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A novel HPLC method for determination of EDTA in a cataract inhibiting ophthalmic drug*

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Abstract: A novel HPLC method for determination of EDTA in a cataract inhibiting ophthalmic drug product has been developed. In this method EDTA was converted to Cu(II)EDTA complex, using Cu^{+2} containing mobile phase, after injection into the chromatographic system. This allowed complexation of EDTA with Cu^{+2} without interference from formulation ingredients. The Cu(II)EDTA complex was separated from drug substance, impurities, degradants and other formulation excipients by a 250 × 4.1 mm anion exchange column and detected at UV wavelength 250 nm. The mobile phase consisted of 2 mM cupric nitrate, 11 mM nitric acid, and 25% (v/v) acetonitrile at pH 3.0. This stability indicating assay has been validated and shown to be specific, linear, precise, accurate and rugged for routine EDTA analysis.

Keywords: *HPLC method; ophthalmic drug; EDTA; Cu–EDTA formation within chromatographic system.*

Introduction

Ethylenediaminetetraacetic acid (EDTA) is widely used in the pharmaceutical industry. It is used as an antioxidant, a chelating agent for heavy metals, and a preservative effectiveness enhancer in many pharmaceutical formulations. EDTA has been frequently used in ophthalmic formulations along with benzalkonium chloride to enhance the antimicrobial effectiveness of this compound. Various methods for determination of EDTA in different matrices have been reported in the literature, including colorimetry [1], titrimetry [2] and high-performance liquid chromatography (HPLC) [3–11]. The HPLC methods are based on complexation of EDTA with Cu^{+2} or Fe^{+3} prior to injection into the chromatographic system. These HPLC methods offer good sensitivity and specificity for a variety of matrices. However, they lack an acceptable specificity and sensitivity for matrices which contain ligands that interfere and compete with EDTA for complexation with Cu^{+2} and Fe^{+3}. The active ingredient of the cataract inhibiting ophthalmic solution (drug product) in this study has a great tendency to form a complex with Cu^{+2} and Fe^{+3}. The concentration of this compound in the formulation is about 3000 times greater than that of EDTA and therefore, it interferes with complexation of EDTA with Cu^{+2} and Fe^{+3}. As a result, the use of HPLC methods mentioned above for determination of EDTA in this formulation is impractical.

In this paper a novel HPLC method for determination of EDTA in a cataract inhibiting ophthalmic drug is described. This method is based on complexation of EDTA with Cu^{+2} after injection into the chromatographic column, which alleviates the interferences of matrix components with this complexation.

Experimental

Equipment and materials

The method development was performed using a Perkin–Elmer (Norwalk, CT, USA) HPLC system consisting of a model 250 pump, a 295 variable wavelength UV detector, and a PE Nelson TurboChrom data acquisition software (version 3.1) loaded onto a model 450/L Dell 486 computer (Austin, TX, USA). For post-column complexation studies, another Perkin–Elmer pump (model 250), a mixing tee (Upchurch Scientific, Inc., Oak Harbor, WA, USA) and a pulse dampener model LP-21 (Scientific System, Inc. State College, PA,

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USA) were added to this chromatographic system. Validation and ruggedness assays were performed using both the Perkin–Elmer instrument described above and a Rainin (Woburn, MA, USA) HPLC system. The latter included a Dynamax HPXL pump, an AI-1 autosampler (20-μl loop), a multi-wavelength model UV-D detector and a Dynamax data acquisition software loaded onto a Macintosh SE/30 computer. Absorbance spectra were generated using a model U-3110 Hitachi spectrophotometer (Hitachi Instrument Inc., Danbury, CT, USA). The columns used for method development were a 25 cm × 4.6 mm, 5 μm particle size, Absorbosphere C_{18} column and a 25 cm × 4.1 mm, 10 μm particle size, Anion/R column purchased from Alltech (Deerfield, IL, USA). Disodium EDTA dihydrate, cupric nitrate, cupric disodium EDTA, were provided by the Sigma Chemical Co. (St Louis, MO, USA). Tetrabutylammonium phosphate (TBAP) and cupric sulphate were supplied by Aldrich (Milwaukee, WI, USA). HPLC grade methanol (MeOH), acetonitrile (ACN), and deionized water as well as nitric acid and sodium hydroxide were supplied by Fisher Scientific (Fair Lawn, NJ, USA).

Mobile phase

The mobile phase consisted of 25% (v/v) ACN in a solution of 2 mM cupric nitrate, 11 mM nitric acid in deionized water (pH 3.0). The reagents were dissolved in deionized water and pH was adjusted to 3.0 with 10 N sodium hydroxide prior to mixing with ACN (75:25 v/v). This solution was filtered (through a 0.45-μm filter) and degassed prior to use. The chromatographic separations were performed isocratically at room temperature while the flow rate was maintained at 1 ml min^{-1}.

Preparation of standards and samples

A stock EDTA solution was prepared by dissolving 65 mg of disodium EDTA dihydrate in deionized water and diluting to 500 ml. Hereafter, references to concentrations of EDTA in solutions (for both standards and drug product samples) practically refer to concentrations of disodium EDTA dihydrate. Working standards were prepared by pipetting 4.0, 5.0 and 6.0 ml of stock solution into separate 10-ml volumetric flasks. These solutions correspond to 52, 65 and 78 μg ml^{-1} EDTA. EDTA-free drug formulations (hereafter, referred to as EDTA-F in this text) were prepared according to manufacturing formula. These solutions contained the active drug substance and other excipients (except EDTA) present in the formulation at 100% level of label claim. The spiked EDTA-F solutions were prepared by pipetting 4, 5 and 6 ml of EDTA stock solution into separate 10-ml volumetric flasks. These EDTA solutions correspond to 80, 100 and 120% of label claim. Samples of drug product were diluted with deionized water (1:1, v/v) prior to analysis. EDTA concentration in the diluted drug product samples was 65 μg ml^{-1}.

Results and Discussion

Method development

The purpose of this work was to develop a method for determination of EDTA for in-process testing, release of drug product as well as stability studies. A primary consideration was simplicity of the assay, so that it could be used for routine testing, using minimal instrumentation, such as an isocratic HPLC pump and a UV detector, which are available in most laboratories.

The applicability of reported HPLC method in the literature [3–6] for determination of EDTA in ophthalmic drug product was studied. EDTA samples were mixed with cupric sulfate solution (molar ratio of EDTA/cupric sulfate was about 1/10) prior to injection into the HPLC system. The copper(II)EDTA complex thus formed was separated from the other drug product components using ion-pair chromatography (IPC). The resulting chromatograms for injection of 20 μl of standard (0.13 mg ml^{-1} EDTA) and drug product (containing 0.13 mg ml^{-1} EDTA), as well as chromatographic conditions, are shown in Fig. 1(a) and (b), respectively. The EDTA peak area in drug product was found to be 19% of that in the standard solution. These data suggest that the use of pre-column complexation of Cu(II)EDTA leads to values which are less than the actual concentration of EDTA in formulation. Complexation of the active ingredient with Cu^{+2} is the most probable explanation of this phenomenon. This active ingredient has a strong tendency to form a complex with Cu^{+2}, and since its concentration in the drug product is about 3000 times more than that of EDTA it interferes with Cu(II)EDTA complexation.

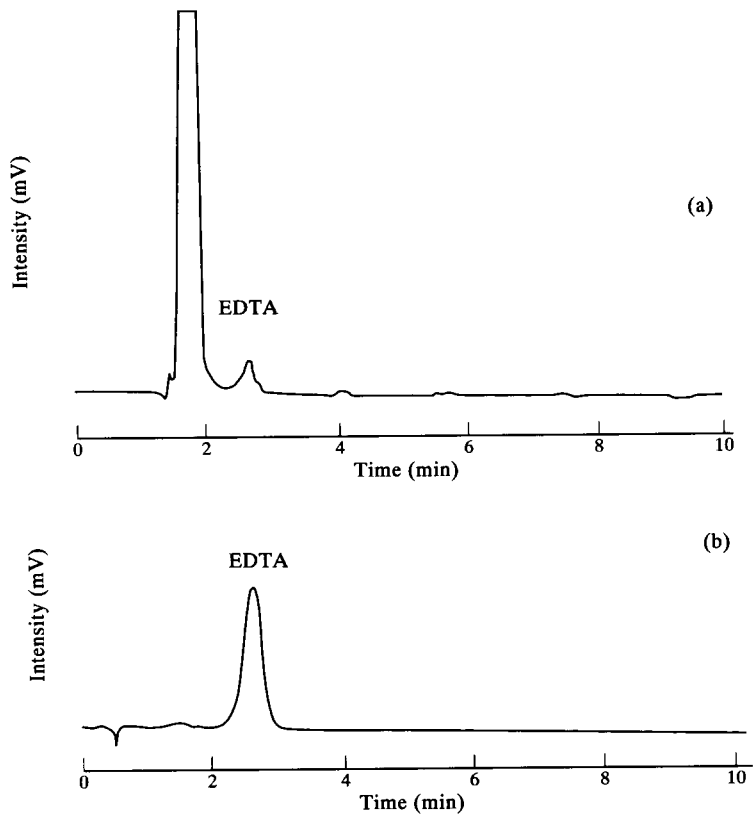

Figure 1
Chromatograms for (A) ophthalmic drug and (B) EDTA standard using the following chromatographic conditions: column; adsorbosphere C18 (25 cm × 4.6 mm, 5-μm particle size), mobile phase; 26% (v/v) ACN in a 5 mM solution of TBAP in water (pH 6.5), flow rate; 1 ml min^{-1}, detection; 254 nm.

One way to overcome this problem was to separate EDTA from the other drug product ingredients on the HPLC column, then mix it with cupric nitrate to form Cu(II)EDTA complex prior to UV detection (post-column complexation of EDTA with Cu^{+2}). The schematic diagram of the apparatus assembled for this purpose is displayed in Fig. 2. Although the results obtained using this approach were satisfactory, it required extra equipment (i.e. HPLC pump, pulse dampener, etc.) which limited its feasibility for use on a routine basis.

As described above both pre- and post-column complexation of EDTA with copper had limitations. Therefore, it was worthy to investigate the feasibility of formation of a copper EDTA complex within the chromatographic system. For this purpose, cupric ions needed to be added to the mobile phase of the HPLC system at a pH of 3.0 to prevent formation of cupric hydroxide (which cause deterioration of chromatographic performance).

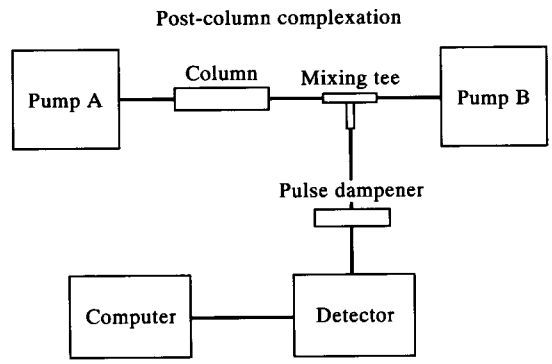

Figure 2
Schematic diagram of instrumentation used for post-column complexation of Cu(II)EDTA. Pump A and B were used for mobile phase and cupric ions at a flow rate of 1 ml min^{-1}, respectively.

In order to determine the optimum UV wavelength for detection of copper EDTA complex in the presence of excess cupric ions (wavelength at which the difference between the absorbance of the cupric ions and

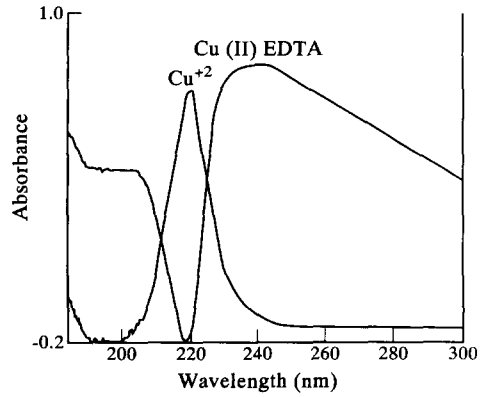

Figure 3
Absorption spectra for Cu^{+2} (7 μg ml^{-1} solution of cupric nitrate in water; pH of this solution was adjusted to 3.0 with HNO_3) and Cu(II)EDTA (7 μg ml^{-1} solution of cupric disodium EDTA in water; pH of this solution was adjusted to 3.0 with HNO_3).

Cu(II)EDTA had a maximum value), the UV spectra of Cu^{+2} (cupric nitrate) and Cu(II)EDTA complex (cupric disodium EDTA) were obtained and are shown in Fig. 3. Visual comparison of these spectra indicated that a wavelength of 250 nm provides good sensitivity for Cu(II)EDTA with no interference from cupric nitrate.

Since inclusion of Cu^{2+} (0.2 mM cupric nitrate) in the mobile phase of IPC (at pH 3.0) adversely affected separation, the use of ion-exchange chromatography was investigated. For this purpose a poly(styrene-divinyl benzene) based anion exchange column was selected. The use of a silica based anion exchange column was avoided to prevent any possible interaction of Cu^{+2} existing in the mobile phase with silica which could lead to poor chromatographic behavior. All the mobile phases studied for this separation contained 0.2 mM cupric nitrate and pH was adjusted to 3.0 for reasons described earlier in this report. Mobile phases containing various types and concentrations of both strong acids (nitric acid and sulfuric acids at concentrations between 1 and 20 mM) and organic solvents (MeOH and ACN at concentrations 0–30%) were studied. The results of these experiments demonstrated that: first, the retention of Cu(II)EDTA complex decreases with increase in acid concentration (this effect was more pronounced with nitric acid than with sulfuric acid). Second, varying organic modifier types and concentrations had little effect on retention of Cu(II)EDTA while retention of most of the formulation ingredients decreased with increase in organic solvent concentration. The reduction in retention of these compounds was more pronounced with increase in the concentration of ACN than that of MeOH. Finally, a good separation within a reasonable run time (about 10 min) can be achieved using a mobile phase composed of 0.2 mM cupric nitrate, 11 mM nitric acid, 25% (v/v) ACN with a pH adjusted to 3.0 with sodium hydroxide. To determine the optimum Cu^{+2} concentration for complete complexation and best sensitivity, mobile phases containing various concentrations of cupric nitrate were used.

The peak area for injection of 20 μl of 0.13 mg ml^{-1} samples of EDTA were observed to be constant for mobile phases containing 1–3 mM of cupric nitrate. Therefore, 2 mM cupric nitrate was used in the mobile phase for subsequent studies. This provides a molar ratio of Cu^{+2} to EDTA of about 25 at 0.13 mg ml^{-1} EDTA level (this quantity corresponds to the EDTA level in the ophthalmic solution). The resulting chromatograms for EDTA in standard solutions and ophthalmic formulation using a mobile phase consisting of 2 mM cupric nitrate, 11 mM nitric acid, 25% (v/v) ACN (pH 3.0) are illustrated in Fig. 4(a) and (b), respectively. These chromatographic conditions were chosen for subsequent studies.

Method validation

Method validation included the parameters of specificity, linearity, precision, accuracy and ruggedness.

Chromatograms resulting from injections of samples of EDTA-F and standard EDTA were compared. The results showed that there were no interfering peaks from ophthalmic formulation ingredients with the EDTA peak. This confirmed the specificity of the assay for determination of EDTA.

To investigate stability indicating potential of this assay, samples of drug product as well as EDTA-F were subjected to acidic, basic and oxidation conditions in order to promote degradation. Then, these samples were analysed using the HPLC method and the resulting chromatograms were compared with the chromatograms obtained for EDTA standard samples. The results demonstrated that there were no interfering peaks from drug product ingredients and degradants with the peak of EDTA. Therefore this method qualified as a stability indicating assay for

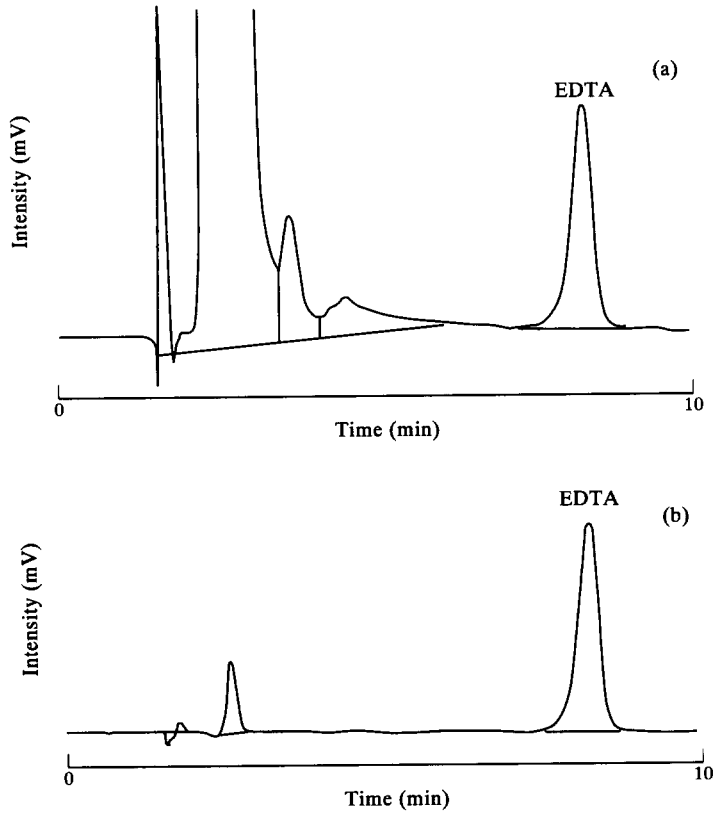

Figure 4
Chromatograms for (A) drug product and (B) EDTA standard.

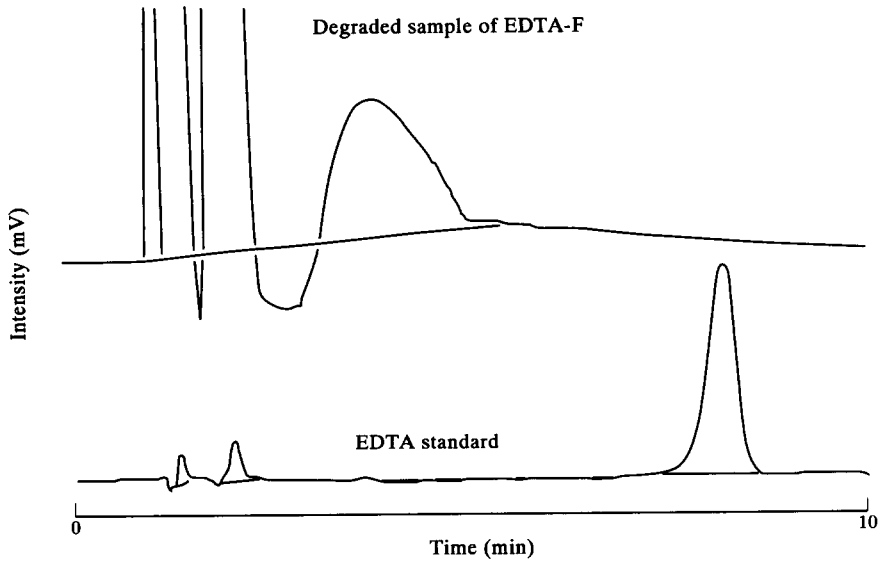

Figure 5
Chromatograms for EDTA standard and degraded sample of EDTA-F.

determination of EDTA in ophthalmic formulations.

Figure 5 illustrates the chromatograms obtained for EDTA-F samples which had been severely degraded (about 10% degradation of active ingredient) and EDTA standard samples. As shown, the window for the EDTA peak is clearly free from degradation products and other formulation ingredients.

To investigate the linearity of the method, three standard solutions were prepared over the concentration range of 52–78 μg ml^{-1} EDTA, corresponding to 104–156 ppm in the ophthalmic formulation (80–120% of EDTA concentration in drug product). The correlation coefficient of the least squares line of EDTA peak area vs concentration was found to be $r = 0.9999$.

To study the precision of this assay, EDTA standard solutions at varying concentrations level (80, 100 and 120% of EDTA concentration in drug product) were prepared. These solutions were assayed in seven replicates. Relative standard deviations (RSDs) of 0.22, 0.28 and 0.11% were obtained for 80, 100 and 120% levels, respectively.

The accuracy of the method was studied using EDTA-F samples spiked with EDTA standards at 80, 100 and 120% of label claim. These spiked solutions were assayed and compared to standards at these concentration levels. Results expressed as percent recovery were 98.0, 101.0 and 101.0% for 80, 100 and 120% levels, respectively.

Ruggedness testing was performed on two samples of drug products of the same lot. These samples as well as standard samples (at 100% level of label claim) were assayed in quadruplicate injections by two analysts on two instruments using separately prepared standards and mobile phases. The results obtained for drug products were compared to those obtained for standard. These results expressed as percent recovery are shown in Table 1.

The validation described above showed that the assay is qualified for determination of EDTA in ophthalmic formulation on a routine basis.

Table 1
Ruggedness

	Per cent recovery of EDTA from ophthalmic formulation	
	Analyst 1	Analyst 2
	101.08	102.46
	100.77	100.15
	99.38	99.70
	99.62	101.15
Mean	100.21	100.86
RSD	0.84	1.22

Conclusion

A simple and reliable HPLC method for determination of EDTA in a cataract inhibiting formulation was developed. This assay is specific and sensitive, suitable for detection and quantitation of EDTA in ophthalmic formulation. Test parameters commonly used for method validation were found to be acceptable. Furthermore, a new approach for detection of EDTA based on complexation of this compound with Cu^{+2} ions within chromatographic system was developed and proved to be successful. This approach can be used for detection of EDTA in other complex matrices.

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