6
Column Temp. (°C)	28	5.37	+0.6	2049481	-2.3
	30	5.34		2096370	
	32	5.34	0	2082004	-0.7
Potential (mV)	90	5.41	+0.1	2000676	-2.6
	100	5.40		2052575	
	110	5.41	+0.2	2031076	-1.1
MP Buffer Conc. (mM)	45	5.21	-1.1	1931672	-0.6
	50	5.27		1944162	
	55	4.91	-7.0	1947727	+0.2
MP Buffer PH	7.8	5.31	-0.4	1598867	+1.7
	8.0	5.33		1572411	
	8.2	5.26	-1.3	1561939	-0.7

Criteria: RT window: 5-7.2 min; peak area % difference: ≤5%

Ruggedness (Intermediate Precision)

Analytical method yields comparable results when different analysts run the same samples on different instruments with different solution preparations.

Sample No.	Analyst 1 Instrument 1 Choline (µg/mL)	Analyst 2 Instrument 2 Choline (µg/mL)	Percent Difference
1	0.794	0.764	1.0
2	0.642	0.625	0.7
3	0.547	0.537	0.5
4	0.439	0.427	0.7
5	0.285	0.279	0.5
6	0.032	0.031	1.3

Criteria: Percent Difference ≤2%

Multiple Injections

Multiple injections do not significantly deteriorate the performance of the column/post column reactor/modified glassy carbon electrode combination.

Injection #	Retention Time (min)	Peak Area (µV*sec)
1	**5.27**	**1441432**
101	**5.31**	**1414244**
Average	5.28	1414244
%RSD	0.57	1.60

Conclusions

The results indicate that a rugged, selective, and sensitive method has been developed and validated for determining the concentration of choline at low-levels (ppb) in an ophthalmic formulation containing carbachol in a stability program.