Determination of Chelator and Cu^{2+} in Aqueous Formulations Using High Performance Liquid Chromatography with UV Absorbance Detection and Atomic Absorption Spectroscopy

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1 ABSTRACT

Purpose: To develop an HPLC/UV method for the determination of chelator and an AA (atomic absorption) method for the determination of Cu^{2+}. The selectivity of the HPLC method allowed for the separation of the chelator from a liposome matrix and citrate buffer. The AA method could then be used to confirm the concentration of Cu^{2+} in the aqueous formulations.
Method: An Agilent 1100 HPLC coupled with a variable wavelength detector was used for the quantitation of a chelator compound in aqueous formulations containing excipient liposomes and Cu^{2+} in citrate buffer. Reproducible chromatography was achieved using a Synergi Polar-RP, 250 x 4.6 mm, 4-µm particle size column maintained at a temperature of 25°C. A gradient method was devised using two mobile phases: 0.1% trifluoroacetic acid in de-ionized water, and acetonitrile.
A Perkin Elmer Analyst 800 AA Spectrometer equipped with a THGA furnace was used for the quantitation of Cu^{2+} in formulations containing chelator and liposomes in citrate buffer. Reproducible absorbances were obtained using a Perkin Elmer Lumina Hollow Cathode Lamp for Copper and a temperature gradient.
Results: The HPLC method was validated from 0.100 to 5.00 µg/mL. In addition, a quality control sample at 9.30 µg/mL showed inter-session variability of 1.5 to 7.7% RSD and inter-session accuracy ranging from 2.5-8.1% RE.
The AA method demonstrated linearity from 10.0 to 50.0 ng/mL. In addition, a quality control sample at 377 ng/mL showed inter-session variability of 1.3 to 18% RSD and inter-session accuracy ranging from 7.7 to 22% RE.
Conclusion: An accurate method was developed and validated for the quantitation of chelator using HPLC/UV. An additional method was developed to confirm the concentration of Cu^{2+} in the aqueous formulations.

2 PURPOSE

- Develop both HPLC/UV and AA methods to quantify a copper chelator and total copper.
- Develop a prep to accurately replicate chelation and loading of liposomes.

3 BACKGROUND

The chelator used in this study was able to form a complex with copper. This complex was then loaded into excipient liposomes as a way to improve biodistribution of the compound to target sites. This provides a unique challenge to develop a method that may be used to quantitate both the chelator and copper, and eliminate interferences caused by the liposomes.

https://www.researchgate.net/figure/265387798_fig1_Figure-1-Synthesis-of-4-DEAP-ATSC-and-formation-of-64-Cu4-DEAP-ATSC-complex

http://europepmc.org/abstract/med/25200610

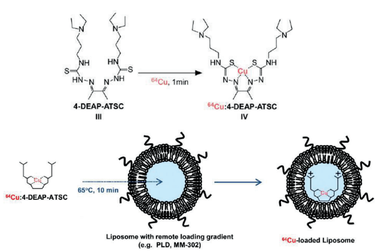

Figure 1: Structure of copper chelator and liposome

4 MATERIALS AND METHODS

HPLC Conditions	Instrument: Agilent 1100 high performance liquid chromatograph equipped with a variable wavelength detector, autosampler, and Dionex Chromeleon® software version 6.8			
Column:	Synergi® Polar-RP, 250 × 4.6 mm, 4-µm particle-size			
Guard Column:	SecurityGuard Polar-RP, 4 × 3.00 mm			
Mobile Phase A:	0.1% TFA in DI water			
Mobile Phase B:	ACN			
Needle Wash:	90:10 (v/v) DI water:MeOH			
Gradient:	Time (minutes)	A(%)	B(%)	Flow Rate (mL/min)
	0	73	27	1.0
	2.0	73	27	1.0
	8.0	48	52	1.0
	9.0	73	27	1.0
	14.0	73	27	1.0
Detector:	UV at 290 nm for Chelator			
Column Temperature:	25°C			
Autosampler Temperature:	4°C			
Injection Volume:	50 µL			
Retention Time:	Approximately 5.1 minutes for Chelator			

Table 1: HPLC Method Conditions

AA Conditions	Instrument: Perkin Elmer Analyst 800 AA Spectrometer equipped with a THGA furnace and WinLab32 for AA software					
Lamp:	Perkin Elmer Lumina Hollow Cathode Lamp - Copper					
Wavelength	324.8 nm					
Slit Width:	0.7 nm					
Sample Injection Volume:	20 µL					
Instrument Mode:	THGA Furnace					
Gradient:	Step	Temp (°C)	Ramp Time (seconds)	Hold Time (seconds)	Internal Flow (mL/min)	Read Step
	1	110	1	30	250	No
	2	130	15	30	250	No
	3	1200	10	20	250	No
	4	2000	0	5	0	Yes
	5	2450	1	3	250	No
Number of Replicates:	3					
Injection Temperature:	90°C					

Table 2: AA Method Conditions

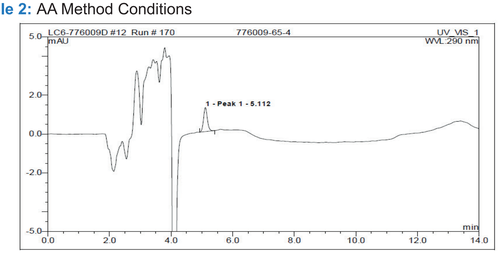

Figure 2: Chromatogram of 0.5 µg/mL chelator standard

Solutions
Mobile phases A and B were 0.1% (v/v) TFA in DI water, and ACN. The needle wash was 10:90 (v/v) MeOH: DI water. The HPLC diluent was 99:1 (v/v) MeOH: Acetic acid. 0.05M Citric acid and sodium citrate buffer pH 6 were used to prepare the working calibration stocks for the chelator. 0.1M HCl was used to prepare a copper (II) chloride stock to be combined with the chelator. 1% Nitric acid was use to prepare copper standards for AA. The matrix modifier solution was prepared by diluting 0.5 mL of palladium matrix modifier and 0.05 mL of magnesium matrix modifier up to 10 mL with DI water.

HPLC/UV Method

Calibration and Quality Control Samples: On each day of analysis, a primary stock solution was prepared at a concentration of 10.0 mg chelator/mL in 0.05 M citric acid solution. From this stock, calibration and quality control stock solutions were prepared at 0.100 and 0.200 mg/mL by diluting with sodium citrate buffer, pH . Calibration standards were prepared at various concentrations by diluting the stock with HPLC diluent. A primary copper (II) chloride stock solution was prepared at a concentration of 2.725 nmol copper (II) chloride/µL in 0.1 M HCl. A secondary copper (II) chloride stock was prepared at a concentration of 0.375 nmol copper (II) chloride/µL by diluting the primary stock. Quality control samples were prepared at a concentration of 9.30 µg chelator/mL by combining chelator stock and copper (II) chloride stock, then heating the solution at approximately 65°C for 1 minute, then chilling in an ice bath for 30 seconds. An aliquot of this solution was then added to excipient liposomes, heated again for 10 minutes, then chilled for 1 minute. Quality control samples were prepared at additional concentrations of 4.0, 2.0, and 1.0 µg chelator/mL by diluting with 10 mM HEPES. A representative blank sample was also prepared for analysis. Conditions for analysis are listed in Table 1.

Atomic Absorption Method

Calibration and Quality Control Samples: On each day of analysis, a copper calibration stock was prepared at a concentration of 1996 ng copper/mL in 1% nitric acid. Calibration standards were prepared at various concentrations from the stock by diluting with 1% nitric acid. Quality control samples were prepared the same as stated above, then diluted 14 fold with 1% nitric acid. Conditions for analysis are listed in Table 2.

5 RESULTS AND DISCUSSION

The HPLC/UV method to determine chelator was validated in this study with 3 validation sessions, and later the range was extended with an additional validation session. The chelator peak areas and concentrations were used to construct a calibration curve using least-squares regression analysis to a quadratic function with 1/x weighting. The mean back calculated standard concentrations had intersession variability ranging from 2.4% to 9.4% RSD and RE ranging from -1.9% to 1.6%. Assay precision and accuracy was verified by QC samples.
The AA method was similarly validated. The copper standard peak areas were used to construct a linear calibration curve. The intersession variability of the standard concentrations ranged from 0.94% to 1.9% RSD and RE ranging from -0.7 to 0.41%. Assay precision and accuracy was verified by QC samples.

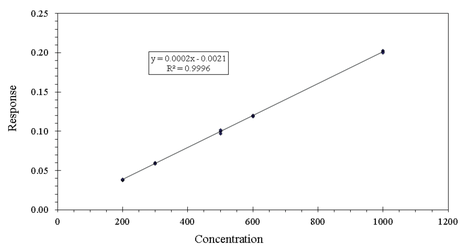

Figure 3: Calibration curve for atomic absorption standards

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