Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 792 (2003) 205–215
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Monitoring EDTA process residuals in recombinant protein manufacturing using liquid chromatography

Miao-Fang Lin[a], Mabel Royal[b], Kirk Hayenga[c], Greg Conn[b,*]

[a]*Department of Purification Process Development, Diosynth-RTP, 3000 Weston Parkway, Cary, NC 27513, USA*
[b]*Department of Pharmaceutical Sciences, Diosynth-RTP, 3000 Weston Parkway, Cary, NC 27513, USA*
[c]*Hyseq Pharmaceuticals Inc., Sunnyvale, CA 94085, USA*

Received 6 August 2002; received in revised form 29 October 2002; accepted 24 March 2003

Abstract

We have developed a chromatographic method for the high sensitivity quantitation of EDTA process residuals in recombinant protein manufacturing validation studies. The reversed-phase HPLC method is based upon the detection of Cu^{2+}/EDTA complexes at 254 nm, and has been qualified for use on intermediates from a purification process for a recombinant protein expressed in *E. coli*. Quantitation of EDTA in recombinant protein process intermediates is linear in the range of 0.2 to 64 μ*M* with LOD/LOQ values below 2.0 μ*M*. The assay is suitable for use in process backgrounds containing Tris, HEPES, MES, NaCl, hexanediol, NH_4SO_4, and PEG. EDTA spike recovery values in all process samples tested were greater than 90% at the 4.0 μ*M* concentration. System suitability parameters for the chromatographic method were developed based upon peak area and retention time precision, column efficiency and USP tailing. Peak area precision and intermediate precision values across the linear range of the assay exhibited C.V. values less than 15% at any concentration tested in all sample backgrounds. The assay robustness was tested by transfer of the assay to a second laboratory and analyst with use of multiple process intermediate lots, reagent/column lots, and HPLC systems.

Keywords: Process residuals; Ethylenediaminetetraacetic acid; Recombinant proteins

1. Introduction

Manufacturing processes developed to produce recombinant protein therapeutics for human use must be validated for clearance of any residual potentially toxic process additives [1,2]. A common process additive during recombinant protein isolations is the metal ion chelator, ethylenediaminetetraacetate (EDTA). EDTA is widely used in recombinant protein manufacturing to inhibit product degradation by host cell-derived metalloproteases. A successful method for EDTA quantitation suitable for use in recombinant protein manufacturing process residual clearance validation studies must be sensitive, accurate and applicable to measurements in process intermediates which vary in buffer components, pH, salts and protein concentration and contain *E. coli* contaminants.

A variety of analytical methods have been developed for the quantitative and qualitative determination of EDTA or metal ion/EDTA complexes. They include UV spectroscopic methods for metal

*Corresponding author. Tel.: +1-919-388-5651; fax: +1-919-677-0937.
E-mail address: greg.conn@diosynth-rtp.com (G. Conn).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00260-5

ion/EDTA estimation in water and serum and EDTA in commercial purified protein [3–5], colorimetric assays [6,7], titrimetic analyses [8–12], thin layer chromatography [13,14] and atomic absorption for EDTA in blood or urine [15]. More sophisticated methods for determination of EDTA utilize polarography [16], coupling tandem mass spectrometry with capillary electrophoresis of plasma, urine and environmental water samples or ion chromatography of plant xylem or bloodstains [17–22], and isotachophoretic rare earth and Fe(III)/EDTA complex measurements with particle-induced X-ray emission [23]. Chromatographic assays for EDTA include quantitation of palmitic esters of EDTA by gas–liquid chromatography [24], determination of Cu^{2+}/EDTA complexes in ophthalmic drug preparations by anion-exchange chromatography [25] or isocratic variations on the USP nitrilotriacetic acid assay [26], and ion-exchange chromatography of divalent metal ion complexes of EDTA in waste water [27,28]. Fluorescent complexation analysis of EDTA with terbium/salicylate in serum or urine samples [29,30] has also been reported.

Our choice of an analytical method to validate EDTA clearance during recombinant protein manufacturing was predicated upon several considerations. The method would be utilized both during process validation and GMP product release testing, and therefore should involve technology readily available and familiar in most quality control environments, ensuring ready transferability of methods from analytical development to manufacturing and testing sites. Methods involving use of instrumentation or techniques which may not be familiar and routinely used in most quality laboratories, such as tandem CE–MS, isotachophoresis, polarography, etc. could not be used. Samples for analysis would be heterogenous as to buffer, pH, salts, organic modifiers, protein concentration and *E. coli* cell-derived material. Potential assay interferences due to sample complexity necessitated use of a method with high specificity for EDTA, precluding use of simple spectrophotometric, colorimetric or titrimetric assays. Studies in our laboratory attempting to apply such an assay, the recently reported fluorescent terbium/salicylate/EDTA method [29,30], to our samples yielded very low sensitivities in most process intermediates compared to results reported with serum and urine (data not shown). Additionally, techniques requiring processing of samples by chemical derivatization, digestions or precipitation were considered inappropriate not only because of laboratory to laboratory method reproducibility concerns but also since the efficiency of chemical or physical sample processing might be affected by sample heterogeneity.

Our experience is that the most reliable, cost effective and successful analytical methods applied in a quality environment are often the simplest for operators to understand and execute; we therefore chose a technique familiar to most analytical laboratories, HPLC. Although several chromatographic methods for EDTA determination have been reported in the literature, none reported application to EDTA determinations in *E. coli* lysates or manufacturing intermediates. HPLC methods reported in the literature involved analysis of water samples, urine, serum or highly purified samples such as ophthalmic preparations. We chose the most commonly applied chromatographic method, the isocratic reversed-phase determination of nitrilotriacetic acid in EDTA, referenced in the USP [31], as the basis for our assay. Based upon the formation of a Cu(II)/EDTA complex by treating process samples with cupric nitrate, the EDTA complex is separated from the biological products and other interfering substances and quantitated using reversed-phase chromatography, with detection by UV absorbance at 254 nm.

Dual concerns in applying a modification of this method to our process were ensuring adequate sensitivity to meet acceptance criteria for residual clearance and providing full resolution of EDTA from process intermediate components to ensure assay specificity. EDTA is typically used in recombinant drug manufacturing at low m*M* concentrations. Due to the chelating and anticoagulant activities of EDTA, toxicity during drug delivery can lead to thrombocytopenia, kidney toxicity, convulsions, insulin shock and at high levels, death [6]. Our client set maximum residual levels for EDTA at process steps subsequent to the initial two-phase extraction at 12 ppm, or 12 μg/ml, ~36 μ*M*. The LD_{50} (intravenous) for EDTA in the mouse is ~28 mg/kg, or 2 g for a 70 kg adult human; for a 10-ml injection a residual level of 12 μg/ml will yield an EDTA injection ~16,000-fold below the LD_{50}. Our target

Table 1
Process intermediate compositions

Sample	Protein (mg/ml)	Buffer composition	pH
A	1	6.25% NH_2SO_4, 6.25% PEG 75 m*M* Tris	7.2
B	1	25% Hexanediol, 50 m*M* Tris	7.2
C	5	25 m*M* HEPES	7.0
D	1	25 m*M* MES, 45 m*M* NaCl	6.4
E	10	100 m*M* HEPES	7.7

LOQ was therefore set during assay development at least 10-fold below this value to ensure the assay would meet ICH validation guidelines [1,2], or 100 ng/0.1 ml injection. In order to meet requirements for both high sensitivity and specificity, we converted the standard isocratic reversed-phase USP method to a microbore gradient method intended to allow increased sample injection volume for high sensitivity and full resolution of the Cu^{2+}/EDTA complex peak from process and *E. coli*-derived materials. For the development and qualification of this method for use in process clearance validation, we used a series of buffers from recombinant protein process manufacturing intermediates and actual manufacturing process intermediates (Table 1).

2. Experimental

2.1. Materials

EDTA disodium dihydrate, tetrabutyl ammonium hydroxide, Tris, MES and HEPES were from Sigma (St. Louis, MO, USA). Cupric nitrate trihydrate was from Fluka (Milwaukee, WI, USA). EDTA, methanol, and phosphoric acid were from J.T. Baker (Phillipsburg, NJ, USA). HPLC methods were run on an Agilent 1100 series HPLC system, consisting of a G1312A binary pump, a G1316A column compartment and a G1329A autosampler with an on-line vacuum degasser (G1322A) and a variable injection capacity from 1 to 100 μl. A G1315A UV–visible diode array detector was used (254 nm) for analyte detection. The column used was an Eclipse 2.1×150 mm XDB-C_8 column with 5 μm particle size (Agilent) with a precolumn filter (0.5 μm, Phenomenex). The column temperature was controlled at 60 °C. Chromatograms were recorded using HP Chemstation software version 8.03.

2.2. Chromatographic parameters

All chromatographic data analysis was done using Agilent Chemstation software version 8.03 in extended performance format. Peak areas were calculated and reported as mAUs. No internal control for retention was used; retention time values in minutes are reported from time of injection. Peak widths were determined at half-height and column efficiencies were calculated by the half-width method. Peak tailing was reported as USP tailing. Peak symmetry was calculated as the ratio of the sum of the retention time of the peak apex plus that of the front peak inflection point divided by the sum of the retention time of the peak apex plus that of the rear peak inflection point. All mathematical formulae used for chromatographic parameter calculations are detailed in Agilent Technologies ‘‘Evaluating System Suitability, CE, GC, LC and A/D Chemstation, Revisions: A.03.0–A.08.0’’.

2.3. HPLC method

The HPLC method we developed to quantitate residual EDTA in recombinant protein samples is derived from basic concepts described in the US Pharmacopeia for the determination of nitrilotriacetic acid in EDTA [31]. A 3.2 m*M* EDTA stock solution and a 5 m*M* cupric nitrate solution were prepared in MilliQ water. Serial dilutions of the EDTA stock solution were made using MilliQ water or process buffers (listed in Table 1) and 5 m*M* cupric nitrate to obtain a final concentration of cupric nitrate of 0.1 m*M*.

HPLC mobile phase A consisting of 0.25% (v/v) tetrabutyl ammonium hydroxide (TBAH) and 4.4% methanol (pH 7.5) was prepared by adding 6.25 ml of TBAH and 3.75 ml of methanol to 190 ml of MilliQ water and titrating to pH 7.5 using 1 *M* phosphoric acid, adding 40 ml methanol, and bringing the final volume to 1 l with MilliQ water. Mobile phase B, 0.25% TBAH, 79.4% methanol at pH 7.5, was prepared the same as mobile phase A, except the final volume was brought to 1 l using methanol. The mobile phases were filtered and degassed prior to

use. The chromatographic separation was performed using a gradient at 1 ml/min flow-rate (0–8 min 0–50% B, 8–11 min at 50% B, 11–12 min 50–100% B, 12–20 min at 100% B, 20–21 min 100–0% B, and 21–35 min at 0% B). The PDA detector was set at 254 nm.

2.4. Study design

A set of process intermediate buffers and actual process intermediates from a recombinant protein manufacturing process were chosen to study the sensitivity and accuracy of the EDTA test method using spike/recovery analysis. The recombinant protein purification process utilized product expression in *E. coli* host cells as a soluble periplasmic protein. Five purification process steps were chosen for study. The first process pool tested was from a two-phase product extraction of membrane-solubilized cells in a buffer containing polyethylene glycol, ammonium sulfate and Tris. The second intermediate was a reversed-phase purification eluate containing the organic hexanediol in Tris buffer. The final three process intermediates were an anion-exchange product pool in HEPES buffer, a cation-exchange process pool in MES buffer and the final bulk intermediate product in HEPES buffer (Table 1).

The range of pH values for the five purification process buffers was 6.4–7.7. Final concentrations of process-derived buffer salts in the Cu^{2+}-treated samples ranged from 12.5 to 50 m*M* with no preparatory sample dilutions. Total protein concentrations including the recombinant product ranged from 1 to 10 mg/ml in the process intermediates. We added a series of process buffer and intermediate sample dilutions to the EDTA spike/recovery studies to identify and ameliorate any buffer or protein assay interferences.

This recombinant protein purification process was chosen for study as an initial addition of 5 m*M* EDTA is made at the *E. coli* cell wall permeabilization step to inhibit host cell metalloproteases. An acceptable method suitable for use in establishing EDTA process clearance validation would be required to yield accurate EDTA measurements at each process step from the initial EDTA concentration of 5 m*M* to values 3–4 logs lower in concentration (μ*M* levels). We set our EDTA spike/recovery test levels in the range of 1 to 16 μ*M*. We studied process intermediates from the process run both with and without the initial EDTA addition.

Subsequent to completion of assay development, initial chromatographic maximization and sample treatment studies, we initiated a method qualification study following ICH validation guidelines (1,2) using the same protein process buffers and sample intermediates.

3. Results and discussion

3.1. HPLC method development

The USP assay from which we developed our HPLC method specifies quantitation of EDTA/Cu^{2+} complexes by isocratic reversed-phase chromatography on a reversed-phase column using a TBAH–methanol buffer system. We investigated appropriate maximum sample loading volume for the isocratic USP method to establish the assay sensitivity limits. Fig. 1 shows an overlay of a 10 and 50 μl injection of sample containing 5 m*M* EDTA/Cu^{2+} complex. The serious peak width increases noted with sample volumes above 10 μl limit sample loading and assay

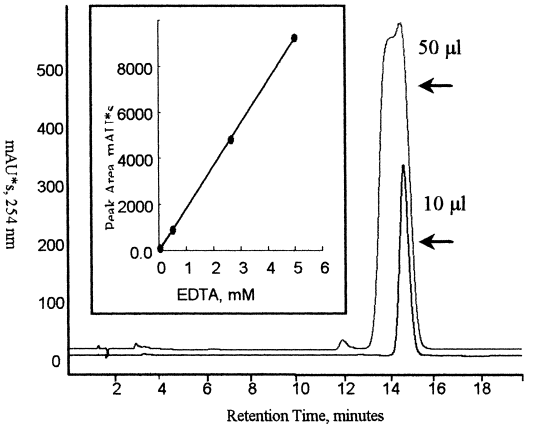

Fig. 1. EDTA/Cu^{2+} assay sample load volume comparison using isocratic reversed-phase elution. EDTA/Cu^{2+} complex at 1 m*M* was analyzed according to the USP method for the determination of nitrilotriacetic acid in EDTA (32) at 10 and 50 μl injections. The isocratic assay linearity over the test range of 0 to 5 m*M* is shown (insert) at the 10 μl injection volume (R^2>0.99, LOD/LOQ 44/134 μ*M*).

sensitivity for the isocratic method. Fig. 1 (insert) shows an assay linear calibration curve for the isocratic method (R^2>0.99). We calculated the limit of quantitation (LOQ) and limit of detection (LOD) from the equations below, where S is the slope of the calibration curve and d is the standard deviation of y-intercepts of the regression line. LOD and LOQ values for the isocratic assay were 44 and 134 μM, respectively

$$\mathrm{LOQ} = \frac{10 \times d}{S} \quad (1)$$

$$\mathrm{LOD} = \frac{3.3 \times d}{S} \quad (2)$$

An additional limitation for this method was the difficulty we found in column cleaning for protein-containing samples between runs. Inadequate protein removal between analyses led to short column lifetimes and chromatographic peak distortions (data not shown). These factors necessitated identification of a more suitable column packing amenable to repetitive injections and cleanings of protein sample pools from *E. coli* extractions and a modification of the reversed-phase system for gradient sample elution to increase sample load and sensitivity. We decided to retain the TBAH buffer system and methanol as the organic eluant for gradient reversed-phase analysis.

Screening numerous reversed-phase packings for the ability to resolve the EDTA/Cu^{2+} complex from *E. coli* process pool contaminants using gradient elution in the TBAH buffer led to use of the Eclipse XDB C_8 2.1×150 mm column. The packing had the additional advantage of low levels of proteinaceous carryover run to run, resulting in longer column lifetimes analyzing *E. coli* extracts for EDTA. The EDTA metal complex elutes in our method at ~7 min retention time (Fig. 2), allowing fast chromatographic cycling time including column cleaning.

The relatively steep gradient slope (3.75% organic/min) and an elevated column temperature of 60 °C were chosen to minimize the EDTA complex peak width, increasing sensitivity compared to the USP isocratic method. An additional advantage compared to the USP method is that sample load volumes for the gradient analysis can reach at least

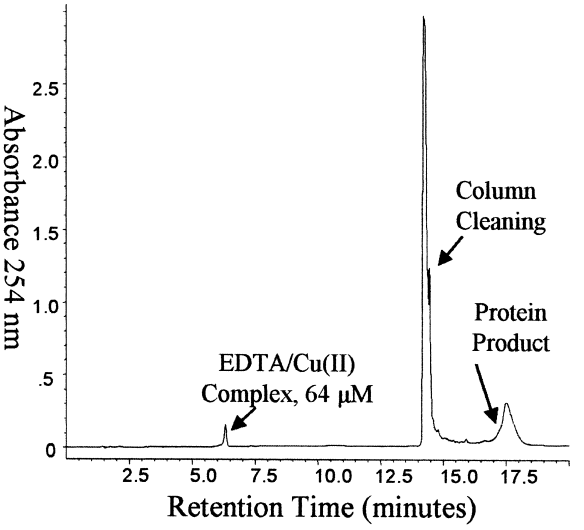

Fig. 2. EDTA/Cu(II) complex analysis by gradient reversed-phase analysis. A sample from the final recombinant protein purification process step (step E) was spiked with EDTA at 64 μM, treated with cupric nitrate and analyzed by gradient reverse chromatography. The sample was injected in a volume of 0.1 ml.

0.1 ml with no apparent degradation in peak shape, increasing assay sensitivity.

3.2. Chromatographic system suitability parameters

To establish appropriate system suitability standard and acceptance criteria for the assay a series of 49 injections of 0.1 ml of 16 μM EDTA prepared in process buffer C (25 mM HEPES, pH 7.0) were statistically analyzed. These samples were analyzed on four HPLC systems, using three column lots, by two operators over a period of several weeks. The resultant chromatograms were analyzed for EDTA peak retention time, peak area, peak asymmetry, peak width (half-height method), column efficiency (half-width method) and USP tailing (Table 2). Mean peak retention time measured was 6.6 min with a standard deviation of 0.2 min and a % C.V. of 3.3%. It was noted that new columns that had not been used to analyze actual process samples exhibited peak retention times at the low extreme of the range (6.0 to 6.8 min). After several cycles of sample analysis and column cleaning, peak retention times stabilized at the mean value for the assay. The

Table 2
Chromatographic system suitability study

Chromatographic parameter[a]	Mean value ($n=49$)	SD	% C.V.
Retention time	6.6 min	0.2	3.3
Peak area	297 mAUs	16.2	5.4
Peak width (half-height)	0.94	0.004	5.0
Peak asymmetry	1.46	0.26	18.0
Column efficiency (half-width method)	27 292	1170	4.2
USP tailing	0.78	0.12	15.0

[a] 16 μ*M* EDTA, 0.1 ml injections. System suitability acceptance criteria were set at ±2 SD from the mean for peak area, retention time, column efficiency, peak width and USP tailing.

average peak area determined was 297 mAUs for a 16 μ*M* sample with a % C.V. of 5.4%. The mean peak width at half height was 0.94 (5.0% C.V.). Peak asymmetry measurements yielded a value of 1.5 with a C.V. of 18% due to a front shoulder on the peaks noted on columns heavily treated with proteinaceous samples. Column efficiency measured as plate number yielded a mean value of 27 512 with a % C.V. of 4.2%. USP tailing for all analyses was determined to be 0.775 with a % C.V. of 15%. These measurements and values were used to establish assay system suitability acceptance criteria for all subsequent assay qualification studies (Table 2).

Since peak retention time and peak area are dependent upon HPLC system mixing and post column volumes and detector sensitivities, these system suitability parameters were specified for use only for analyses performed within our organization on identically configured Agilent HPLC systems subjected to GMP IQ/OQ/PQ and maintenance. The range for acceptance criteria for system suitability parameters, including USP tailing, peak width and column plate number were set at two standard deviations from the mean values determined in our study. Degradation in column performance, as indicated by failure to meet minimum system suitability criteria, was normally attributed to extensive processing of samples of the crude *E. coli* extract leading to column fouling. Columns that failed system suitability criteria after use were ordinarily discarded and replaced by a new column rather than attempting cleaning to bring their performance back within specification, as a matter of cost control. Peak symmetry values, although not specified as system suitability acceptance criteria for the process validation study, were monitored for trend analysis since changes in the EDTA/Cu^{2+} complex peak shape had been noted during assay development as columns were exposed to crude *E. coli* samples.

3.3. Assay qualification linearity and limits of quantitation and detection

The linear range of the assay was determined by analyzing EDTA spikes in the five process intermediate buffers and process intermediates at concentrations of 0.0, 0.2, 0.5, 1.0, 4.0, 16.0 and 64 μ*M*. All EDTA concentrations were analyzed in triplicate. The method shows very good linearity at low concentrations of EDTA (0.2 to 64 μ*M*) in all five process buffers and intermediates studied, with correlation coefficient values ≥0.999 and % C.V. for the mean correlation coefficient below 0.01%. We calculated the limit of quantitation (LOQ) and limit of detection (LOD) from the residual standard deviation in the *y* intercepts using Eqs. (1) and (2). In any process sample tested, LOQ and LOD were ≤2.0 and 0.7 μ*M*, increases of 17- and 48-fold, respectively, over the LOD/LOQ values for the USP assay. The mean slope of the linear EDTA calibration in the process intermediates was 18.17 with a standard deviation of 0.67 and a % C.V. of 3.69 ($n=3$ for each buffer or pool). The mean *y*-intercept was −2.15 with a standard deviation of 4.50 and a % C.V. of 2.12 ($n=3$ for each buffer or pool).

3.4. Assay peak area and retention time precision/intermediate precision

EDTA was spiked into each of the process buffers and actual process intermediates at the 1, 4 and 16 μ*M* levels and analyzed. Table 3 summarizes peak area and retention time precision data from six replicate analyses performed by a single analyst on a single HPLC system in 1 day for the three process intermediates A, B and C. The % C.V. for analysis of EDTA peak area in the top phase process intermediate (A) ranged from 3.7 to 8.7 across the range of concentrations tested; the % C.V. for peak area in

Table 3
(a) EDTA/Cu^{2+} peak area precision, analyst #1 day #1; (b) EDTA/Cu^{2+} retention time precision, analyst #1 day #1

Process intermediate	EDTA (μM)	Mean peak area	SD	C.V. (%)
(a)				
A, $n=6$	1	18.6	0.69	3.7
B, $n=6$	1	21.0	1.82	8.7
C, $n=6$	1	20.3	0.80	3.9
Mean ABC[a]	1	19.9	1.53	7.7
A, $n=6$	4	77.9	0.69	0.9
B, $n=6$	4	79.8	2.22	2.8
C, $n=6$	4	76.9	0.86	1.1
Mean ABC[a]	4	78.2	1.83	2.3
A, $n=6$	16	301.2	1.89	0.6
B, $n=6$	16	299.9	1.25	0.4
C, $n=6$	16	308.2	2.46	0.8
Mean ABC[a]	16	303.1	4.19	1.4
(b)	Mean retention time (min)			
A, $n=18$	6.59		0.02	0.3
B, $n=18$	6.80		0.01	0.1
C, $n=18$	6.43		0.04	0.6
Mean ABC[b]	6.61		0.03	0.5

[a] $n=18$.
[b] $n=54$.

Table 4
Retention time and peak area intermediate precision, process pools A, B and C, at 1, 4 and 16 μM

Process pool	Mean retention time (min)	SD	C.V. (%)	
A, $n=30$[a]	6.6	0.02	0.3	
B, $n=30$[a]	6.7	0.19	2.8	
C, $n=30$[a]	6.4	0.07	1.1	
Mean ABC $n=90$[b]	6.6	0.15	2.3	
	EDTA (μM)	Peak area (mAUs)	SD	C.V. (%)
A, $n=10$	1	18.4	0.87	4.7
B, $n=10$	1	20.0	2.82	14.1
C, $n=10$	1	20.3	0.80	3.9
Mean, $n=30$	1	19.6	1.90	9.7
A, $n=10$	4	77.9	0.77	1.0
B, $n=10$	4	78.9	3.43	4.3
C, $n=10$	4	76.5	0.98	1.3
Mean, $n=30$	4	77.8	2.30	2.9
A, $n=10$	16	302.0	1.82	0.6
B, $n=10$	16	298.7	2.45	0.8
C, $n=10$	16	307.0	7.04	2.3
Mean, $n=30$	16	302.6	5.50	1.8

[a] Retention time mean, all 30 analyses at 1, 4 and 16 μM.
[b] Overall retention time mean from pools A, B and C, analyses at 1, 4 and 16 μM.

the more highly purified UF/DF #1 (C) process intermediate ranged from 0.4 to 0.8 across the concentration range tested. The overall mean peak area at each of the three test concentrations of 1, 4 and 16 μM was also calculated by averaging the peak areas at each test concentration from process intermediates A, B and C (Table 3). The % C.V. values for these mean peak areas ranged from 7.7% at the lowest concentration (1 μM) to 1.4% at the high concentration (16 μM). The overall % C.V. for retention time based upon the average of all 54 sample injections in all three process intermediates at the three test concentrations was 0.5% (Table 3).

Intermediate precision for EDTA peak area and retention time was estimated by averaging a total of 10 injections at each of the three EDTA test concentrations in each of three process intermediates (Table 4). Three different lots of each process intermediate were utilized for these studies, which were performed by two analysts on two HPLC systems on 3 separate days. Data for peak retention time from all three test EDTA concentrations for each of the three process intermediates were pooled for analysis (Table 4). An overall mean retention time of 6.6 min with a % C.V. of 2.3% was determined for all 90 injections at 1, 4 and 16 μM in all three process intermediates, indicating good intermediate precision for retention time. All 10 injections for each process intermediate at each of the three test concentrations were pooled for peak area statistical analysis. Table 4 shows a comparison of the mean EDTA peak areas for process intermediates A, B and C. C.V. values for peak area at all three EDTA concentrations tested for all process intermediates are less than 15.0%. Overall mean peak areas were calculated by combining data at each test concentration for all three process intermediates ($n=30$). The % C.V. for peak area at the three test

concentrations was 9.7, 2.9 and 1.8% for the 1, 4 and 16 μ*M* concentrations, respectively. The data indicate good intermediate precision in peak area determinations even at the lowest test concentration.

3.5. Assay robustness

Analytical robustness was studied during the assay qualification by replicating qualification tests in two different laboratories. Each laboratory performed identical sequences of test analyses, including spike/recovery and standard EDTA linearity determinations. Chromatographic sequences in both laboratories included identical standards and system suitability tests. Each laboratory utilized an identically configured Agilent HPLC system, but used three different column lots, and testing was performed by two analysts. Three manufacturing lots of each process intermediate were used for the testing. Testing ranged over a period of several weeks. Data from both laboratories were pooled for analysis. The system suitability data presented in Table 2, derived from the pooled data from both laboratories using EDTA standard in buffer at the 16 μ*M* test level, indicates peak retention at 6.6 min with a % C.V. of 3.3%. This compares well with the mean data from both laboratories on EDTA in actual process intermediates in Table 4, where peak retention was 6.6 min with a % C.V. of 2.3%. Similarly, the EDTA standard peak area data at 16 μ*M* derived from both laboratories in Table 2 of 297 mAUs with a % C.V. of 5.4% compares well with the intermediate precision data from both laboratories in actual process intermediates in Table 4 at 16 μ*M* of 303 mAUs with a % C.V. of 1.8%. The good intermediate precision data from both laboratories in all the process pools and with the EDTA standard in buffer alone, including process intermediate A which is heavily contaminated with *E. coli*-derived material, indicates that the assay is transferable from laboratory to laboratory with confidence in good comparability of results.

3.6. Assay sensitivity/accuracy by EDTA spike recovery testing

Spike/recovery tests of EDTA/Cu^{2+} complex formation and quantitation in the five process buffers at low μ*M* concentrations indicated sample dilutions were necessary in both the two-phase extraction buffer and the reversed-phase buffer (process intermediates A and B). A 25-fold dilution of the top phase process intermediate was required to bring EDTA levels down from the process concentration (0.8 m*M* after process dilution) into the linear range of the assay. Top phase samples diluted in this manner reached >90% EDTA spike recovery at the 4 μ*M* spike concentration (Fig. 3A). Using the minimum LOQ value of 2.0 μ*M* EDTA determined from the residual standard deviation of the *y*-intercepts from the assay linearity determinations, the assay can establish a process step clearance of 400-fold from the initial *E. coli* extract EDTA concentration of 0.8 m*M* (after process dilution, starting concentration, 5 m*M*).

The presence of an organic modifier in the second process intermediate buffer necessitates a sample dilution at this step of 1:4 to ensure proper EDTA/Cu^{2+} complex binding to the analytical reversed-phase column (Fig. 3B). HPLC analysis of reversed-phase intermediate samples (intermediate B) without at least a 1:4 dilution to lower the content of organic modifier show poor or irreproducible retention of the EDTA peak on the analytical column (data not shown). This required dilution lowers sensitivity at this step from the maximum 2.0 μ*M* LOQ established by the assay linearity testing to 8.0 μ*M*, still below the 32 μ*M* minimum residual level specified by our client for samples from this step. Standard curves (see Section 3.3) prepared in process buffers A (25× dilution), B (5× dilution) and C (no dilution) show good linearity across the test range from 0 to 64 μ*M* EDTA, with very similar slopes and good intermediate precision. These three standard curves are comparable to curves generated at the same EDTA levels prepared in MilliQ water, and process buffers D and E (see Section 3.3, no dilutions). The maximum calculated limit of detection under these conditions for any of the five process buffers was 0.7 μ*M* and the LOQ was 2.0 μ*M*.

EDTA spiking studies to evaluate the recovery of EDTA in actual process sample intermediates A, B and C, spiking at 1, 4 and 16 μ*M*, are shown in Table 5. The data summarizes the percent EDTA spike recovery for three different lots of each sample

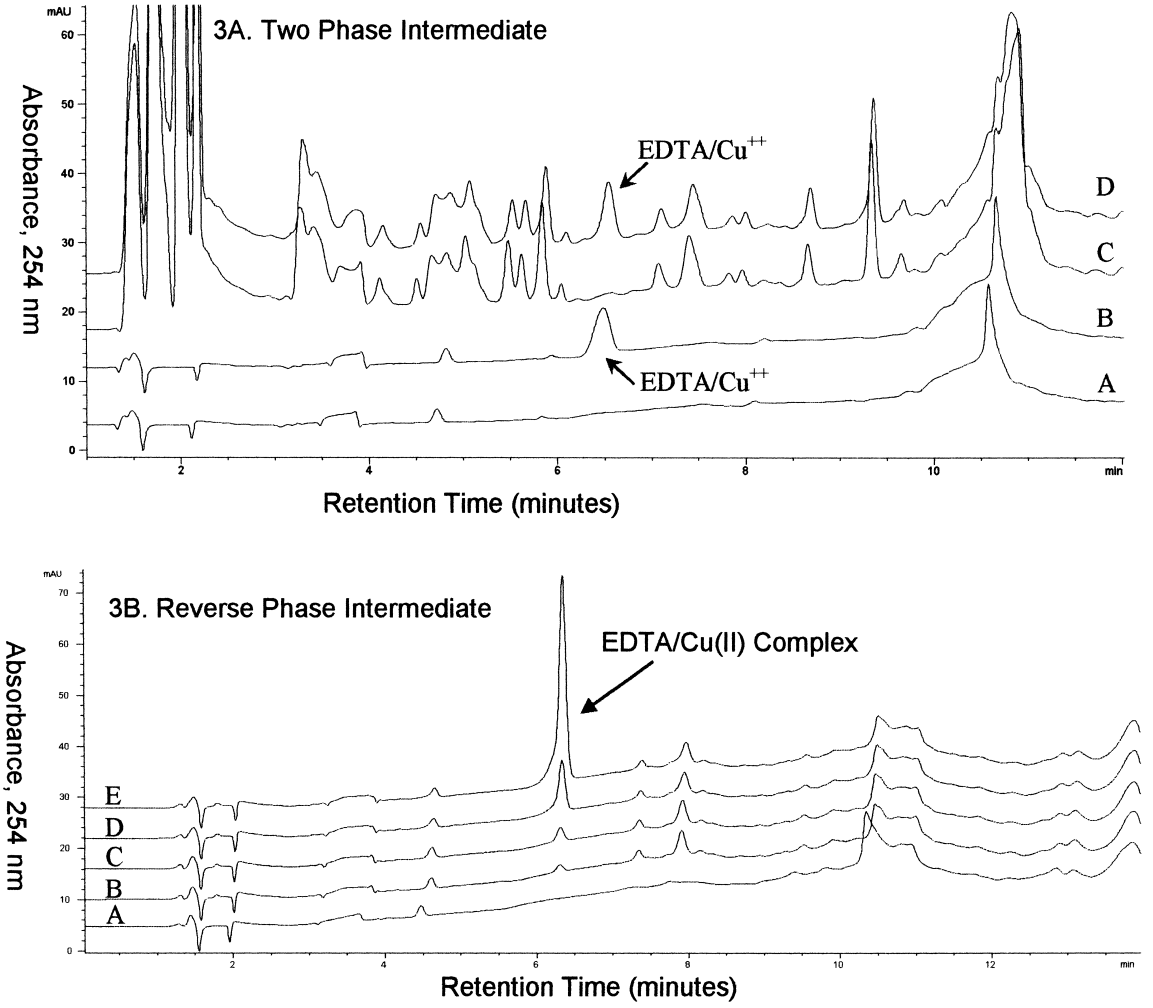

Fig. 3. EDTA/Cu(II) complex analysis by gradient reversed-phase chromatography. Process intermediate derived from two-phase extraction (intermediate A) was spiked with EDTA at 4 μ*M*, treated with cupric nitrate and analyzed by reversed-phase HPLC (plot A, buffer, no EDTA; plot B, buffer plus EDTA; plot C, intermediate A, no EDTA; plot D, intermediate A plus EDTA). (B) Process intermediate derived from reversed-phase purification (intermediate B) spiking with EDTA. Samples at 0.5, 1.0, 4.0 and 16.0 μ*M* EDTA, treated with cupric nitrate and analyzed by reversed-phase HPLC (plot A, buffer, no EDTA; plot B, 0.5 μ*M* EDTA; plot C; 1.0 μ*M* EDTA; plot D, 4.0 μ*M* EDTA; plot E, 16.0 μ*M* EDTA).

intermediate analyzed in triplicate. Residual background EDTA measured in sample A was 0.8 m*M* (the initial process step was at 5 m*M* EDTA, and was subjected to a dilution during the manufacturing process). There was no background EDTA detected above the LOQ for the assay in the subsequent down-stream process product intermediates without spiking. Similarly high EDTA spike/recovery values were obtained for the other two process intermediates (D and E, data not shown). These results indicate that this method is suitable for the analysis of EDTA in samples containing up to 10 mg/ml recombinant protein, including early stage process samples containing significant percentages of *E. coli*-derived host cell proteins and contaminants. The data in Table 5 on EDTA spike/recovery indicate good assay accuracy at least down to the 1 μ*M* test concentration.

Table 5
Percent EDTA spike recovery in process intermediates

Process intermediate	Lot	% EDTA spike recovery		
		1 μ*M*	4 μ*M*	16 μ*M*
A	1	93	91	97
	2	101	102	97
	3	97	101	98
B	1	80	98	106
	2	85	94	104
	3	104	97	101
C	1	92	95	97
	2	97	97	98
	3	92	96	98

4. Conclusions

The HPLC assay we developed to validate recombinant protein manufacturing process clearance of residual EDTA demonstrates an ~20-fold enhancement in sensitivity over the USP method for nitrilotriacetic acid determination we used as a basis for the assay. The method was designed to allow large sample volume injections with narrow peak width to improve assay sensitivity. It has been shown to be useful in process samples containing Tris, HEPES, and MES buffers, NH_4SO_4 and NaCl salts, PEG and high levels of *E. coli*-derived and recombinant protein. The assay yields accurate measurements of EDTA at low μ*M* concentrations and allowed us to establish process clearance of residual EDTA to 3 logs below the process starting concentration in the later process steps. In the first process step, consisting of a crude *E. coli* cell extract clarified by a two-phase extraction using NH_4SO_4 and PEG, we could accurately determine EDTA at 2 logs below the EDTA process treatment level of 5 m*M*. In the subsequent reversed-phase eluant pool from the purification process, we were able to measure EDTA at levels 500-fold below the starting process level. The chromatographic assay as designed is rapid, with total cycle time of 30 min per analysis. The reversed-phase column and buffer chosen for the assay were robust, with multiple column lots showing only small changes in peak width and tailing after hundreds of injection/cleaning cycles with complex samples. We are planning to apply the method to monitoring EDTA in protein process pools derived from other typical organisms utilized for recombinant expression such as yeast, baculovirus infected cells or mammalian cell types. The very early elution position of the EDTA/Cu(II) complex should allow routine monitoring of EDTA in most highly purified recombinant protein process pools, since recombinant protein products are expected in general to exhibit greater hydrophobicity and column retention.

5. Nomenclature

C.V.	coefficient of variation
HEPES	hydroxyethyl piperazine ethanesulfonic acid
ICH	International Committee on Harmonization
LOD	limit of detection
LOQ	limit of quantitation
MES	morpholino ethanesulfonic acid
PDA	photo diode array detector
PEG	polyethylene glycol
SD	standard deviation
TBAH	tetrabutyl ammonium hydroxide
Tris	tris hydroxymethylaminomethane

References

[1] The United States Pharmacoepia XXIII: Validation of Compendial Methods, USP Convention Inc, 1990, p. 1710.
[2] A.R. Buick, M.V. Doig, S.C. Jeal, G.S. Land, R.D. McDowall, J. Pharm. Biomed. Anal. 8 (1990) 629.
[3] E. Prenesti, P.G. Daniele, S. Toso, Anal. Chem. Acta 459 (2) (2002) 323.
[4] S. Ahmad, G.S. Rao, J. Anal. Toxicol. 21 (1997) 172.
[5] M. Warner, A.H. Neims, Biochem. Pharmacol. 25 (1976) 1436.
[6] E. Coyne, H.L. Messmore, J.M. Walenga, J. Fareed, W.H. Wehrmacher, Thromb. Res. 90 (1998) 245.
[7] A. Stahlavska, M. Malat, Clin. Chim. Acta 41 (1972) 181.
[8] C. Rinderstsma, J.G.P. Verheij, Voedingsmiddelen Technol. 19 (1978) 17.
[9] S.A. Berger, D.E. Ryan, Mikrochim. Acta 4 (1974) 679.
[10] S.A. Berger, J.B. McKay, Mikrochim. Acta 4 (1974) 665.
[11] B.G. Blijenberg, B. Leijnse, Clin. Chim. Acta 26 (1969) 577.
[12] M.A. Marini, W.J. Evans, R.L. Berger, J. Biochem. Biophys. Methods 12 (1986) 135.
[13] M.V. Camacho, A.K. Banerjee, E. Candela, M. Layrisse, Analyst 110 (1985) 1509.
[14] F.J. Rajabalee, M. Potvin, S. Laham, J. Chromatogr. 79 (1973) 375.

[15] L.J. Zinterhofer, P.I. Jatlow, A. Fappiano, J. Lab. Clin. Med. 78 (1971) 664.
[16] F. Belal, F.A. Aly, M.I. Walash, A.O. Mesbah, J. Pharm. Biomed. Anal. 17 (1998) 1249.
[17] R.L. Sheppard, J. Henion, Electrophoresis 18 (2) (1997) 287.
[18] R.L. Sheppard, J. Henion, Anal. Chem. 69 (1997) 2901.
[19] R.L. Sheppard, J. Henion, Anal. Chem. 69 (1997) 477A.
[20] P. Blatny, F. Kvasnicka, E. Kenndler, J. Chromatogr. A 757 (1997) 297.
[21] R.N. Collins, B.C. Onisko, M.J. McLaughlin, G. Merrington, Environ. Sci. Technol. 35 (2001) 2589.
[22] M.L. Miller, B.R. McCord, R. Martz, B. Budowle, J. Anal. Toxicol. 21 (1997) 521.
[23] T. Hirokawa, K. Nishimoto, Y. Jie, K. Ito, F. Nishiyama, N. Ikuta, S. Hayakawa, J. Chromatogr. A 919 (2001) 417.
[24] M.L. Blank, F. Snyder, J. Chromatogr. A 170 (1979) 379.
[25] A.S. Kord, I. Tumanova, W.L. Matier, J. Pharm. Biomed. Anal. 13 (1995) 575.
[26] J. Bauer, D. Heathcote, S. Krogh, J. Chromatogr. 369 (1986) 422.
[27] M. Sillanpaa, K. Raimo, S. Marja-Liisa, Anal. Chem. Acta, 303(2–3) (1995) 187–192.
[28] W.W. Bedworth, D.L. Sedlak, J. Chromatogr. A. 905 (2001) 157.
[29] Y.C. Lee, Anal. Biochem. 293 (2001) 120.
[30] E.S. Lianidou, C.I. Pinelopi, C.K. Polydorou, C.E. Efstathiou, Anal. Chem. Acta 320 (2001) 107.
[31] The United States Pharmacoepia XXIII, 1995, p. 571.