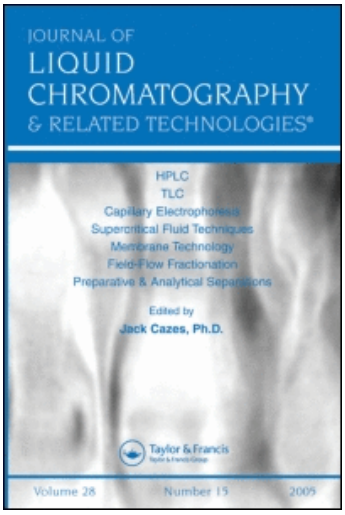

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Quantitative Analysis of Residual EDTA in Vaccine Products by HPLC with Luminescence Detection of Terbium(III) Complexation

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Quantitative Analysis of Residual EDTA in Vaccine Products by HPLC with Luminescence Detection of Terbium(III) Complexation

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Abstract: To facilitate the investigation of EDTA clearance in a protein purification process, a relatively simple, sensitive, and rapid method for EDTA analysis was developed using HPLC with pre-column complexation of EDTA and terbium(III) (Tb^{3+}). The limit of detection of this method is about 87 ng/mL, and the limit of quantitation is about 260 ng/mL. The accuracy, intra- and inter-assay variability of this method were evaluated. The method was applied to determine the residual EDTA concentrations in various stages of the purification process for vaccine products. Examples of evaluating EDTA clearance in a protein purification process will be discussed.

Keywords: Residual EDTA, Terbium, HPLC, Vaccine products, Luminescence, Process intermediates

INTRODUCTION

Ethylenediaminetetraacetic acid (EDTA) and its salt form are widely used in industries, agriculture products, and pharmaceutical applications. For example, it is used as an additive in household detergent, color preservative and flavor in foods, blood anticoagulant, and metal poisoning in medicine. In biological purification process applications, EDTA is primary used as a

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chelating agent for various metal ions, or sometimes it is used as a product stabilizer. In Merck vaccine programs, EDTA is normally added in the intermediate purification processes or at the final formulation, depending on its needs. Therefore, a very sensitive and specific method was developed to detect EDTA clearance during processes or at the final formulation.

EDTA analysis can be performed using various analytical techniques such as potentiometric, chromatographic, or capillary electrophoresis (CE) methods. EDTA analysis using CE was recently described.[1,2] Although this technique provides good sensitivity, the instrument set up is not readily adaptable to any regular laboratory. Gas chromatography is also available for EDTA analysis with low detection limits,[3] but this method normally requires a long procedure for the esterification step in addition to matrix interference. Therefore, the HPLC method is still the most commonly used for EDTA analysis with UV detection. Ion-exchange and reverse-phase in-pair mode HPLC are normally the methods of choice with satisfactory detection limits.[4–7]

EDTA analysis with HPLC is commonly performed using UV either at 220 nm for direct detection of EDTA or at 258 nm using iron (III) as a metal chelator at low pH (<4).[8,9] The detection at 220 nm has major disadvantages with matrix interferences, which most likely absorb at this wavelength, and tailing due to different forms of EDTA.[8] The addition of iron(III) metal to EDTA removes the tailing peak and the EDTA complex can be detected at a longer wavelength of 258 nm, which removes the number of matrix interference peaks, since fewer compounds absorb at this wavelength. However, there are some draw backs of using this iron(III) metal chelator. The Fe(III)-EDTA complex formation is very slow at room temperature.[10] The Fe(III) can form its hydroxide easily at pH >5.[10] Furthermore, this Fe(III)-EDTA complex is not stable in the presence of light, conditions under which the sample solution is not stable over a period of time.[11] Therefore, an alternative method of HPLC detection was sought.

This report describes the development of EDTA detection using lanthanide metal, terbium (III), luminescence with reverse-phase HPLC. The major advantage here is that free terbium ion has negligible luminescence, but strong luminescence once it binds to EDTA or other carboxylate groups. Since the luminescence intensity is detected, there is less matrix interference. This method of HPLC detection is developed to analyze residual EDTA in Merck vaccine processes.

EXPERIMENTAL

Chemicals and Reagents

All chemicals and solvents were analytical purity grade. The 3-(N-Morpholino)-propanesulfonic acid (MOPS) buffer was purchased from Sigma. Sodium

chloride and hydrogen chloride were obtained from Fisher. Acetonitrile and terbium chloride ($TbCl_3$) were purchased from Aldrich. EDTA was bought from GibcoBRL. Buffer A (containing 1 M NaCl, 2 mM $CaCl_2$, and 50 mM sodium citrate pH 6.2) was purchased from Hyclone. Milli-Q water (Millipore, Bedford, MA, USA) was used thoroughout all experiment procedures.

Instrumentation

Titration of EDTA with Tb(III) was performed using a spectrofluorimeter from Jobin Yvon-Spex instrument (Fluorolog-3 FL3–33). The experiments were done in a 0.2 × 1 cm quartz cuvette. The excitation wavelength was set to 230 nm with an excitation slit width of 5 nm. The emission was scanned from 500 to 600 nm to determine the emission wavelength maxima for Tb(III)-EDTA complex with a slit width of 10 nm. The Dionex HPLC system was used including GP40 gradient pump, autosampler AS3500, and UI20 Universal interface. Eluent Degas Module from Thermal Separation Products was used. The fluorescence detector from Shimadzu was employed for terbium luminescence detection. The above system was controlled by Peaknet software Version 5.1 from Dionex. The pH measurements of mobile phase and samples were done by Fisher Accumet basic pH meter. The 10 kDa NMWL filter was obtained from Amicon.

Chromatographic Conditions

An Aquasil C_{18} reverse-phase column (250 × 4.6 mm, 5 μm particle size) from Thermo-Keystone was used including a guard column (Aquasil C_{18}, 5 μm particle size). The HPLC was run isocratically for 10 min and the mobile phase contained 40 mM MOPS, 0.5 M NaCl with pH 6.1 adjusted by adding HCl. The column temperature was ambient (21–25°C) with a flow rate of 1 mL/min. The injection volume was 100 μL. The luminescence signal was detected with excitation at 240 nm and emission at 544 nm. The concentration of EDTA in the samples was determined by comparison with a calibration curve method using the peak area.

Sample Preparation

The terbium chloride stock solution was prepared by dissolving 1 g $TbCl_3.6H_2O$ in 5 mL of Milli-Q water and adding 2 μL of 1 N HCl. This stock gives approximately 0.536 M $TbCl_3$. The HCl was added to maintain the acidic condition of $TbCl_3$ solution, which otherwise would give white precipitates at pH >7. From this stock solution, a 25 mM $TbCl_3$ solution was prepared to be used for subsequent experiments.

An EDTA tetra salts (1 mM) stock solution was prepared in water and adjusted to pH to 6.5 by adding HCl. The EDTA standard solutions were prepared in buffer A with concentration range from 0.63–10 μM and a final $TbCl_3$ concentration of 0.75 mM in each EDTA standard. These standards were used for the establishment of linearity range, calibration curve, and the precision of the proposed method.

The vaccine product samples analyzed were from three intermediate manufacturing process called D0, D2, and D3 steps. The D0 sample was diluted appropriately in buffer A so it falls within the calibration curve, while D2 and D3 samples containing buffer A were neat. Terbium chloride (0.75 mM final concentration) was added to each sample. The recovery studies of the developed method were performed in D3 sample by spiking 1 μM or 3 μM EDTA final concentration.

All samples, including standards, were transferred to microcentrifuge with Ultrafree 10 kDa filter and centrifuged at 16000 × g for 50 min at room temperature to remove the vaccine product. The EDTA recovery before and after filtration was between 90–100%.

RESULTS AND DISCUSSION

EDTA Complexation with Tb^{3+}

Lanthanide ion, Tb^{3+}, emits a very weak luminescence due to a weak absorption band, but its luminescence quantum yield is greatly enhanced when it binds to a compound containing polycarboxylic acids groups such as EDTA or citrate. The kinetic of pre-column reaction of Tb^{3+} with EDTA is very fast and its complex, Tb(III)-EDTA is very stable. Figure 1 illustrates the titration curve of EDTA with Tb^{3+}, with the inset graph the luminescence spectrum of Tb(III)-EDTA complex, which has emission maximum at 544 nm with 230 nm excitation. These similar excitations and emissions are also employed in HPLC methods. The titration curve indicates that Tb^{3+} binding to EDTA is formed with 1 : 1 stoichiometry. All buffer matrices used for these experiments contain Ca^{2+} ions that also bind to EDTA but with 8–9 order of magnitudes lower in binding constant than Tb^{3+}. Therefore, a relatively high $CaCl_2$ (2 mM) in buffers does not interfere with EDTA measurement using Tb^{3+}.

HPLC Characteristics

Chromatographic separation of EDTA was performed on a C_{18} Aquasil column. MOPS buffer (40 mM) containing 0.5 M NaCl at pH 6.1 was used as a mobile phase with isocratic elution. A typical chromatogram of Tb(III)-EDTA complex in a standard EDTA sample is illustrated in Figure 2a. The

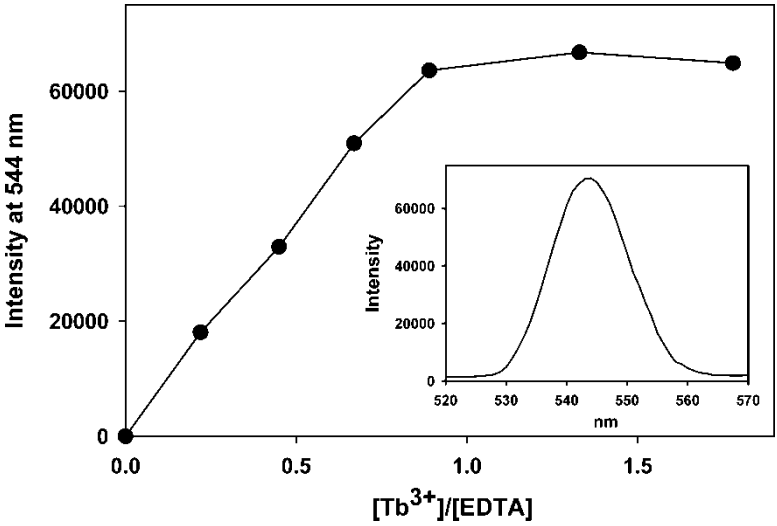

Figure 1. Titration curve of Tb(III) with EDTA indicates the formation of 1 : 1 complex of Tb(III)-EDTA. The typical luminescence spectrum of the Tb(III)-EDTA complex is illustrated in the inset.

Tb(III)-EDTA complex is eluted at 3.68 min, while a large preceding peak at 2.81 min is due to Tb(III)-citrate complex. The standard EDTA was dissolved in the same buffer as in a sample matrix that contains 50 mM sodium citrate, 1 M NaCl, and 2 mM $CaCl_2$. A Tb^{3+} concentration dependence study indicated that 0.75 mM of Tb^{3+} was more than sufficient to chelate all the EDTA available in all of our analyzed samples (data not shown). Even though the buffer matrix has a very high citrate concentration relative to residual EDTA, the Tb(III)-citrate stability constant ($\log K_{Tb\text{-}citrate} = 9.8$) is much lower than Tb(III)-EDTA ($\log K_{Tb\text{-}EDTA} = 18$).[12] Therefore, EDTA should all be chelated with Tb^{3+} even at very low concentration. Similarly, high concentration of $CaCl_2$ did not interfere with the Tb^{3+} binding to EDTA, since the stability constant of Ca^{2+} to EDTA ($\log K_{Ca\text{-}EDTA} = 10.6$)[12] is much lower than Tb^{3+} to EDTA.

Quantification Aspects of the Assay

Standard linear calibration curves were obtained for EDTA and showed linearity over the selected concentration range (0.63–10 μM) with slope ($(161.7 \pm 2.6) \times 10^5$), y-intercept ($(-1.85 \pm 1.9) \times 10^5$), numbers of data points on each calibration curve ($n = 5$), and correlation coefficients ($r^2 > 0.999$) throughout all experiments.

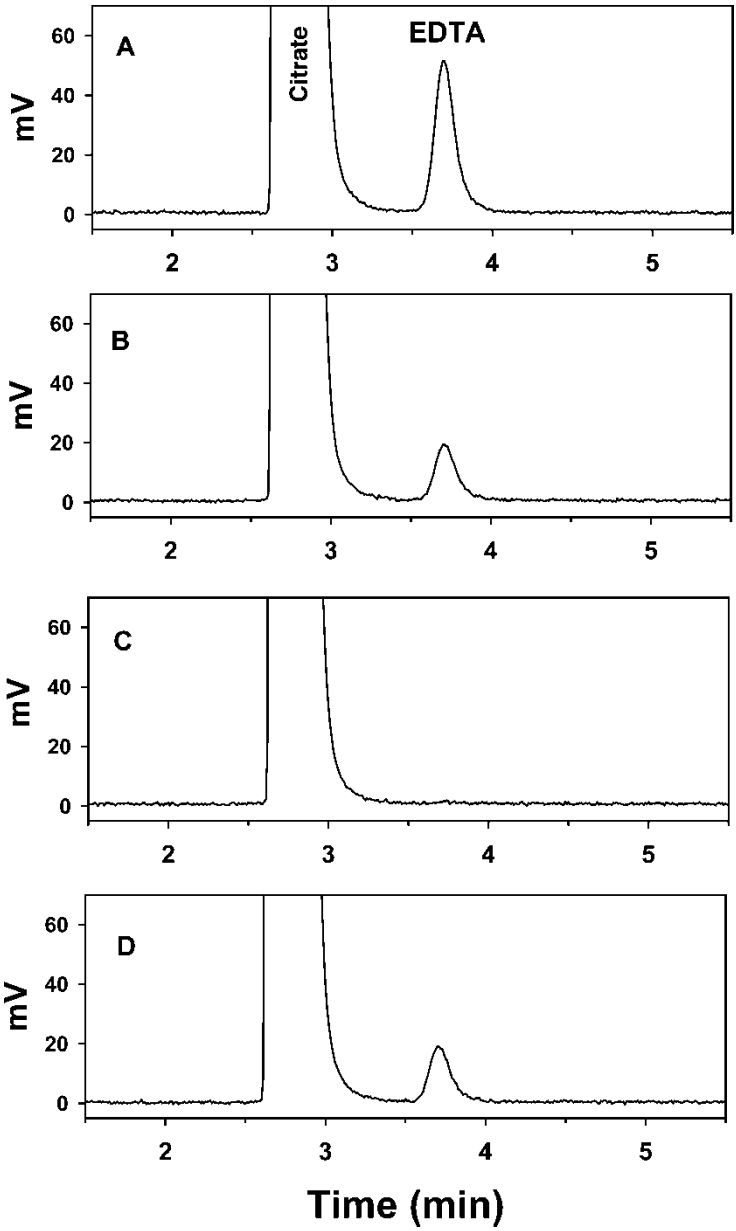

Figure 2. EDTA clearance in one set of samples from a single purified lot (lot X) was monitored, (A) EDTA standard at 2.5 μM; (B) EDTA analysis at the beginning of diafiltration (D0); (C) EDTA analysis after second diafiltration (D2); (D) EDTA analysis after the third diafiltration (D3) but in this example, the sample has been spiked with 1 μM EDTA.

The precision of the proposed new HPLC method for EDTA was obtained by performing intra- and inter-day assays for both standard and known EDTA-spiked sample (D0). The relative standard deviations of EDTA standard and sample were 1.1 and 0.92% for intra assay and 2.7 and 1.4% for inter assay, respectively. The accuracy of this method was determined by measuring the recovery of 1 and 3 μM EDTA spike samples that give 104% and 103% for mean recovery, respectively. The precision and accuracy are summarized in Tables 1 and 2. The limit of detection (LOD) of EDTA with this new method was about 87 ng/mL (0.21 μM) at a signal to noise ratio of 3, and the limit of quantification (LOQ) of EDTA was 260 ng/mL (0.63 μM) at a signal to noise ratio of 10.

Determination of EDTA in the Samples

Using the method described above, the residual EDTA was analyzed in many process intermediate samples. EDTA is added as part of vaccine process manufacturing for metal chelators. The EDTA clearance is performed through several diafiltration steps. Figure 2 shows typical EDTA analysis in sample matrix obtained from various manufacturing process steps. The initial step of adding EDTA into a sample matrix is called D0 (Figure 2b). EDTA is then removed through several diafiltration steps (D1, D2, D3, D4). The residual EDTA was analyzed at D2 and D3 steps (Figure 2c and 2d). Since, in almost all cases, the EDTA was cleared after the D2 step (Figure 2c), the spike recovery study of EDTA was performed at the D3 step (Figure 2d). Occasionally, residual EDTA is still observed in the D2 step or even D3 step as illustrated in Figure 3. Table 3 shows the typical EDTA results for some process intermediate samples.

Table 1. Precision of this EDTA HPLC assay

	Determined [EDTA] in μM from assay	
[EDTA] in μM	Mean ± SD	RSD (%)
Intra-day (n = 3)		
1	1.05 ± 0.012	1.1
2	1.97 ± 0.018	0.92
Inter-day (n = 3)		
1	1.02 ± 0.027	2.7
2	1.99 ± 0.028	1.4

Table 2. Accuracy of this EDTA HPLC assay

Sample	Spiked in μM	Determined in μM (n = 6)	Mean recovery (%)
D3	1	1.04	104 ± 2.5
D3	3	3.08	103 ± 2.7

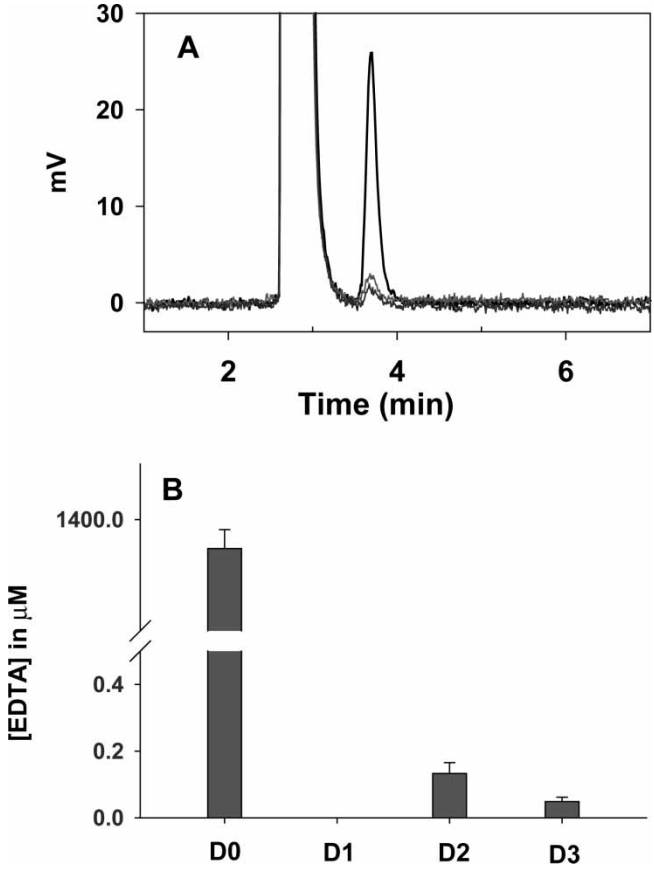

Figure 3. Another example of EDTA clearance from lot Z in which the EDTA was not completely removed even after D3. (A) EDTA clearance from initial D0 (black trace), D2 (red trace), and D3 (blue trace). The chromatogram intensities for D2 and D3 have been increased several fold for illustration purposes; (B) The bar graph representation for the chromatograms described in (A), note that EDTA analysis was not performed at D1 step.

Table 3. Typical EDTA analysis in some lots

Lot #	Diafiltration steps	[EDTA] in μM
X	D0	1250
	D2	<LOD
	D3	<LOD
Y	D0	1100
	D2	<LOD
	D3	<LOD
Z[a]	D0	1374
	D2	LOD
	D3	<LOD

[a]See figure 3 for the chromatograms and bar chart illustration.

CONCLUSIONS

The relatively simple new proposed EDTA assay using the terbium-sensitized luminescence method in pre-column HPLC proves to be very selective, sensitive, accurate, and precise. The new assay has a comparable sensitivity with Fe(III)-EDTA detection HPLC using absorbance at 258 nm, but Tb(III)-EDTA complex is stable in room temperature and light for at least one week, while Fe(III)-EDTA is unstable in the presence of light. Furthermore, the luminescence detection removes many potential matrix interferences as compared to absorbance detection at 258 nm, which is normally performed for Fe(III)-EDTA complex. Furthermore, the assay requires minimal sample manipulation and yields relatively fast chromatographic elution (<4 min), resulting in high sample throughput to allow monitoring of the clearance of residual EDTA during bioprocess manufacturing.

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