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Determination of EDTA in vancomycin by liquid chromatography with absorbance ratioing for peak identification

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Abstract: An LC method is described for the determination of EDTA in vancomycin formulations. EDTA is complexed with iron and the $Fe(EDTA)^-$ complex is separated from vancomycin components on a reversed-phase column using an ion pair mobile phase. Quantitation is achieved using UV detection, with absorbance ratioing employed to discriminate between the analyte and vancomycin-related compounds. The complexity of the sample matrix and the trace levels of EDTA that are of interest dictate unique development considerations. This method offers good specificity and precision over the range 20–300 ppm EDTA in vancomycin formulations, while maintaining a degree of simplicity. Wavelength selection is optimized to demonstrate the potential application of absorbance ratioing to trace determinations. This method has been effectively applied to vancomycin formulations containing a wide range of chemical impurities and is not affected by vancomycin degradation products.

Keywords: *Vancomycin; EDTA; absorbance ratioing; liquid chromatography.*

Introduction

Ethylenediaminetetraacetic acid (EDTA) is widely used in the pharmaceutical industry as a chelating agent for heavy metals. These heavy metals exhibit catalytic activity even at trace levels, often requiring low levels of EDTA in formulated products. In Vancocin™ Vials-For-Injection, EDTA is present at levels up to 200 ppm. A method was sought for the determination of EDTA in vancomycin formulations that was accurate and precise, and could be applied throughout the shelf-life of the product.

Several methods for the determination of EDTA are described in the literature. The spectrophotometric method based on colour reduction of the zirconium–xylenol complex [1] suffers from phosphate interference and exhibits inaccuracy due to light-absorbing sample components. Gas chromatographic [2] and ion chromatographic [3] methods have also been developed. Liquid chromatographic methods have been developed for EDTA based on the formation of a copper complex [4–7] or an iron complex [8–10]. These HPLC methods offer good specificity and sensitivity, while maintaining a degree of simplicity.

The complexity of the vancomycin chromatographic profile requires confirmation of the analyte identification throughout the product shelf-life. The significant spectral differences between the EDTA complex and the vancomycin chromophore can be exploited using absorbance ratioing. Since the early reports of its use [11, 12], absorbance ratioing has demonstrated widespread application for solute identification [13–15]. Multichannel UV detectors have increased its use for routine applications [16, 17].

This paper describes the development of an HPLC method for the determination of EDTA in vancomycin formulations. EDTA is complexed with iron and the $Fe(EDTA)^-$ complex is separated from vancomycin components using an octadecylsilane column and a mobile phase containing tetrabutylammonium ion. The chromatographic parameters were adopted from Venezky and Rudzinski [10]. The significance of this work, however, is the separation and quantitation of EDTA in a complex sample matrix. Vancomycin degradation products provide a special separation challenge throughout the shelf-life of the product. Previous LC applications presented for EDTA determinations describe less com-

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plex separations, while multichannel detection techniques such as absorbance ratioing have faced sensitivity limitations. Design considerations and performance characteristics to overcome these limitations are described herein.

Experimental

Apparatus

Method development was performed on a Varian 5560 gradient HPLC with integrated UV-200 variable wavelength detector (Varian, Walnut Creek, CA, USA) and a Perkin–Elmer ISS-100 autosampler (Perkin–Elmer, Norwalk, CT, USA). Absorbance ratiograms and spectral scans were generated with a Beckman 167 rapid-scan UV detector (Beckman, San Ramon, CA, USA). Data collection and reduction were performed on a central chromatography computer system with data storage, manipulation, and graphics capabilities. The column was a Beckman Ultrasphere™ ODS (4.6 mm i.d. × 25 cm; 5 μm). A sample loop of 20 μl nominal volume was used for all injections. Initial method development measurements were made at 254 nm.

Reagents

Water was obtained from a Millipore water purification system (Millipore, Bedford, MA, USA). HPLC-grade methanol was purchased from Mallinckrodt (Mallinckrodt, Paris, KY, USA). Tetrabutylammonium hydroxide was purchased from J.T. Baker as a 25% solution in methanol (J.T. Baker, Phillipsburg, NJ, USA). All other reagents were of analytical reagent grade. The stock iron(III) chloride solution was prepared by adding 1.2 g of iron(III) chloride hexahydrate to 10 ml of 1 N HCl and sufficient water to produce 500 ml total solution.

Mobile phases

A 0.1 M acetate buffer solution was prepared by dissolving 4.1 g sodium acetate in 1 l water and adjusting the pH to 4.5 with dropwise addition of glacial acetic acid. Mobile phase A was prepared by adding 17 ml tetrabutylammonium hydroxide (TBAH) (25% in methanol) to 1 l acetate buffer. Mobile phase B was prepared by adding 17 ml TBAH and 600 ml methanol to 400 ml acetate buffer. Each solution was mixed thoroughly and briefly degassed before use.

Chromatographic conditions

A flow rate of 1.5 ml min^{-1} was used throughout the method development process. The gradient profile was defined as 100% A for 5 min, a linear ramp from 100% A to 100% B over 5 min, 100% B for 5 min, and a linear ramp from 100% B to 100% A over 5 min. Equilibrium was re-established after 26 min from injection. A thorough rinse of the system with water before and after use was found to eliminate pressure increases over time. The detector was set at 0.1 AUFS. All quantitation was based on peak areas.

Preparation of standards and samples

A stock EDTA solution was prepared by dissolving 635 mg $Na_2EDTA{\cdot}2H_2O$ in water and diluting to 500 ml. This solution has a concentration of about 1 mg ml^{-1} EDTA. Appropriate dilutions were made to produce a standard solution of about 20 μg ml^{-1} EDTA. Working standards were prepared by pipetting 1, 2 and 5 ml of the 20 μg ml^{-1} EDTA solution into separate 10-ml volumetric flasks. One ml of the iron(III) solution was added to each flask and the flasks were diluted to volume with water. These solutions correspond to 2, 4 and 10 μg ml^{-1} EDTA. Sample solutions were prepared by placing about 500 mg of vancomycin HCl sample in a 10-ml volumetric flask, adding 1 ml of the iron(III) solution, and diluting to volume with water.

Results and Discussion

Separation considerations

Vancomycin hydrochloride is a complex mixture of closely related components. Chromatographic conditions developed to profile vancomycin products have been described previously [18]. Initial development of a method for the determination of EDTA was based on the use of an ion pairing reagent to provide adequate retention of the $Fe(EDTA)^-$ complex. After elution of the complex peak, the organic composition of the mobile phase was rapidly increased to elute all vancomycin factors. The gradient profile was optimized for a wide range of vancomycin materials including samples that have been severely degraded. An initial detection wavelength of 254 nm was selected, providing adequate sensitivity.

The performance of this method is illustrated by the chromatograms in Fig. 1 for a blank, a 10 μg ml^{-1} EDTA standard, and a

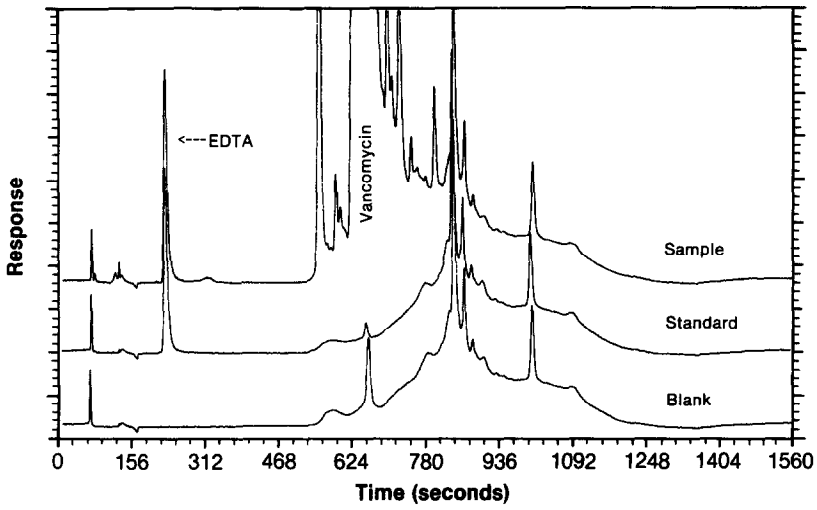

Figure 1
Typical chromatograms for: solution blank, 10 μg ml^{-1} EDTA standard, and vancomycin sample containing 200 ppm EDTA.

sample containing 200 ppm EDTA. Baseline resolution of the $Fe(EDTA)^-$ peak from vancomycin peaks is clearly demonstrated with excellent peak shape. Attempts to further reduce the assay time resulted in compromised resolution, especially for the vancomycin degradation products.

Method optimization

The iron complex was selected instead of the copper complex because of the greater stability of $Fe(EDTA)^-$ compared with $Cu(EDTA)^{2-}$ [9]. The photosensitivity of $Fe(EDTA)^-$ has not been observed throughout these studies. No degradation was observed in solutions analysed 24 h after preparation. No special precautions for limited light exposure have been employed over the 18 months of routine application of this method.

To determine if sufficient iron(III) is available for complete complexation, varying volumes of the iron(III) solution were added to standard solutions containing 4 μg ml^{-1} EDTA and spiked sample solutions containing 300 ppm EDTA (15 μg ml^{-1}). Constant peak areas were observed for those solutions containing 0.5 to 2.0 ml iron(III) solution. Therefore, 1.0 ml iron(III) solution was used in all subsequent assays. This quantity corresponds to 50 μg ml^{-1} Fe^{3+} or 0.9 mM Fe^{3+} in the sample solutions. This provides a molar ratio of Fe^{3+} to EDTA of about 20 at the 300 ppm EDTA level.

Method validation included the parameters of linearity, sensitivity, and precision. Ten standard solutions were prepared over the concentration range 0.5–10 μg ml^{-1} EDTA, corresponding to 10–200 ppm in the vancomycin formulation. A coefficient of determination of 0.999 and a log–log slope of 0.95 were obtained. A detection limit of about 0.25 μg ml^{-1} or 5 ppm was estimated. Additional testing indicates that calibration curve linearity extends to at least 20 μg ml^{-1} or 400 ppm. The reproducibility of this method was determined by assaying a set of standards, spiked samples, and samples. The spiked sample solutions were prepared by the addition of various amounts of EDTA corresponding to 10–200 ppm to 10 sample weighings from a single vancomycin lot which contained an initial concentration of about 80 ppm EDTA, and calculated by the technique of standard addition. The samples represented 10 replicate weighings from a single sample lot containing about 180 ppm EDTA. Relative standard deviations of 2.14, 2.36 and 2.08% (n = 10) were obtained for the standards, spiked samples, and samples, respectively. The consistency in precision among the sample sets suggests that the method is limited by the separation and detection steps rather than the sample preparation.

Finally, method performance was evaluated for severely degraded samples. Figure 2 illustrates the chromatograms obtained for vanco-

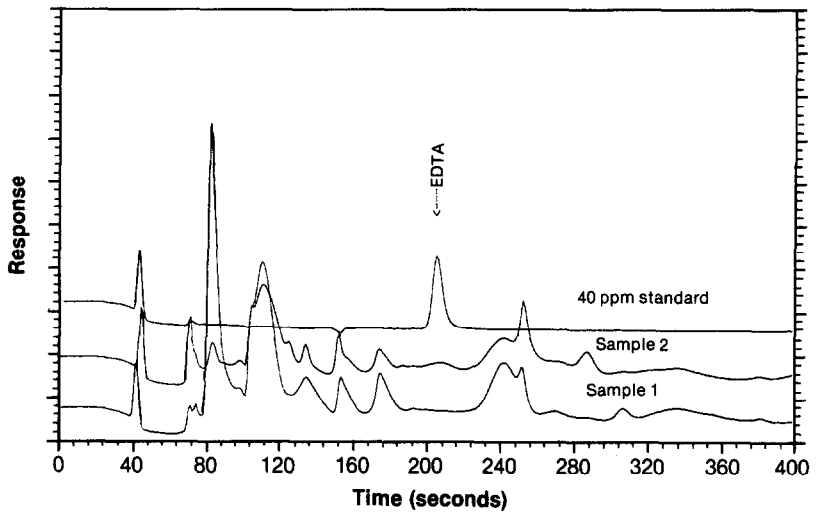

Figure 2
Chromatograms of vancomycin samples that have been degraded.

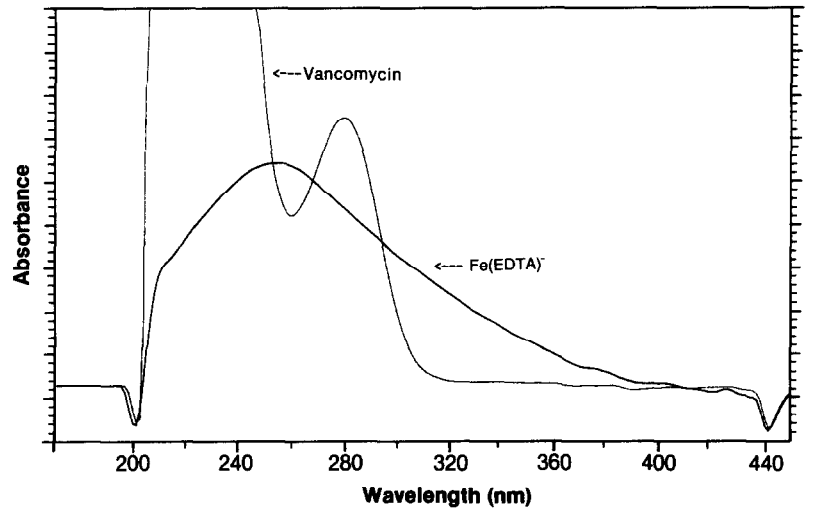

Figure 3
Absorption spectra for vancomycin and $Fe(EDTA)^-$.

mycin samples that were free of EDTA with chemical purities <80%. These have been compared to the 40 ppm standard, indicating that the EDTA window is relatively free of interference from vancomycin degradation products.

Detection considerations

While vancomycin hydrochloride contains a number of related factors and degradation products, the chromophore remains unchanged and the spectral characteristics are nearly identical for these factors [18]. The UV spectra of vancomycin and the $Fe(EDTA)^-$ complex are shown in Fig. 3. Visual comparison of these spectra indicated that a wavelength of 260 nm provides good sensitivity for EDTA with minimal interference from vancomycin.

These spectral differences can be further exploited to provide additional qualitative information for peak identification. The ratiogram, a point-by-point ratio of chromatograms detected at two different wavelengths, provides additional identification information to supplement the retention time data. For these applications, one is interested in obtaining sufficient information to classify the peak without requiring complete identification. That is, if an unknown peak has spectral character-

istics that are similar to the $Fe(EDTA)^-$ complex or vancomycin, the analyst can propose that the peak is due to analyte, a vancomycin cofermentation or degradation product, or is unrelated, protecting against false positives. The absorbance ratio is characteristic of the chromophore, yet independent of its concentration. This general classification process can then suggest the direction of additional investigation, if desired. Thus, for routine applications of this method, the ratiogram provides an effective screen.

Chromatograms for a typical standard and sample are shown in Fig. 4(A). The corresponding ratiograms, shown in Fig. 4(B), can be divided into three general regions. The $Fe(EDTA)^-$ complex provides a 260:280 nm ratio of 1.2 ($R = 0.2$), resulting in a positive peak during the isocratic portion (Region 1) of the chromatogram for those samples containing EDTA. Vancomycin factors yield 260:280 nm ratios of 0.64 ($R = -0.6$), resulting in negative peaks. This is due to the ratio algorithm built into the detector electronics so that:

$$R = A_1/A_2 - 1, \text{ when } A_1 \geq A_2 > \text{threshold} \quad (1)$$

$$R = 1 - A_2/A_1, \text{ when } A_2 > A_1 > \text{threshold} \quad (2)$$

$$R = 0, \text{ when } A_1 \leq \text{threshold or } A_2 \leq \text{threshold} \quad (3)$$

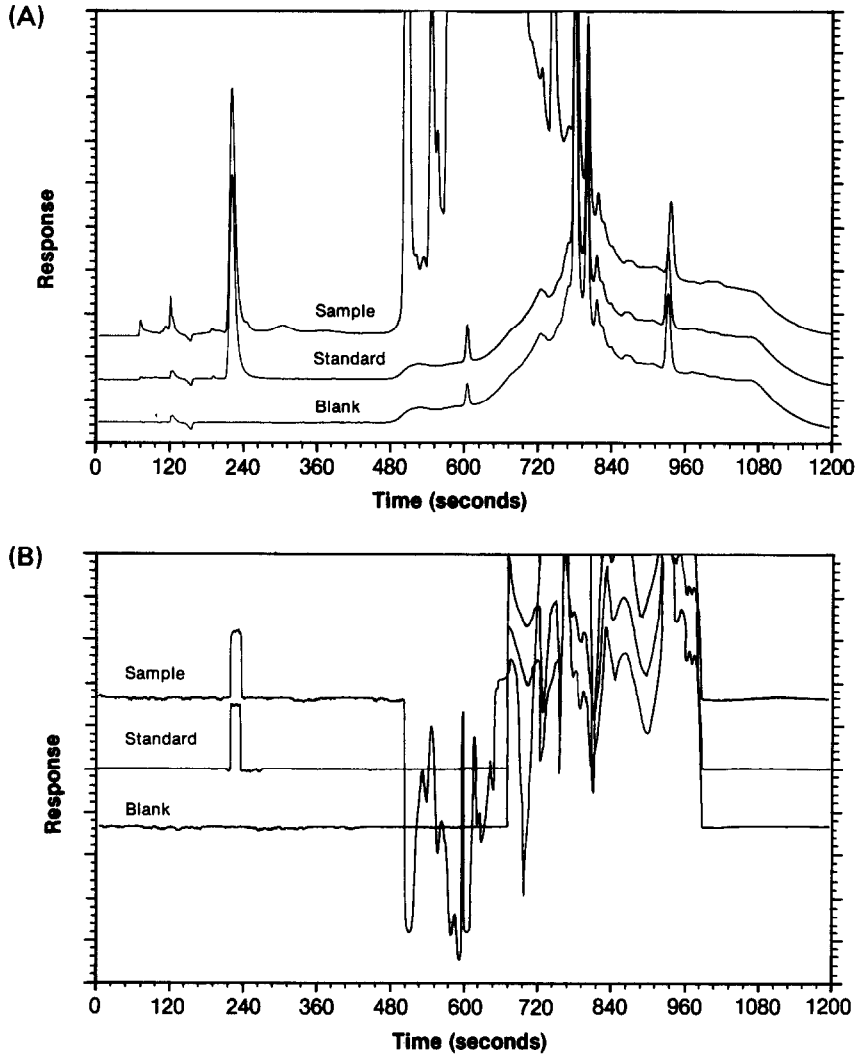

Figure 4
(A) Chromatogram of a sample blank, 10 μg ml^{-2} EDTA standard, and a sample containing 200 ppm EDTA. (B) Ratiograms for solutions described in (A).

where R is the ratio output, A_1 and A_2 are absorbances at wavelengths 1 and 2, and threshold is a user defined lower absorbance limit to reduce the impact of detector noise. Under these chromatographic conditions, most vancomycin-related peaks appear in the ratiogram between 500–700 s (Region 2) and appear in the negative direction. The ratiogram band shapes were not ideal in this region because the gradient baseline contributed to both A_1 and A_2, distorting the value of R. The third region, present in all ratiograms beyond 700 s, provided little identification information as baseline drift again contributed to absolute absorbance. The limitations caused by baseline artifacts have been previously described [14]. Thus the ratiogram was most effective in the isocratic portion of the chromatogram.

Parameter selection must be carefully considered to maximize the effectiveness of the ratiogram as a screen for peak identity. Selection of wavelengths 1 and 2 must complement the adjustment of the absorbance threshold for a number of practical reasons. Each peak of interest must exhibit sufficient absorption at both wavelengths to yield a non-zero output. This is especially important for trace applications where sensitivity is often a major concern. Directionality provides an additional parameter for peak differentiation, where absorption peak maxima can be exploited. Detector noise can limit one's ability to identify analyte-specific information if a minimum signal-to-noise ratio is not established. The selection of absorbance ratioing parameters must be designed for the specific application of interest.

The set of detector parameters used in this application illustrated the selection process. The EDTA complex and vancomycin-related compounds exhibited absorbance maxima at 260 and 280 nm, respectively, with sufficient absorbance at the off-peak wavelength. In this way, EDTA yielded a positive peak while vancomycin and related compounds yielded negative peaks. A threshold was set to coincide with the analyte standards employed, with sufficient sensitivity for detection of the 2 μg ml^{-1} EDTA standard in the ratiogram. This adjustment was detector specific and highly dependent on the selection of the absorbance threshold and the off-peak wavelength. Thus one should identify and characterize the components from which the analyte is to be differentiated.

Judicious adjustment of detection parameters provides another potential screening mechanism. The ratiogram not only provides analyte identification information, but it also provides a quantitative screen. As illustrated in Figs 5(A) and 5(B), the ratiogram detection limit can be adjusted to coincide with an appropriate standard. Samples with analyte concentrations below this level are quickly identified. In this way, an effective limit test can be designed. Also note the effectiveness of the ratiogram for the two large peaks eluting before the EDTA peak. The peak that appeared at about 80 s in sample 1 is spectrally unrelated to either vancomycin or the EDTA complex due to its direction and magnitude in the ratiogram. The peaks that appear in samples 1 and 2 at about 120 s are probably vancomycin degradation products as indicated by the ratiogram. Thus, qualitative and quantitative classification of the peaks in the chromatograms for three samples is illustrated.

Perhaps the greatest potential for absorbance ratioing as a chromatographic screening tool rests in its ease of automation of the data evaluation process. While multichannel UV detectors provide the capability for generation of voluminous quantities of data, data evaluation often limits full utility of the data. Real-time absorbance ratioing reduces data collection requirements compared to full spectral scanning while providing an additional detection dimension for analyte identification. Automation of the generation and evaluation of ratiograms bridges the gap between single wavelength detection and complete spectral scanning. Full evaluation of the automation of absorbance ratioing is beyond the scope of this discussion.

Conclusions

The stability and UV absorptivity of the $Fe(EDTA)^-$ complex has been exploited for the LC determination of EDTA in vancomycin formulations. Reversed-phase separation is achieved with the addition of an ion pairing reagent, tetrabutylammonium hydroxide. Chromatographic conditions have been optimized for adequate resolution from the many vancomycin related factors, especially degradation products, and sensitivity for determinations at the 20–300 ppm EDTA levels. Absorbance ratioing provides qualitative information, providing an effective

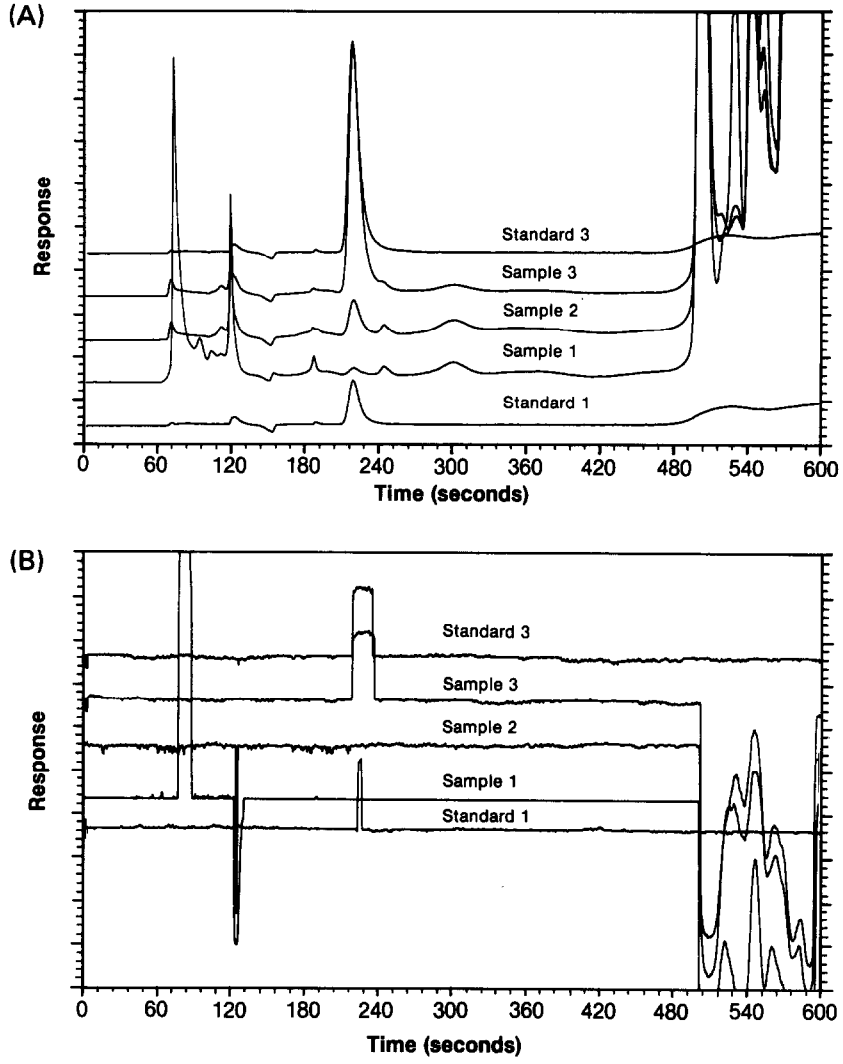

Figure 5
(A) Chromatograms from vancomycin samples containing varied EDTA concentrations. Standards 1 and 3 contain the equivalent of 40 and 200 ppm EDTA. (B) Ratiograms from the vancomycin samples described in (A). Note that the threshold is set to detect EDTA peaks at 40 ppm.

screen for differentiating the analyte peak from vancomycin-related compounds and other peaks. This application of absorbance ratioing for trace determinations also provides a quantitative screen when a level of significance has been identified. The effectiveness of this methodology suggests that more widespread applications are possible for trace determinations in complex matrices.

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