RESEARCH PAPER

Pre-column Derivatization Combined with HILIC-HPLC Method for Determining the Content of Quercetin

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ABSTRACT
A convenient, rapid, and accurate pre-column derivatization combined with HILIC-HPLC method was developed for determining the content of quercetin tetrahydrochloride (triethylenetetramine tetrahydrochloride). In this study, the chelation reaction between triethylenetetramine and copper ions was utilized to form a substance that absorbs in the ultraviolet region. Chromatographic conditions included a Luna® 5μm HILIC 200A column (4.6×250 mm, 5μm), with acetonitrile: 0.3% triethylamine-acetate (pH=4.00) (40:60, v/v) as the mobile phase for isocratic elution at a flow rate of 1 mL/min. The detection wavelength was set at 260 nm, and the column temperature was maintained at 30°C. The results indicated that the standard curve of triethylenetetramine exhibited excellent linearity with an R^2 of 0.9998. The limit of detection (LOD) was 30 ng/mL, and the limit of quantification (LOQ) was 100 ng/mL. The recovery rate was 100.54%, and the relative standard deviation (RSD) was 0.75%. The derivatized product remained stable at room temperature for up to 12 hours. This method demonstrates excellent selectivity, linearity, and accuracy, with stable target compounds, making it suitable for the determination of quercetin tetrahydrochloride content and providing support for its quality control.

Keywords: *Quercetin Tetrahydrochloride, HILIC-HPLC Method, Content Determination, Pre-column Derivatization1.*
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INTRODUCTION

Trientine tetrahydrochloride (Trientine-4HCl) is a copper ion chelator primarily used for the treatment of Hepatolenticular degeneration (HLD), also known as Wilson disease (WD)[1], a recessive hereditary copper metabolism disorder caused by mutations in the ATP7B gene[2]. The core mechanism of action involves the formation of a stable soluble complex with free copper ions in the body, which facilitates the excretion of copper through urine and reduces intestinal absorption of copper, thereby effectively lowering pathological copper deposition in tissues such as the liver, brain, and cornea[3]. This drug is especially suitable for adult patients with stable Wilson disease who are intolerant to penicillamine (approximately 20%-30% of patients cannot tolerate penicillamine due to allergies, nephrotoxicity, or neurological side effects) or experience relapses. It significantly improves clinical symptoms caused by copper overload, including liver dysfunction (such as elevated transaminases, cirrhosis), neurological symptoms (such as tremors, dystonia, psychiatric disorders), and the characteristic Kayser-Fleischer rings in the cornea[4, 5]. Compared to the traditional first-line drug penicillamine, trientine tetrahydrochloride has a milder chelation property and better safety profile, especially with good tolerance in children and patients with neurological symptoms. Its lower urinary copper excretion (274 mcg/24h vs. 511 mcg/24h for penicillamine) suggests that it may reduce the risk of treatment-related tissue damage through a more physiological copper elimination mechanism[6-8]. Trientine hydrochloride (Trientine-2HCl) primarily exists in this form; however, due to the exposed nitrogen atoms in its structure, it is prone to degradation when exposed to water and oxygen, potentially converting into a less soluble trientine dihydrate[9]. The formulation requires refrigeration and storage in sealed containers, significantly limiting its portability[10]. In 2022, the FDA approved Cuvrior® (trientine tetrahydrochloride) based on Phase III CHELATE study data, confirming its equivalence to penicillamine in maintaining serum non-copper-bound ceruloplasmin (NCC) levels (56 mcg/L vs. 46 mcg/L)[11], providing a safe and effective alternative for penicillamine-intolerant patients.

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Trientine tetrahydrochloride as a second-line therapeutic agent for Wilson's disease, has made significant progress in its global market approval[1]. However, research on the quality control and analytical methods for this drug is still in its early stages. None of the international pharmacopeias (USP, EP, ChP) have established content determination standards for trientine tetrahydrochloride, resulting in a lack of standardized analytical techniques for raw material synthesis process optimization, formulation quality control, and clinical pharmacokinetic studies. These development stages urgently require the establishment of a dedicated, sensitive, and accurate quantitative analysis method for TETA to support content determination, process development, stability evaluation, and monitoring of in vivo exposure.

The trientine tetrahydrochloride used in this study was synthesized via the following process: Ethylenediamine (EDA) and dichloroethane (EDC) were used as the starting materials. The core of the process involves constructing a linear triethylenetetramine backbone through a nucleophilic substitution reaction: the amino group of ethylenediamine attacks the carbon-chlorine bond of dichloroethane, and through alkylation reactions, the structure is gradually linked to ultimately form the core product—triethylenetetramine (TETA). Excess EDA is used to suppress side reactions, which could otherwise lead to the formation of by-products such as diethylenetriamine (DETA), 1-(2-Aminoethyl)piperazine (AEP), and tris(2-aminoethyl)amine (TREN). After purification of TETA, it undergoes a salt formation reaction with hydrogen chloride (HCl) in solution, converting all four amino groups in the molecule into hydrochloride salts, yielding the final stable product, trientine tetrahydrochloride (Figure 1). This method avoids the use of other special, expensive reagents (e.g., 2,2-(2,2-(ethane-1,2-diyl-bis(benzyl)-bis(ethane-2,1-diyl))diisoindole-1,3-dione)) and hazardous materials (e.g., Raney nickel), making it cost-effective and highly safe.

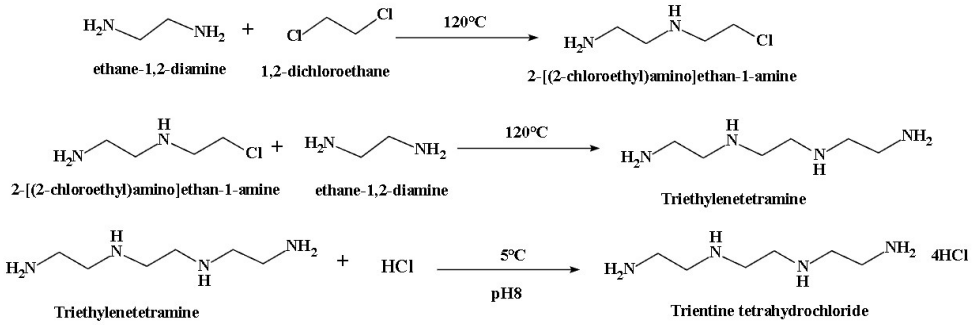

Figure 1. Synthesis Route of Trientine Tetrahydrochloride

The core difficulty in chromatographic analysis of polyamine compounds such as triethylenetetramine lies in their strong polarity and strong basicity: the strong polarity results in weak retention in reversed-phase chromatography, while the strong basicity leads to intense interactions with the silica hydroxyl groups of the chromatographic column, causing peak tailing, irreproducible retention times, and reduced column efficiency[12, 13]. Since these compounds do not absorb at ultraviolet wavelengths, liquid chromatography analysis typically involves reacting them with derivatizing reagents to generate products with ultraviolet/fluorescent groups[14-16]. However, due to the multiple amine derivatization sites in the triethylenetetramine molecule, commonly used derivatizing agents such as o-phthalaldehyde (OPA), dansyl chloride (Dns-Cl), and others often generate multiple derivatized products, resulting in non-singular chromatographic peaks, making them unsuitable for accurate content determination[4, 17-19]. Studies have shown that triethylenetetramine can react with copper to form a single product, triethylenetetramine copper (TETA—Cu) (Figure 2)[20-22], which exhibits a maximum UV absorption at 260 nm. In this study, based on the strong polarity of triethylenetetramine copper, hydrophilic interaction chromatography (HILIC-HPLC) was employed to effectively analyze the compound[23-25]. A pre-column derivatization combined with HILIC-HPLC analytical method was developed. This method is convenient, rapid, and accurate, making it suitable for the content determination of trientine tetrahydrochloride and can be extended to its process development, stability evaluation, and monitoring of in vivo exposure.

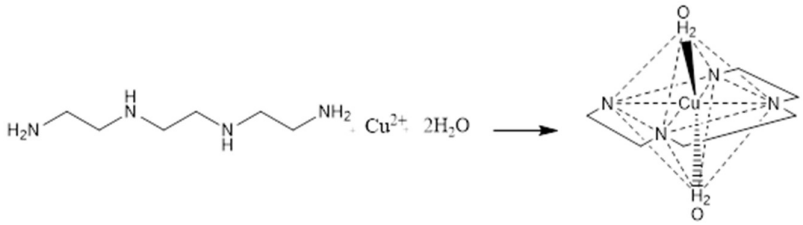

Figure 2. Reaction of Triethylenetetramine with Copper

MATERIALS AND METHODS

Materials and Reagents

Acetonitrile (HPLC grade) was purchased from Honeywell (Charlotte, USA). Glacial acetic acid (GR grade), ammonium acetate (GR grade), sodium hydroxide (AR grade), copper sulfate pentahydrate (98%), ethylenediamine monohydrate (≥98%), diethylenetriamine (GC grade), N-(2-Aminoethyl)piperazine (≥99%), and tris(2-aminoethyl)amine (≥96%) were purchased from Aladdin (Shanghai, China). Trientine tetrahydrochloride was synthesized by Jiangsu Institute of Pharmaceutical Research, with batch numbers 20240605 and 20240606. The purity of each batch was determined by silver nitrate titration, with batch 20240605 having a purity of 98.2% and batch 20240606 having a purity of 99.8%. Batch 20240605 was used as the test sample, and batch 20240606 was used as the reference standard.

Instrumentation

The LC-2030C high-performance liquid chromatography (HPLC) system, online vacuum degasser, DAD detector, and LC-Solution workstation were obtained from Shimadzu (Japan). The Luna® 5μm HILIC 200A (4.6×250 mm, 5μm) column was purchased from Phenomenex (USA). The QUINTIX35-1CN electronic analytical balance was from Sartorius (Germany). The FiveEasyPlus pH meter (Mettler, Switzerland) was used for pH measurement. The PURELAB flex2 ultrapure water system was from Veolia (France). The HH-6 thermostatic water bath was from Guohua Electric Co., Ltd. (China).

Preparation of Solutions

Preparation of Derivatizing Reagents

Accurately weigh copper sulfate pentahydrate, dissolve it in water, and prepare a solution containing 2 mg of copper ions per 1 mL, to be used as a derivatizing reagent.

Preparation of Test Solution

Weigh an appropriate amount of trientine tetrahydrochloride test sample, dissolve it in water, and prepare a solution containing 2 mg of triethylenetetramine per 1 mL. Take 1 mL of the above solution and transfer it into a 25 mL volumetric flask. Add 1 mL of 0.1 mol/L NaOH, followed by 1 mL of the derivatizing reagent. Shake well, let it stand at room temperature for 5 minutes, then dilute with water to the mark. Shake well and filter the solution through a 0.45 μm membrane filter before performing HPLC analysis.

Preparation of Reference Solution

Weigh an appropriate amount of trientine tetrahydrochloride reference sample, dissolve it in water, and prepare a solution containing 2 mg of triethylenetetramine per 1mL. Take 1 mL of the above solution and transfer it into a 25 mL volumetric flask. Add 1 mL of 0.1 mol/L NaOH, followed by 1 mL of the derivatizing reagent. Shake well, let it stand at room temperature for 5 minutes, then dilute with water to the mark. Shake well and filter the solution through a 0.45 μm membrane filter before performing HPLC analysis.

Chromatographic Conditions

Chromatographic Column: Luna® 5μm HILIC 200A (4.6×250 mm, 5μm);Mobile Phase: Acetonitrile - 0.3% Triethylamine - Acetate (pH=4.0) (40:60);Flow Rate: 1.0 mL/min; Injection Volume: 10 μL ;Column Temperature: 30°C;Detection Wavelength: 260 nm; Mode: Isocratic Elution.

Method Validation

Specificity

An appropriate amount of TETA reference sample, EDA, DETA, AEP, and TREN were derivatized according to section " Preparation of Test Solution ". A suitable volume of the resulting derivatized solutions, along with a blank derivatized solution, was injected into the liquid chromatograph for analysis to evaluate the specificity of the method.

Linearity and Sensitivity

Accurately weigh an appropriate amount of trientine tetrahydrochloride reference sample, dissolve it in water, and prepare a standard solution with a concentration of 2 mg/mL (based on triethylenetetramine). Dilute the standard solution with water to prepare test solutions containing triethylenetetramine at concentrations of 40, 60, 80, 100, and 120 μg/mL. Derivatize the solutions according to section " Preparation of Test Solution ", filter through a 0.45 μm membrane filter, and take 10 μL for HPLC analysis. Plot the peak area of the target compound (y) as the vertical axis and the concentration of the target compound (x, μg/mL) as the horizontal axis to construct a standard curve and perform linear regression.

The sensitivity of the method for detecting triethylenetetramine is evaluated using the detection limit (LOD) and quantitation limit (LOQ). The reference solution with low concentration is sequentially diluted with water, filtered, and then analyzed by injection. When the signal-to-noise ratio (S/N) is 3, the corresponding

concentration is defined as the limit of detection (LOD). When the S/N is 10.0, the corresponding concentration is defined as the limit of quantitation (LOQ). The sensitivity is calculated using the following formula：

D=3N/S

Where D is the detection limit, N is the noise, and S is the sensitivity of the detector.

S=I/Q

Where S is the sensitivity, I is the signal response value, and Q is the injection volume.

Precision and Repeatability

Accurately weigh an appropriate amount of trientine tetrahydrochloride reference sample, prepare the solution according to section " Preparation of Test Solution ", filter the solution, and inject 10 μL into the liquid chromatograph. Perform five consecutive injections to assess the instrument's injection precision.

Accurately weigh an appropriate amount of six aliquots of trientine tetrahydrochloride test sample, prepare the solutions according to section " Preparation of Test Solution ", filter the solutions, and inject 10 μL into the liquid chromatograph to assess the repeatability of the method.

Solution Stability

Accurately weigh the trientine tetrahydrochloride reference sample and test sample solutions, prepare the solutions according to section " Preparation of Test Solution ", and place them at room temperature for 0, 1, 2, 3, 4, 6, 8, and 12 hours. After each time interval, filter the solutions and inject 10 μL into the liquid chromatograph to assess the stability of the test and reference sample solutions.

Accuracy

Accurately weigh appropriate amounts of trientine tetrahydrochloride test and reference sample, and dilute them with water to prepare test and reference solutions containing 1 mg/mL of triethylenetetramine. Transfer nine aliquots of the test solution into volumetric flasks, and quantitatively add the trientine tetrahydrochloride reference solution. Perform recovery tests at three concentration levels (80%, 100%, and 120%). The recovery rate is calculated using the following formula:Recovery rate (%) = (Measured amount - Sample background) / Theoretical added amount, to evaluate the accuracy of the method.

Robustness

Accurately weigh an appropriate amount of trientine tetrahydrochloride test sample, prepare the solution according to section " Preparation of Test Solution ", filter the solution, and inject 10 μL into the liquid chromatograph. Change the single variables in the chromatographic conditions under section " Chromatographic Conditions", including column temperature (30 ± 2°C), flow rate (1.0 ± 0.1 mL/min), and mobile phase ratio (60% ± 5% aqueous phase), and perform an external standard method to calculate the content and RSD based on the peak area, to evaluate the ruggedness of the method.

RESULTS AND DISCUSSION

Due to the lack of UV absorption and the strong polarity of triethylenetetramine, it is challenging to use conventional RP-HPLC for content determination. Therefore, this study established an analytical method combining pre-column derivatization and HILIC-HPLC. By systematically optimizing the detection wavelength, mobile phase type and ratio, and derivatization conditions, a convenient, rapid, and accurate method for determining the content of trientine tetrahydrochloride was successfully developed.

Optimization of Detection Wavelength

The triethylenetetramine copper derivative was scanned over a wavelength range of 200-400 nm using a DVD detector. The results showed that the triethylenetetramine copper derivative exhibited maximum absorption at 260 nm, and thus the detection wavelength was ultimately selected as 260 nm, as shown in Figure 3.

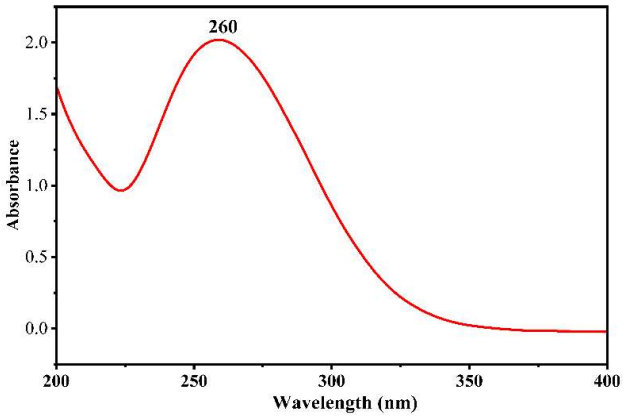

Figure 3. UV Spectrum of the Triethylenetetramine Copper Derivative with Characteristic Peak

OPTIMIZATION OF MOBILE PHASE

Acetonitrile-water, acetonitrile-ammonium acetate-acetate buffer solution, and acetonitrile-triethylamine-acetate buffer solution were selected as mobile phases, and the effects of pH and ratio of acetonitrile-ammonium acetate-acetate buffer solution and acetonitrile-triethylamine-acetate buffer solution on the separation of triethylenetetramine copper derivative were investigated (Figure 4).

Table I. Separation Efficiency of Triethylenetetramine Copper Derivative under Different Chromatographic Conditions

Chromatographic Condition	Phase A	Phase B	Flow rate (mL/min)	Results
Chromatographic Condition I	Acetonitrile	Water	1	Severe peak tailing and broadening, with a low theoretical plate number
Chromatographic Condition II		50 mM ammonium acetate-acetic acid buffer (pH = 5.00)		The main peak cannot be effectively separated from diethylenetriamine and tris(2-aminoethyl)amine
Chromatographic Condition III		50 mM ammonium acetate-acetic acid buffer (pH = 4.00)		Severe peak tailing and broadening, with a low theoretical plate count
Chromatographic Condition IV		0.3%Triethylamine (pH = 5.00)		Peak tailing, with a low theoretical plate count
Chromatographic Condition V		0.3%Triethylamine (pH = 4.00)		The main peak has a good shape, with no interference from impurity peaks

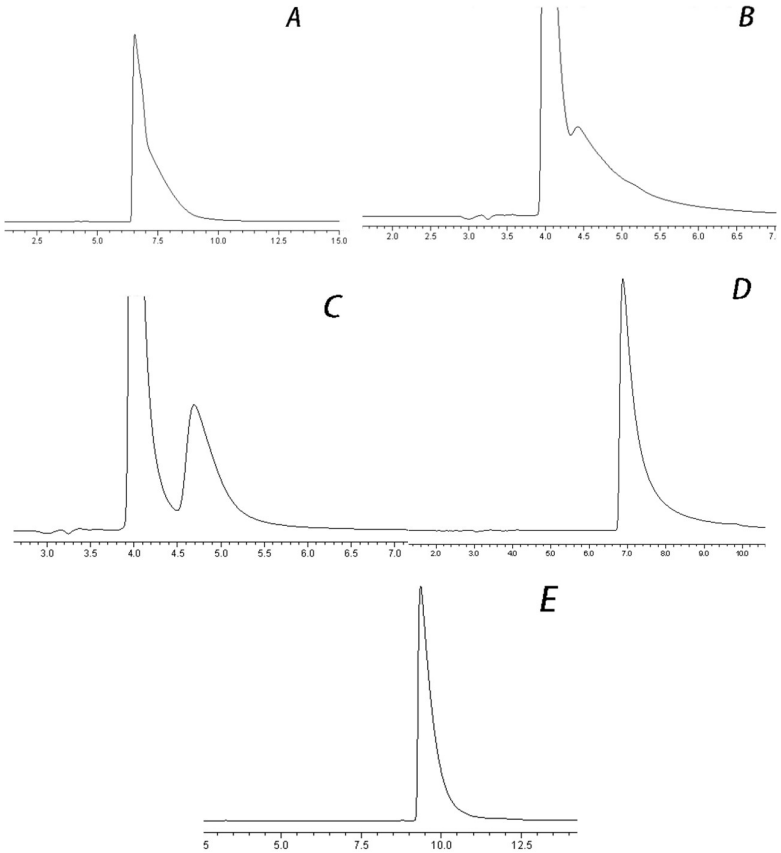

Figure 4. Chromatograms of Triethylenetetramine Copper Derivative under Different Chromatographic Conditions(Figure 2A: B phase is water; Figure 2B: Chromatogram of the derivatized solution of triethylenetetramine

and diethylenetriamine mixture under 50 mM ammonium acetate (pH=5.00) conditions; Figure 2C: Chromatogram of the derivatized solution of triethylenetetramine and diethylenetriamine mixture under 50 mM ammonium acetate (pH=4.00) conditions; Figure 2D: B phase is 0.3% triethylamine (pH=5.00); Figure 2E: B phase is 0.3% triethylamine (pH=4.00)).

Optimization of Derivatization Conditions

The aqueous solution of trientine tetrahydrochloride is acidic and not easily chelated with copper. It is usually necessary to adjust the pH to alkaline conditions to deprotonate the primary and secondary amines in triethylenetetramine. The effect of different pH values on the response of the target derivative was investigated under a target compound concentration of 80 μg/mL. As shown in Figure 5A, the derivatization reaction was most complete at a pH of 9, and pH=9 was selected as the optimal pH condition for derivatization.

The effect of reaction temperature (25°C-45°C) on the response of the target derivative was investigated, and the results are shown in Figure 5B. The reaction temperature had no significant effect on the derivatization reaction. Considering the convenience of the experiment, room temperature was ultimately chosen as the reaction condition.

The derivatization reaction time (0-30 minutes) was investigated and optimized. The results are shown in Figure 5C. The peak area of the derivatized product became stable after 5 minutes, so the derivatization time was chosen as 5 minutes.

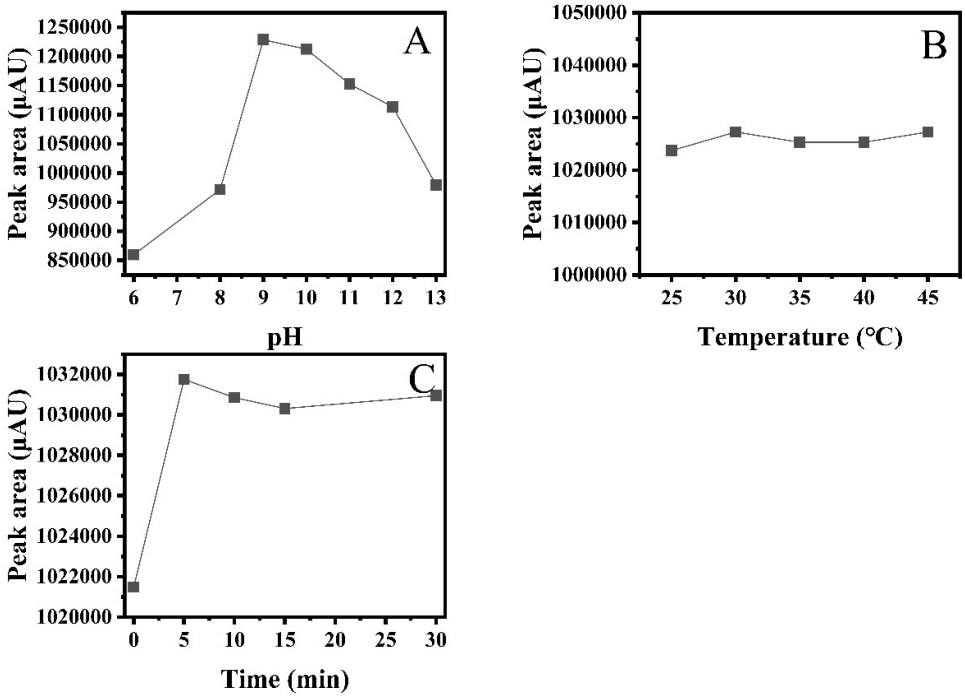

Figure 5. Effect of Derivatization pH (A), Derivatization Temperature (B), and Derivatization Time (C)

Method Validation

The method was validated according to the International Council for Harmonisation (ICH Q2) guidelines for the validation of analytical methods.

Specificity

The chromatograms of the blank derivatized solution, the reference sample derivatized solution, and known impurities (EDA, DETA, AEP, TREN) are shown in Figure 6. The known impurities and the blank derivatized solution showed no response under these conditions and did not interfere with the detection of the main peak, indicating that the specificity of the method is good.

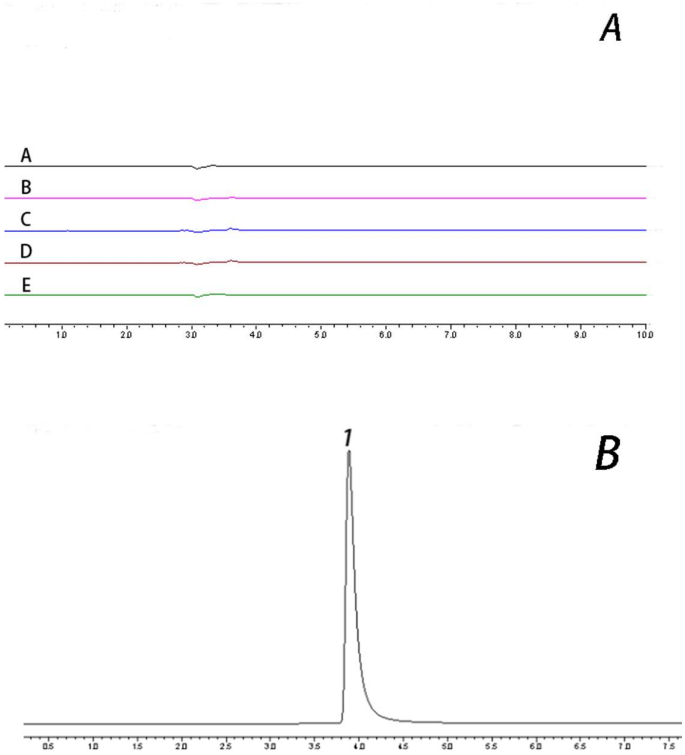

Figure 6 . Chromatograms of Known Impurities and Triethylenetetramine Copper under These Conditions(Figure 6A: A = TREN, B = AEP, C = EDA, D = Blank Derivatized Solution, E = DETA; Figure 6B: 1 = TETA-Cu)

Linearity and Sensitivity

The linearity of the method was investigated using trientine tetrahydrochloride reference solution in the concentration range of 40 μg/mL to 120 μg/mL. A standard curve was constructed with the peak area of the target compound (y) as the vertical axis and the concentration of the target compound (x, μg/mL) as the horizontal axis, and linear regression was performed. The linear regression equation was y = 9408x - 57201 (r = 0.9998). The results showed that triethylenetetramine exhibited a good linear relationship in the range of 40–120 μg/mL.

The reference solution with low concentration was sequentially diluted with water, filtered, and then analyzed by injection. When the signal-to-noise ratio (S/N) was 3.0, the corresponding concentration was defined as the limit of detection (LOD). When the S/N was 10.0, the corresponding concentration was defined as the limit of quantification (LOQ). The LOD value of this method was 30 ng/mL, and the LOQ value was 100 ng/mL.

3.4.3 Precision and Repeatability

Accurately pipette the derivatized reference solution and perform five consecutive injections. Measure the peak area of trientine tetrahydrochloride and calculate the RSD. The results are shown in Table 2. The RSD of the peak area of trientine tetrahydrochloride was 0.21%, indicating that the instrument's precision is good.

The precision of the method was evaluated through repeatability testing. Six parallel preparations of the trientine tetrahydrochloride test solution were made, and the peak areas were recorded. The content of trientine tetrahydrochloride in the six test solutions was calculated using the external standard method to assess the repeatability of the method. The results are shown in Table 2. The RSD of the measurements was 0.54% (n=6), indicating that the method has good repeatability.

The intermediate precision of the method was evaluated by analyzing trientine tetrahydrochloride reference standard solutions on different days, by different analysts, and using different instruments. The test results showed an intermediate precision RSD of 1.32%, indicating good intermediate precision of the method.

Table II. The results of linearity, sensitivity, and precision tests

Name	Results
LOD(ng/mL)	30
LOQ(ng/mL)	100
Slope(b)	9408
Intercept(a)	-57201
r^2	0.9998
Precision (%RSD)	0.21
Repeatability (%RSD)	0.54
Intermediate Precision(%RSD)	1.32

Stability

The peak areas of the test sample solution and reference sample solution were analyzed at 0h, 1h, 2h, 3h, 4h, 6h, 8h, and 12h under the given chromatographic conditions, and the results are shown in Table 4. Compared to the peak area at 0h, there were no significant changes in the peak areas of the test and reference sample solutions. The RSD for the test sample (n=12) was 0.23%, and the RSD for the reference sample (n=12) was 0.43%, indicating that the test and reference samples remained stable at room temperature for 12 hours.

Table IV. Stability Test Results

Time	Peak Area of Test Sample Solution	Peak Area of Reference Standard Solution
0h	1020807	1000155
1h	1019428	990776
2h	1016298	991247
3h	1014593	985794
4h	1015795	988414
6h	1014366	988609
8h	1015476	990009
12h	1015811	991325
均值	1016572	990791
RSD	0.23%	0.43%

Accuracy

The accuracy of the method was evaluated using a spiking recovery test. Three different levels of reference solution (80%, 100%, and 120%) were added to the trientine tetrahydrochloride test solution, followed by derivatization, and then analyzed according to the chromatographic conditions under section " Chromatographic Conditions". The test results are shown in Table 5. The average recovery rate of trientine tetrahydrochloride was 100.54% (n=9), with an RSD of 0.75%.

Table V. Accuracy Test Results

Concentration(%)	Original(μg/mL)	Added(μg/mL)	Found (μg/mL)	Recovery(%)	Average recovery(%)	RSD(%)
80	57.40	34.44	92.03	100.54%	100.54%	0.75%
			91.98	100.41%		
			92.04	100.58%		
100		57.40	114.51	99.49%		
			114.64	99.72%		
			115.40	101.05%		
120		80.36	137.68	99.90%		
			139.05	101.60%		
			139.00	101.54%		

Ruggedness

By changing single variables such as column temperature (30 ± 2°C), flow rate (1.0 ± 0.1 mL/min), and mobile phase ratio (60% ± 5% aqueous phase), the content and RSD of trientine tetrahydrochloride test solution were calculated using the external standard method based on peak area. The results shown in Table 6 indicate that when any parameter was slightly varied, the RSD of the detected amount of trientine tetrahydrochloride did not exceed 1.24%, demonstrating that the method has good ruggedness.

Table VI. Ruggedness Test Results

Conditions	Parameter	Minimum theoretical plate number	Trientine tetrahydrochloride content (mg/mL)	Mean value (mg/mL)	RSD
Flow rate	1.0 mL/min	6202	1.999	2.008	0.48%
	0.9 mL/min	6215	2.018		
	1.1 mL/min	6345	2.007		
Column temperature	30 ℃	6217	1.999	2.022	1.24%
	32 ℃	6058	2.049		
	28 ℃	6325	2.017		
Mobile phase ratio	60% Phase B	6542	1.999	2.024	1.11%
	55% Phase B	6289	2.030		
	65% Phase B	6358	2.043		

CONCLUSION

Based on this study, a fast and accurate analytical method for determining the content of trientine tetrahydrochloride, combining pre-column derivatization with HILIC-HPLC, has been successfully developed. By optimizing the derivatization and chromatographic conditions, the issue of triethylenetetramine's strong polarity and basicity, which complicates its analysis by conventional reversed-phase chromatography, has been addressed. The method demonstrated good specificity, linearity, precision, repeatability, and accuracy, meeting the requirements for content determination of trientine tetrahydrochloride, while also exhibiting good solution stability and ruggedness. Experimental results indicate that this method is suitable for quality control of trientine tetrahydrochloride and provides an effective tool to support its process development, stability evaluation, and monitoring of in vivo exposure. Moreover, the method's sensitivity and stability ensure its reliability in practical applications. Overall, this study provides a simple and efficient approach for the quantitative analysis of trientine tetrahydrochloride, with broad potential for future applications.

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