

Quantitative Analysis of Pilocarpine in Human Plasma using LC-MS/MS

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Introduction

Pilocarpine belongs to a class of medications known as cholinergic parasympathomimetics and is a naturally occurring alkaloid in the leaves of South American shrub *Pilocarpus jaborandi*. Its chemical structure is presented in Figure 1.

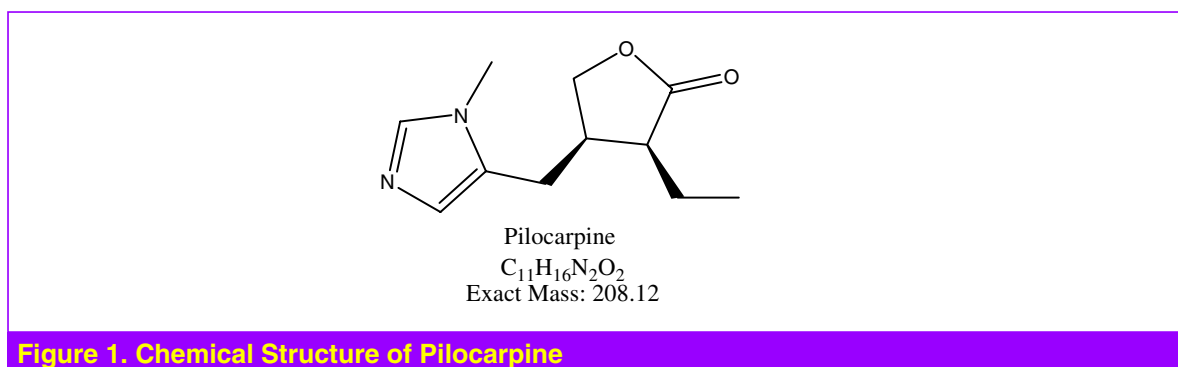


Figure 1. Chemical Structure of Pilocarpine

Methods

Sample cleanup was accomplished using an automated liquid-liquid extraction of 0.2 mL aliquots of human plasma. An isocratic HPLC method (5-6 minutes run time, 0.25-0.50 mL/min flow rate, acetonitrile:buffered aqueous mobile phase) was used. A structural analogue of pilocarpine was selected as the internal standard. A Micromass Quattro tandem mass spectrometer was operated in the positive ion mode via electrospray. Data was collected using multiple reaction monitoring.

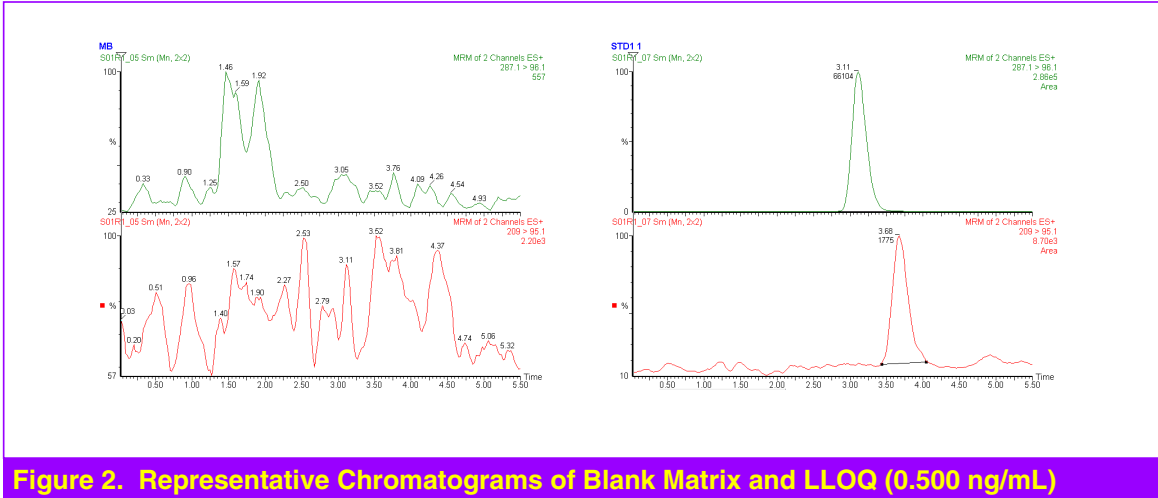
Results and Discussion

The between-run precision and percent bias of the method ranged from 6.23 to 10.11% and from 1.80 to 5.88% respectively (Table 1).

Table 1. Between-Run Precision and Percent Bias Data for a Liquid Chromatography-Tandem Mass Spectrometry Method for the Quantification of Pilocarpine in Human Plasma. Quality Control Samples. Three-Run Validation.

Run Date	Curve Number	Low (0.500 ng/mL)	Mid (1.00 ng/mL)	Mid (20.0 ng/mL)	Mid (425 ng/mL)	High (2000 ng/mL)
Mean		0.509	1.05	20.4	450	2040
S.D.		0.0387	0.0919	1.34	45.5	127
%CV		7.60	8.75	6.57	10.11	6.23
%Theoretical		101.80	105.00	102.00	105.88	102.00
%Bias		1.80	5.00	2.00	5.88	2.00
n		18	18	17	18	17
Overall %CV		7.85				

Figure 2 illustrates representative chromatograms of a human plasma blank and standard containing 0.500 ng/mL.



To ensure specificity of the extraction procedure, six lots of plasma were tested. Aliquots of each plasma lot containing 20.0 ng/mL of pilocarpine were extracted and analyzed. The coefficient of variation was 9.21% and the accuracy was 102.00% for pilocarpine. The results of the specificity studies are shown in Table 2.

Table 2. Specificity Data for Analysis of Pilocarpine in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry Method.

Lot	Theoretical Concentration 20.0 ng/mL	Mean Concentration ng/mL	Accuracy %
1	17.5	18.0	89.75
	18.4		
2	19.4	21.7	108.50
	24.0		
3	20.9	21.1	100.25
	19.2		
4	20.4	21.1	105.25
	21.7		
5	22.5	21.9	109.25
	21.2		
6	20.6	19.5	97.50
	18.4		
Mean Measured Concentration		20.4	
Precision (% C.V.)		9.21	
Theoretical Value (%)		102.00	
N		6	

The stability of spiked human plasma samples following three freeze/thaw cycles was determined. Triplicate samples spiked at concentrations of 1.00 and 425 ng/mL of pilocarpine were subjected to three freeze/thaw cycles. The samples were then extracted and injected. The mean concentrations of the stability samples were compared to the theoretical concentrations.

The stability of pilocarpine in spiked human plasma after 24 hours at ambient temperature was determined. Triplicate samples spiked at concentrations of 1.00 and 425 ng/mL of pilocarpine were kept at room temperature for 24 hours before extraction. The samples were then extracted and injected. The mean concentrations of the stability samples were compared to the theoretical concentrations.

The stability of the extracted samples on the autosampler was measured. Half of the extracted quality control samples at the concentrations of 1.00 and 425 ng/mL pilocarpine were reinjected after 43.5 hours at room temperature. The mean concentration at each level was then compared to the theoretical concentration of pilocarpine.

Long term and frozen storage stability was demonstrated. Quality control samples at concentrations of 1.00 and 425 ng/mL pilocarpine stored at -20 °C were quantitated against a freshly prepared, never frozen, standard line and quality controls. Frozen storage stability was demonstrated for at least 39 days, since the test samples quantitated within 15% of theoretical. The results for all these stability tests can be found in Table 4.

Table 4. Stability Data

1- For Pilocarpine in Human Plasma Following One, Two and Three Freeze/Thaw Cycles.

2- For Pilocarpine in Human Plasma at Room Temperature for 24 Hours.

3- For Extracted Pilocarpine Control Samples on the Autosampler for 43.5 Hours at Ambient Temperature. 4- For Pilocarpine in Human Plasma for 39 Days at -20 °C.

Replicate Number	Pilocarpine Stability											
	Freeze Thaw Cycle 1		Freeze Thaw Cycle 2		Freeze Thaw Cycle 3		Room Temperature (24 Hours)		Autosampler (43.5 Hours)		Long Term Frozen Storage (39 Days)	
	Theoretical Concentration		Theoretical Concentration		Theoretical Concentration		Theoretical Concentration		Theoretical Concentration		Theoretical Concentration	
	1.00	425	1.00	425	1.00	425	1.00	425	1.00	425	1.00	425
#1	1.02	436	0.951	460	1.11	445	1.00	437	1.02	384	0.954	385
#2	1.02	441	1.03	437	1.08	442	1.03	441	1.01	396	0.963	412
#3	1.12	447	1.03	444	1.09	459	1.06	462	1.06	399	0.922	410
Mean Measured Concentration	1.05	441	1.00	447	1.09	449	1.03	447	1.03	393	0.946	402
Precision (%CV)	5.50	1.25	4.56	2.64	1.40	2.02	2.91	3.01	2.57	2.02	2.27	3.73
Stability (%)	105.00	103.84	100.00	105.18	109.00	105.58	103.00	105.11	103.00	92.47	94.60	94.59
Number of Samples	3	3	3	3	3	3	3	3	3	3	3	3

To verify the extraction in the presence of hemolyzed blood, an amount of hemolyzed blood was added to the plasma so that the plasma contained 2.00% hemolyzed blood. The plasma containing hemolyzed blood was then extracted; one blank with internal standard, one blank without internal standard and triplicate spiked samples at concentrations of 1.00 and 425 ng/mL of pilocarpine. In the presence of 2% hemolyzed blood, the mean recovery of pilocarpine was 100.80%. The results of the hemolysis experiments are shown in Table 3.

Table 3. Effect of 2.00% Hemolyzed Blood on the Pilocarpine Liquid Chromatography-Tandem Mass Spectrometry Method in Human Plasma.

Replicate Number	Pilocarpine Hemolysis Stability (ng/mL)	
	Theoretical Concentration	
	1.00	425
#1	1.08	410
#2	1.02	405
#3	1.08	404
Mean Measured Concentration	1.06	406
Precision (%CV)	3.26	0.79
Stability (%)	106.00	95.60
Number of Samples	3	3

Additional experiments were performed in an attempt to lower detection and quantitation levels. A Sciex API 4000 was used. The method demonstrated a predictable response from 5.00 to 5000 pg/mL, as can be seen in Figure 3. Figure 4 illustrates representative chromatograms of a human plasma blank and standard containing 5.00 pg/mL Pilocarpine. Further validation experiments may be done in order to meet lower quantitation requirements.

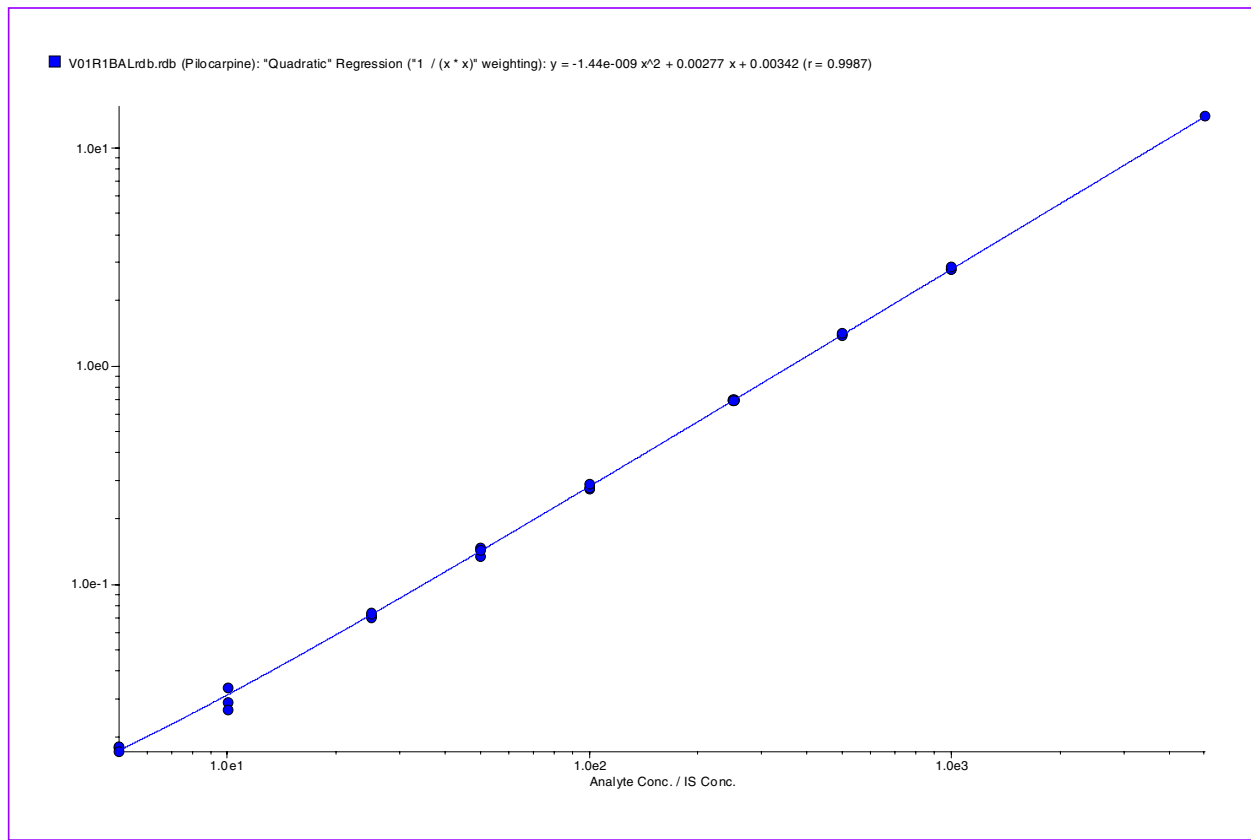


Figure 3. Area Ratio Versus Analyte Concentration, Quadratic Calibration Curve Weighed 1/X² (5.00 – 5000 pg/mL, displayed on log-log axes)

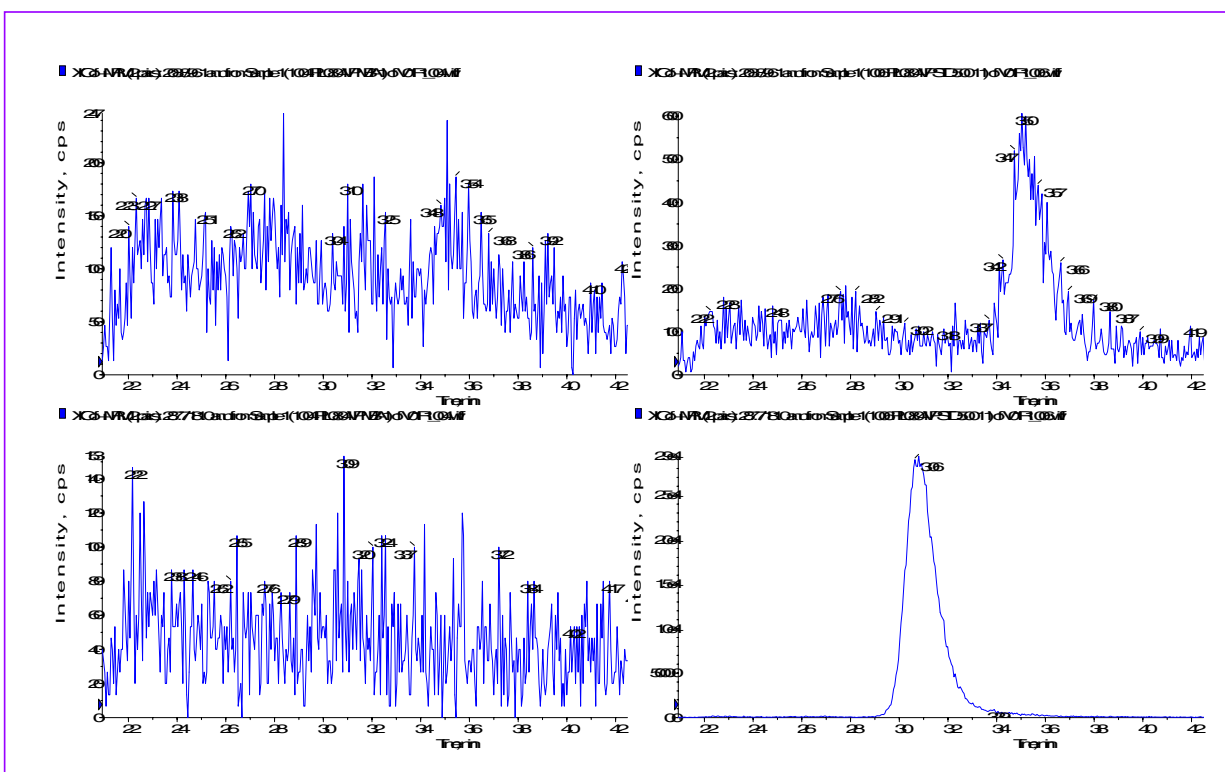


Figure 4. Representative Chromatograms of Blank Matrix and LLOQ (5.00 pg/mL)

Conclusions

The analytical method described is suitable for the analysis of pilocarpine in human plasma. Repeated use of this assay have shown this method to be rugged, sensitive, specific, and accurate. The calibration curve extends from a limit of quantification of 0.500 ng/mL to 500 ng/mL using 0.200 mL of human plasma.

Pilocarpine is stable in human plasma at ambient temperature for 24 hours and stable for at least 3 freeze-thaw cycles. Frozen storage stability of pilocarpine has been demonstrated for at least 39 days at -20 °C. Human plasma extracts containing pilocarpine were found to be stable on the autosampler for a minimum of 43.5 hours at ambient temperature.

The method is acceptable from 5.00 to 5000 pg/mL using a Sciex API 4000 but further testing needs to be done.