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Rapid qualitative and quantitative HPLC/MS analysis of ethylenediaminetetraacetic acid in a pharmaceutical product without prior sample preparation

[Rápido análisis cualitativo y cuantitativo por HPLC/MS del ácido etilendiaminotetraacético en un producto farmacéutico sin preparación previa de la muestra]

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Abstract

Context: Sodium calcium ethylenediaminetetraacetic acid (EDTA) is used to bind metal ions in the practice of chelation therapy, such as treating mercury and lead poisoning. This therapy is used to treat the complication of repeated blood transfusions, as would be applied to treat thalassemia. The work is devoted to the analysis of EDTA in pharmaceutical products.

Aims: To develop and validate a fast method for qualitative and quantitative estimation of EDTA in pharmaceutical products without derivatization.

Methods: The Agilent 6125 C SQ LS/MS instrument was used. Mass Spectrometer was chosen to detect EDTA directly without derivatization and any preliminary sample preparation.

Results: The method was validated in accordance with International Conference on Harmonization guidelines. Total run time was 1.0 min. EDTA was eluted with a retention time of 0.23 ± 0.01 min. The limit of detection was 0.09 µg EDTA. Recovery varied from 100 to 113% and the relative standard deviation varied from 0 to 6%. In positive Electrospray ionization mode, the spectra showed the predominant signals at m/z of 293.2 which corresponds to cation ($EDTAH^+$) and 315.2, which corresponds to cation ($EDTANa^+$).

Conclusions: The developed HPLC/MS method for the determination of EDTA in pharmaceutical preparations containing various other ingredients, including excipients (NaOH and benzyl alcohol), was validated for linearity, accuracy/recovery, precision and selectivity, as well as low detection limit and quantification. The method provides a fast, simple, sensitive and reproducible means of determining the pharmaceutical compositions of EDTA without derivatization and prior sample preparation.

Keywords: EDTA disodium; LC/MS; RP-HPLC; validation.

Resumen

Contexto: El ácido etilendiaminotetraacético de sodio y calcio (EDTA) se usa para unir iones metálicos en la práctica de la terapia de quelación, como el tratamiento del envenenamiento por mercurio y plomo. Esta terapia se usa para tratar las complicaciones de las transfusiones de sangre repetidas, como se aplicaría para tratar la talasemia. El trabajo está dedicado al análisis de EDTA en productos farmacéuticos.

Objetivos: Desarrollar y validar un método rápido para la estimación cualitativa y cuantitativa del ácido etilendiaminotetraacético en productos farmacéuticos sin derivatización.

Métodos: Se utilizó el instrumento Agilent 6125 C SQ LS/MS. Se eligió el espectrómetro de masas para detectar EDTA directamente sin derivatización y cualquier preparación de muestra preliminar.

Resultados: El método fue validado de acuerdo con las pautas de la Conferencia Internacional sobre Armonización. El tiempo total de ejecución fue de 1,0 min. El EDTA se eluyó con un tiempo de retención de 0,23 ± 0,01 min. El límite de detección fue de 0,09 µg de EDTA. La recuperación varió del 100 al 113% y la desviación estándar relativa varió del 0 al 6%. En el modo positivo de ionización por Electrospray, los espectros mostraron las señales predominantes a m/z de 293,2 que corresponde al catión ($EDTAH^+$) y 315,2, que corresponde al catión ($EDTANa^+$).

Conclusiones: El método desarrollado por HPLC/MS para la determinación de EDTA en preparaciones farmacéuticas que contienen varios otros ingredientes, incluidos los excipientes (NaOH y alcohol bencílico), fue validado para linealidad, precisión/recuperación, precisión y selectividad, así como bajo límite de detección y cuantificación. El método proporciona un medio rápido, simple, sensible y reproducible para determinar las composiciones farmacéuticas de EDTA sin derivatización y preparación previa de la muestra.

Palabras Clave: EDTA disódico; LC/MS; RP-HPLC; validación.

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INTRODUCTION

Quality control and potency determination is an important and necessary part of pharmaceutical products manufacturing. Using a modern highly effective analytical instrument such as Agilent HPLC/MS make this task easily done (Agilent Single Quadrupole LC/MS instrument, 2019). The International Conference on Harmonization (ICH) guidelines give necessary analytical criteria for the validation of analytical methods (European Medicines Agency. ICH, 2006). There is an additional and very important consideration for analytical laboratory - time and cost of an analysis.

EDTA is not visible for UV-VIS or Diode Array Detectors (DAD). To make it detectable chemists synthesize stable complexes EDTA with transition metals. Narola at al. (2011) validated a HPLC/UV-VIS method for the quantification of EDTA by derivatizing EDTA with ferric chloride. Hall and Takahashi (1988), prior to analyze EDTA with HPLC/UV method, formed copper-EDTA complexes. Tran at al. (1996), Wang and Tomasella (2016), Harmsen and Van Den Toorn (1982) also synthesized iron-EDTA and copper-EDTA complexes prior to HPLC/UV analysis. Dodi and Monnier (2004) used HPLC coupled with MS to analyze EDTA in industrial waste, authors used $FeCl_3$ as derivatization reagent and detect Fe-EDTA complex ionized in form of single-charged anion [Fe-EDTA$^-$]. Wei et al. (2016) analyzed EDTA in wines and beer using HPLC in combination with MS/MS in positive ion mode. Amines were used as EDTA derivatizing reagents to eliminate interference from Fe (III) and Cu (II) ions. Miller at al. (1997) used HPLC coupled with MS/MS detector in positive and negative ion mode to detect EDTA in dried blood stains. The authors showed the possibility of a qualitative determination of EDTA in the form of ($EDTAH^+$) cation by LC/MS. Lowe et al. (2005) stated that free acid EDTA can be detected using LC/MS, but only at concentrations above 100 µM. To increase the detection limit, the authors derivatized EDTA to its methyl and butyl ether forms.

The purpose of the present work is to elaborate a fast, accurate, precise, selective, and robust procedure to analyze EDTA as qualitatively and quantitively in pharmaceutical products for quality control of fresh prepared drugs and for stability testing of the products containing EDTA. To reach this goal it is necessary exclude the first time consuming, preliminary stage of the analysis — derivatization of EDTA. That simplification of the procedure is possible if the HPLC instrument has as DAD and MSD. Validate the method for linearity, accuracy, precision, repeatability, selectivity, specificity and robustness according to European Medicines Agency. ICH (2006) and FDA guidance for analytical procedures and methods validation (2015).

MATERIAL AND METHODS

Chemicals

Water HPLC grade purchased from Agilent; EDTA calcium disodium (MW=374.27 g/mol), hereinafter simply EDTA, purchased from Medica Inc. USA; HCl aq. 36.5 - 38.0% solution from VWR LLC USA; formic acid 98-100% analytical grade from Merck; sodium bicarbonate and benzyl alcohol from Medica Inc. USA. All the solvents used were of HPLC grade. Branded pharmaceutical and veterinary formulation, in form of injection solution, it was obtained from commercial sources and used as received, without any further purification. The composition of the preparation is as follows: EDTA calcium disodium 200 mg/mL, injection solution 100 mL (Infuserve America St. Petersburg, FL, USA) contained EDTA calcium disodium (20 g), benzyl alcohol (1.0 mL), sodium hydroxide 10% (0.3 mL).

Samples

All the samples where from a fresh prepared product (injection solution). Analysis of EDTA was made for quality control of the product made by Infuserve America St. Petersburg, FL, USA.

Analytical quantification

The instrument Agilent Single Quadrupole LC/MS instrument (2019) includes the following components: OpenLAB CDS Version 2.2. software; single quadrupole (SQ) mass selective detector (MSD) with electrospray ionization (ESI) with 150 V fragmentor, gas flow: 7 L/min, gas temperature 300°C, capillary 4000 V and nebulizer 15psi; reversed-phase (RP) Agilent ZORBAX SB-C18, 2.1 x 50 mm column with particle size:1.8 µm; quaternary pump with flow: 0.6 mL/min, high pressure limit: 600 bar. Isocratic elution was performed with mobile phase: 70% 0.01M HCl, 15% methanol, 15% acetonitrile (ACN), and 0.1% (v/v) formic acid solution in HPLC-grade water.

A qualitative analysis of EDTA was made by the value of mass/charge (m/z). The presence of EDTA was detected by MS spectra that show the predominant signal of 293.2 m/z corresponding to the cation $EDTAH^+$, and/or 315.2 - 315.1 m/z corresponding to the cation $EDTANa^+$.

A quantitative analysis was made based on the area of the detected peaks and calibration curve.

Preparation of the standard

An accurately weighed 50 mg of disodium calcium EDTA was dissolved in 50 mL of a 0.01 M aqueous HCl solution to give an initial concentration of 1.0 g/L (2.67 mM).

The solution was filtered through a 0.45 µm cellulose acetate membrane filter. Several standard solutions of EDTA with various concentrations in the range from 2.67 to 0.0267 mM were prepared from the stock by dilution with 0.01 M HCl.

System suitability

System suitability was examined according to the Center for Drug Evaluation and Research (CDER) (1994) and Evaluating System Suitability CE, GC, LC and A/D ChemStation (2019). Peak area, retention time, number of theoretical plates (N) and tailing factor (T) were considered.

Linearity assay

The sets of dilutions of standard stock solution in range of EDTA concentrations from 0.026 to 2.67 mM, were examined for a linear relationship by plotting the analyte peak areas *versus* the corresponding concentrations (calibration curve) followed by least square regression analysis and calculation of the slope, intercept and coefficient of correlation (r). Each point of the calibration curve was an average of five measurements. Calculations were done automatically by OpenLAB CDS. The concentration range for what the coefficient of correlation was equal or more than 0.999 (CDER, 1994) was taken as the working range.

Accuracy

Accuracy was expressed as mean absolute recovery and percent relative standard deviation (% RSD), for EDTA samples in five copies for each concentration.

As a recovery, the percentage of the measured amount of analyte to the true amount of analyte was taken.

Precision

Precision of the method was determined by measuring five samples under the same experimental conditions. To calculate precision, intra- and inter-day tests were performed and the results were expressed as RSD.

Limits of detection (LOD) and quantitation (LOQ)

The LOD is a characteristic value for the sensitivity of the method, at which the respective compound is just measurable, whereas the LOQ is the lowest concentration with acceptable linearity, accuracy and precision. Limit of detection was calculated based on the standard deviation of the response (σ) and the slope (a) according to the equation [1]:

$$LOD = 3.3\ \sigma/a \qquad [1]$$

and LOQ = 10 σ/a or 3·times LOD. The standard deviation of the response (σ) was determined based on the calibration curve (regression line) as the residual standard deviation of the regression line (European Medicines Agency. ICH 2006).

Repeatability

Repeatability was evaluated in the same tests as for the Accuracy and Recovery. The measure of repeatability was RSD.

Specificity

Specificity of the HPLC method for the quantitative determination of EDTA was investigated in order to obtain indications of possible interference from excipients in the preparations. For the specificity of the method, pairs of 1.32 mM EDTA solutions were prepared, as described above, with and without drug ingredients (sodium bicarbonate, benzyl alcohol) and tested in pairs. The difference between the peak areas in these pairs was expressed as the coefficient of variation (CV%). The peaks with CV<1% were considered as identical.

Robustness

Robustness of the method was evaluated by varying different method parameters such as flow rate, column temperature and mobile phase composition. During robustness testing, the system suitability parameters (T and N) were determined and the results were compared with the acceptable limits.

Statistical analysis

The variation of data is expressed in terms of the standard deviation (S.D.), along with the number of observations (n). The results of $p<0.05$ were considered as statistically significant. To construct the calibration curve, a regression analysis based on the Least Squares method was used. The correlation coefficient (r) and the determination coefficient (r^2) was calculated automatically by OpenLAB CDS version 2.2. during analysis.

RESULTS

System suitability

Test parameters were assessed by injecting 5 µL of EDTA standard solution 5 times, with a concentration of 0.5 µg/mL. Acceptable limit was chosen according to recommendations Bhanot (2013) and Bose (2014). The parameters such as relative standard deviation of peak areas, retention times of EDTA, number of theoretical plates, resolution, and tailing factor were determined and calculated automatically by OpenLAB CDS software and stated in Table 1.

The basic parameters all the tests were as follows: Injection volume: 5.0 µL, column temperature: 40°C, flow speed: 0.6 mL/min, detection time: 1 min. Retention time of EDTA detected by MS detector was 0.23 ± 0.01 min. The chromatograms and extracted MS spectra of EDTA are presented in Figs. 1 and 2.

The linearity was studied in the range of EDTA from 0.026 to 2.6 mM. In the range from 0.027 to 1.32 mM the correlation line has the coefficient of correlation $r > 0.999$. This range was chosen as the working range. Limit of detection was calculated based on the standard deviation of the response (σ) and the slope (a) according to equation [1]. The standard deviation of the response was determined based on the calibration curve (regression line) as the residual standard deviation of a regression line. The range, linearity and LOD are presented in Table 2.

Linearity, range and limit of detection

Taking in to account the injection volume of 5 µL, the LOD of the method should be estimated as 48 µM EDTA.

Accuracy, recovery and precision

The accuracy of the method was confirmed by conducting a recovery study for different concentrations (1.3, 1.0 and 0.26 mM) by five times replicate analysis, in accordance with European Medicines Agency. ICH (2006) guidelines.

Table 1. System suitability.

Test parameters	Mean	± SD	% RSD	Acceptable limit
Peak area (counts·min)	211316	2074	0.98*	RSD ≤2
Retention time (min)	0.23	0.0015	0.67*	RSD ≤2
Theoretical plates (N)	3711*	189	5.09	>2000
Tailing factor (T)	1.2*	0.071	5.90	≤2

EDTA standard solution 0.5 µg/mL, injection volume 5 µL. Values are presented as mean ± S.D, n = 5. *p<0.05.

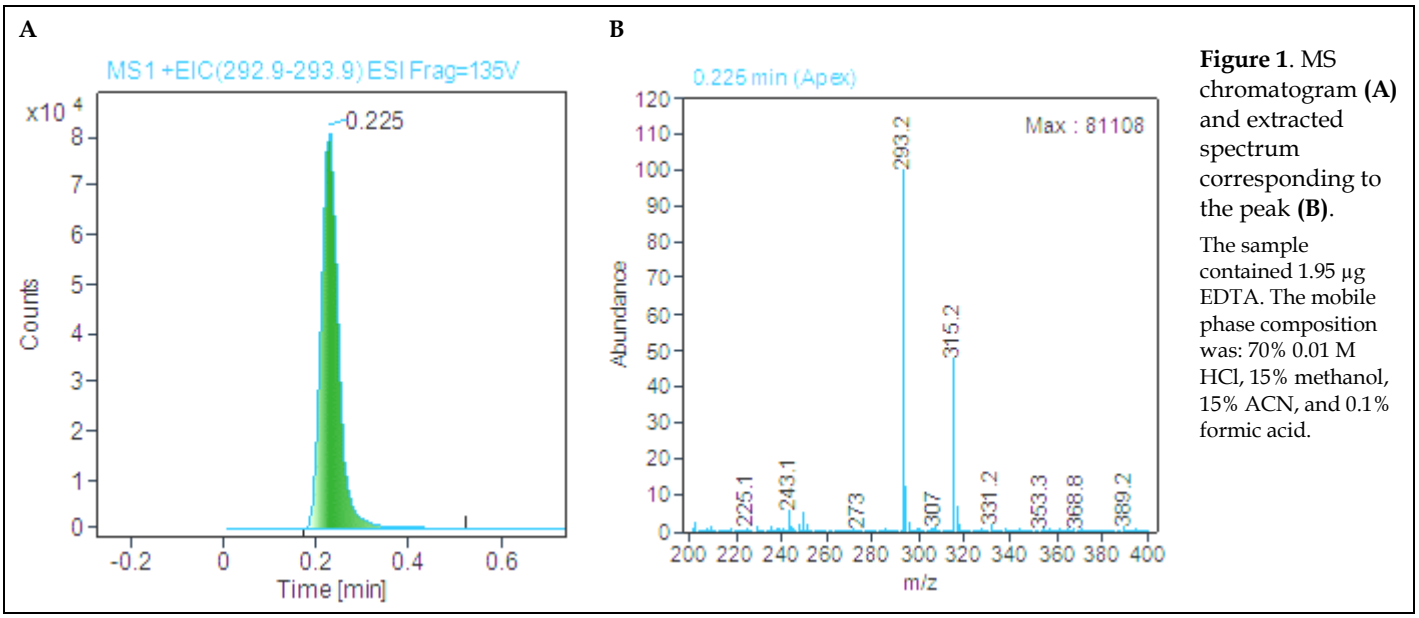

Figure 1. MS chromatogram **(A)** and extracted spectrum corresponding to the peak **(B)**.

The sample contained 1.95 µg EDTA. The mobile phase composition was: 70% 0.01 M HCl, 15% methanol, 15% ACN, and 0.1% formic acid.

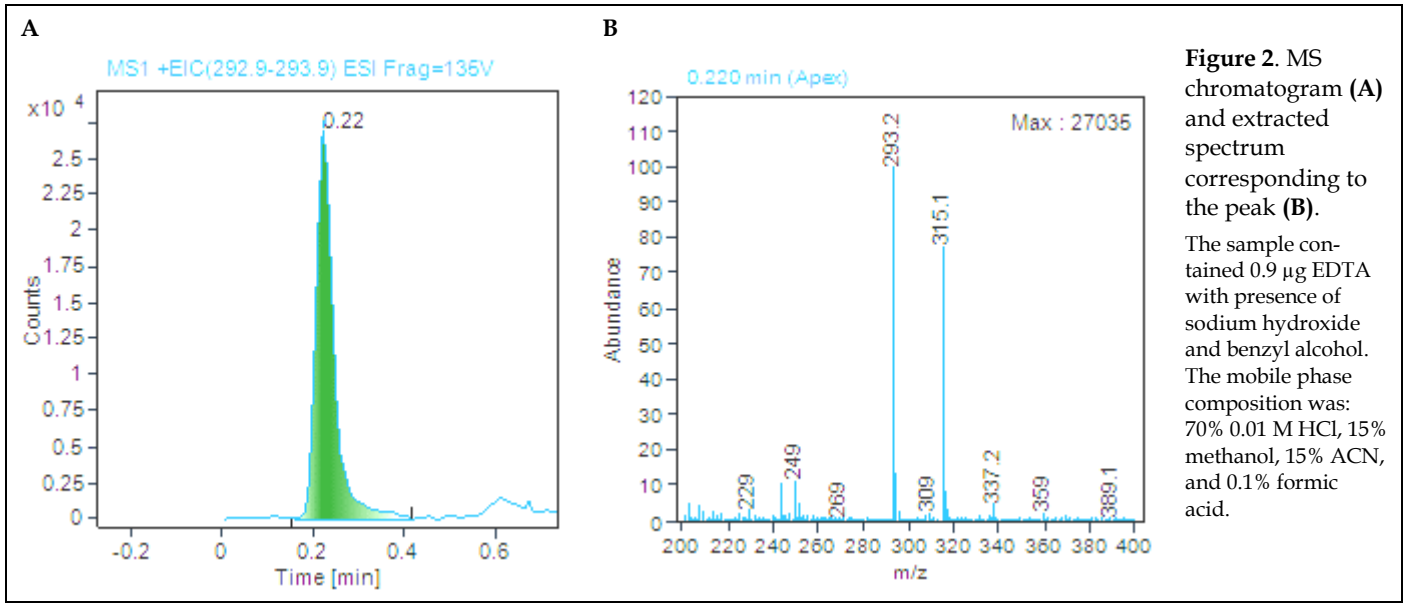

Figure 2. MS chromatogram **(A)** and extracted spectrum corresponding to the peak **(B)**.

The sample contained 0.9 µg EDTA with presence of sodium hydroxide and benzyl alcohol. The mobile phase composition was: 70% 0.01 M HCl, 15% methanol, 15% ACN, and 0.1% formic acid.

The recovery was determined based on the calibration curve (Table 3). LC peak had retention time 0.23 ± 0.01 min and a MS signal of m/z=+293.2 and +315.2. The results of accuracy, recovery and precision experiments are recorded in Table 3. Inter-day analysis (next day test) did not show any degradation of EDTA. Samples were kept overnight in closed vials at 19°C.

Selectivity assay

To demonstrate the specificity and selectivity of the method, EDTA solutions (0.26 mM) were prepared with and without the usual ingredients. The composition of the injection solution was as follows: $CaNa_2EDTA$ 200 g/L (0.53 M), NaOH 0.3 g/L (7.0 mM), benzyl alcohol 10.0 g/L (90.0 mM). All the ingredients were presented in tested solutions in proportional amounts. It was found that the presence of other ingredients in the formulation did not cause any significant effect on the EDTA peak and mass spectra. In particular, the coefficient of variation (CV%) of recovery EDTA between solutions contained all the components and contained only EDTA do not exceeded 0.9%. Thus, the method is specific for EDTA. Chromatogram and MS spectrum are presented in Fig. 2.

Robustness

Robustness test was done by making changes in an assay condition. These changes included: flow rate, ratio of methanol and acetonitrile in mobile phase, column temperature and concentration of HCl in mobile phase. System suitability parameters (T and N) were determined, they were within acceptable values for all changes in assay conditions (Table 4).

DISCUSSION

The EDTA molecule in an acidic (70% 0.01 M HCl) medium has an average positive charge, the RP-C18 column at low pH (≤3) also has a positively charged surface (Loeser, 2008). The electrostatic repulsion leads to the shorter retention time of EDTA and its separation from other uncharged components retained in the column. In the situation considered here, one minute was enough to analyze the sample, but if the composition contains a very highly retained component, the time should be increased to be sure that all components have left the column.

Table 2. Linearity, range and limit of detection (LOD).

X (µg)	Mean Y (n=5)	Y calc.	ΔY
0.05	3411	1280	2131
0.49	36701	39304	-2603
2.475	211316	210843	472
a	86418		
b	-3000		
r	0.9995		
Mean ΔY (n=3)	0.000		
S.D. ΔY (n=3)	2402		
LOD (µg)	0.09		

"X" - the content of EDTA in the sample; "Y"- the peak area; "Y calc." – the calculated peak area; "ΔY" – the residues; "a"- the slope of the regression line; "b"- the intercept; "r" – the correlation coefficient; "S.D. ΔY" – the residual standard deviation of the regression line.

Table 3. Accuracy, recovery, repeatability.

EDTA (µg)	Mean recovery (µg)	± SD	RSD (%)	Recovery (%)
2.475	2.5	0.025	1	100
1.93	1.9	0.021	1	100
0.39	0.4	0.025	6	113
1.93*	2.0	0.010	0.5	113

Recovery data presents an average value of five independent determinations (n=5). *The bottom row corresponds to the inter-day analysis.

Table 4. Robustness. The test results of samples containing 0.49 µg EDTA are presented.

Parameter	EDTA (0.49 µg)			
	T	%RSD	N	%RSD
Flow rate 0.6 mL/min	1.18	5.8	3701	5.1
Flow rate 0.5 mL/min	1.19	5.8	3690	5.1
Temperature 40°C	1.2	6.0	3710	5.2
Temperature 37°C	1.18	5.9	3720	5.0
Mobile phase				
Methanol - acetonitrile 15% - 15%	1.19	5.8	3706	5.0
Methanol - acetonitrile 13% - 17%	1.18	5.8	3705	5.1
70% 0.010 M HCl	1.2	5.8	3721	5.1
70% 0.015 M HCl	1.2	5.8	3735	5.0

T = Tailing factor (mean); N = Theoretical plates (mean); n=5.

It is interesting to compare our results with results of Wei et al. (2016) who used the same but previous generation instrument (Agilent 1200). They analyzed EDTA in wines and beer. Authors reported about very low LOD (0.001 mg/L) it is as low as 6 pg per injection (6 µL). But sample preparation procedure including mixing, heating, shaking, cooling, filtering takes an additional reagent, time, efforts, and also a long time of separation (5 min) makes the analysis more expensive in compare with our method. It must be noted, that a very low LOD is not necessary for method of pharmaceutical products analysis because of high concentration of analytes. Miller at al. (1997) used HPLC coupled with MS/MS detector to detect EDTA in dried blood stains.

For quantitative analysis of EDTA they used ion exchange column, DAD detector and EDTA derivatization by $CuSO_4$. Without derivatization authors can analyze EDTA with help of MS detector only qualitatively (presence/absence). Our method and modern tool give us the opportunity to simplify and speed up the quantitative analysis of EDTA.

CONCLUSIONS

A HPLC/MS technique was successfully developed and applied for the quantitative analysis of EDTA in pharmaceutical products. The development method gives a chance to analyze EDTA without any EDTA derivatization and preliminary sample preparation. The method was validated for

linearity, accuracy, recovery, precision and selectivity, as well as low detection limit and quantification. The method provides a fast (1 minute), simple, sensitive and reproducible means of determining EDTA in the pharmaceutical compositions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

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Concepts or ideas	x
Design	x
Definition of intellectual content	x
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Data acquisition	x
Data analysis	x
Statistical analysis	x
Manuscript preparation	x
Manuscript editing	x
Manuscript review	x

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