

# Development Of Validated Spectrofluorimetric Method For The Estimation Of Thiocolchicoside

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**Abstract:** A simple and sensitive spectrofluorimetric method developed for the estimation of thiocolchicoside in pure and pharmaceutical preparation. This method is based on the oxidation of thiocolchicoside with cerium (IV) to produce cerium (III), whose fluorescence was monitored at 366 nm while excited at 289 nm. The calibration graph was linear over the range of 1-10 $\mu$ g/ml. This is applied successfully for the assay of bulk powder with a mean accuracy of 100.08 $\pm$ 0.2059. Applicability was examined by analyzing dosage form. Recoveries were 99.42 and 99.70 % at 50 and 100% level with the RSD values of 0.227 and 0.044%, respectively.

**Keywords:** Thiocolchicoside, Spectrofluorimetric, fluorescence, excitation, emission, ICH guidelines.

## Introduction

Thiocolchicoside is an antispasmodic drug. Chemically it is N-[3-( $\beta$ -D-glucopyranosyloxy)-1,2-dimethoxy-10(methylthio)-9-oxo-5,6,7,9-tetrahydrobenzo [a] heptalen-7-yl] acetamide<sup>1</sup>. It is used as muscle relaxant, anti-inflammatory and analgesic. The antispasmodic activity is mainly due to the activation of GABA- inhibitory pathways. Thiocolchicoside is not official in any pharmacopoeia. Literature survey reveals that thiocolchicoside can be assayed by UV spectrophotometric<sup>2</sup>, HPLC<sup>3</sup> and HPTLC<sup>4</sup> methods individually or in combination with other drugs. Among the various methods available for the determination of drugs, spectrophotometry and spectrofluorimetry continue to be very popular, because of their simplicity, specificity and low cost. Therefore, the need for fast, low cost, selective method is obvious, especially for the routine quality control analysis of pharmaceutical formulations containing thiocolchicoside. This paper describes a spectrofluorimetric method for the estimation of the cited drug. The method possesses distinct advantages over the existing methods, VIZ. spectrofluorimetric method, with

respect to selectivity, sensitivity, range of determination, speed and simplicity.

## Materials And Methods

Pharmaceutical grade of thiocolchicoside was kindly supplied by Alembic Ltd., (Vadodara, India) and certified to contain > 99% of thiocolchicoside. It was used without further purification. A commercial injection formulation was purchased from the local market (Myoril – 4mg/2ml inj). All other reagents used in this study were of AR grade. Jasco FP-750 spectrofluorimeter connected to computer loaded with spectra manager 1.54.03 version was employed with a 10 mm matched glass cells. All weights were taken on Shimadzu Electronic balance BL-220 H.

### **Reagents and solutions**

#### **Preparation of solutions**

The standard solution of thiocolchicoside was prepared by weighing 10 mg of drug, dissolved in 10 ml of water (1000  $\mu$ g/ml) and further diluted to

get a concentration of 100µg/ml. This solution was protected from light, and stored at 4°C for a week. A concentration of 5µg/ml was used for optimization of reagents and instrumental parameters.

The Ce(IV) solution at concentration of 0.5 mM was prepared by weighing 0.03162 g of Ceric ammonium sulphate in 0.05 M sulphuric acid and was kept at 4°C for two weeks.

### **Selection of wavelength**

The first step involved in fluorimetric analysis was the selection of excitation and emission wavelength. A 5 ml of CAS was added into a standard flask containing 0.5 ml of drug solution of concentration 100 µg/ml and kept aside for 30 minutes and made up to 10 ml with water. It was scanned in the region of 220-525 nm. 3D spectra of excitation and emission of Ce(III) was recorded. Keeping the emission wavelength constant, the excitation spectrum of Ce(III) was measured in spectral measurement mode of the instrument. Similarly, the emission spectrum was again measured with the fixed excitation wavelength.

### **Optimization of experimental variables**

#### **Effect of acid type**

The oxidation of ceric ammonium sulphate is normally performed in acid medium to prevent precipitation of ceric hydroxide. The most suitable acid for the reaction was determined by testing various acids like sulphuric acid, hydrochloric acid, and nitric acid.

#### **Effect of strength of sulphuric acid**

Different concentrations of sulphuric acid ranging from 0.01 to 0.07 M were prepared, and the effect of the sulphuric acid concentration on the reaction yield was examined.

#### **Effect of concentration of CAS**

Different molar solution of CAS ( $2.5 \times 10^{-4}$  M –  $3 \times 10^{-3}$  M) were prepared in 0.05 M sulphuric acid. To 5ml of the above solution, 50 µg/ml of stock was added, and it was made up to 10ml with distilled water. The solutions were kept aside for 30 minutes to complete the reaction. The fluorescence intensities were measured at 366 nm after excitation at 289 nm.

#### **Effect of volume of CAS**

The effect of volume of CAS on fluorescence intensity was studied by keeping the concentration of drug and CAS constant (5 µg/ml and 0.5 mM respectively) and also by using different volumes of CAS (1-7 ml). The procedure was followed as

done earlier, and the fluorescence intensities were measured at 366 nm after excitation at 289 nm.

### **Optimization of time**

After fixing the strength of sulfuric acid, concentration and volume of CAS, the time for completion of the reaction were determined by careful measurement of fluorescence intensity at every ten minutes interval from zero time to 50 minutes.

### **Effect of instrumental variables**

The sensitivity of the method depends not only on the reagent used in the reaction but also on the instrumental parameters such as band width and response time (2).

#### **Effect of bandwidth**

To fix an ideal excitation and emission bandwidth, 5 µg/ml solution of thiocolchicoside was prepared by the method mentioned above. Keeping the excitation bandwidth constant, emission of the solution was measured. Similarly, by keeping emission bandwidth constant and by varying excitation bandwidth, the fluorescence intensity was noted. A combination of excitation and emission band width such as 5, 10, 20 nm were tried, and observed for its effect on fluorescence and smoothness of the curve.

#### **Effect of response time**

Response is the time taken to measure the fluorescence intensity and is highly dependent on the type of instrument. For the present study Jasco FP/750 fluorimeter was used which has different response time such as 0.02 sec, 0.05 sec, 0.1 sec, 0.25 sec, 0.5 sec, 1sec, 2sec, 4sec, and 8 sec. To fix this response factor 5µg/ml of thiocolchicoside was used.

### **Optimized procedure**

The drug solution of thiocolchicoside was added to 5 ml of 0.5 mM of ceric ammonium sulphate which was prepared using 0.05 M sulphuric acid and kept aside for 30 minutes at room temperature to complete the reaction, and then the content was diluted with water to 10 ml and measured against reagent blank.

### **Validation of spectrofluorimetric method**

#### **Linearity**

Under proposed experimental conditions, the relationship between the relative fluorescence intensity and the concentration of thiocolchicoside was studied. 5 ml of 0.5mM ceric ammonium sulphate (prepared using 0.05 M sulphuric acid) was added to 10 ml standard flask containing 0.1 –

1 ml of standard stock solution (100 µg/ml). The contents of the flask were mixed, and kept aside for 30 minutes at room temperature to complete the reaction and made up to the volume with water. Fluorescence intensity was measured at the fixed excitation and emission wavelength of 289 and 366 nm. The calibration curve was plotted between concentrations versus fluorescence intensity.

### Specificity

The prepared standards, sample solutions and the blank solution were scanned from 220-550 nm using the optimized spectrofluorimetric conditions and checked for the change in emission at respective wavelengths.

### Precision

The intraday and interday assay precision were carried out through replicate analysis (n=6) for 3 concentrations (4, 5 and 6 µg/ml). For interday precision assay, the analysis was carried out for three consecutive days at the same concentration level as used in intraday precision.

### Accuracy

To find the accuracy of the method, the recovery experiment was carried out using standard addition technique. To the previously analyzed sample (4 µg/ml), a known amount of standard drug was added at 50 and 100 % level. The contents were reanalyzed with the above described procedure.

### LOD and LOQ

The limit of detection (LOD) and LOQ were calculated according to ICH Q2 recommendation (13) LOD was calculated from the following equation:  $LOD = 3.3 \cdot \text{slope} / \text{slope}$  where ' ' is the standard deviation of intercept. LOQ was calculated from the following equation:  $LOQ = 10 \cdot \text{slope} / \text{slope}$

### Application of proposed method for the estimation of THIO in Formulation

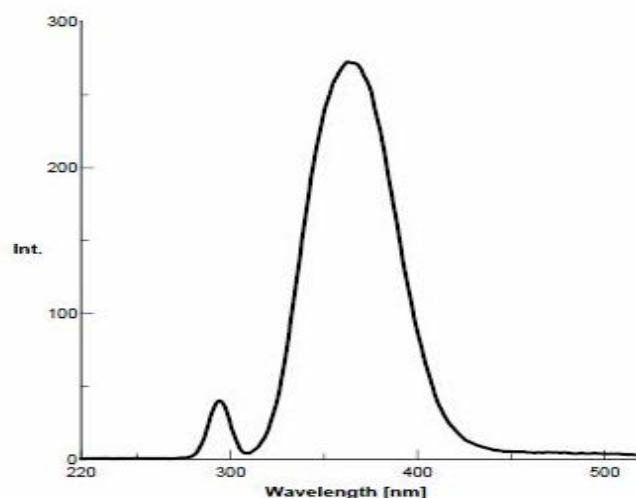
The contents of 5 ampoules (each containing 4 mg/2ml) were quantitatively transferred into a 100 ml volumetric flasks and the volume was made up to the mark with water to get the concentration 200 µg/ml. This resulting solution was used for analysis by the recommended procedure.

### Results and discussion

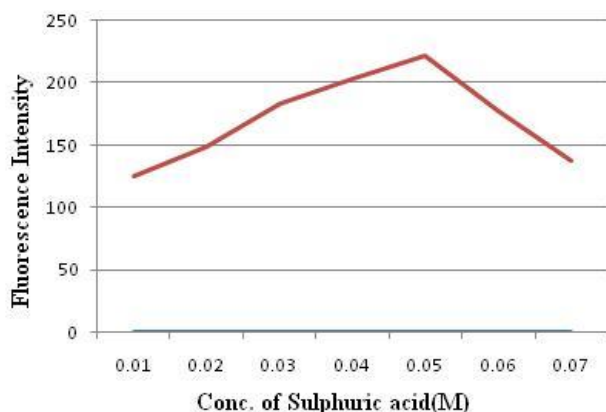
Cerium(IV) is a powerful oxidizing agent and is non-fluorescent under acidic conditions, while its reduced form exhibits native fluorescence<sup>5</sup>. The latest property has been used for the indirect determination of several drugs. The oxidation of

thiocolchicoside with Ce (IV) is the basis of the present analytical procedure developed for determination of thiocolchicoside. The increase in fluorescence intensity due to Ce(III) ions formed after the addition of the drug to an acid Ce(IV) solution was measured at 289nm as excitation wavelength and 366nm as emission wavelength (Fig. 1).

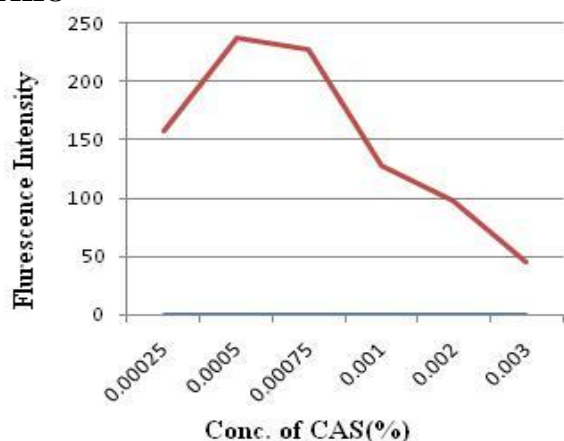
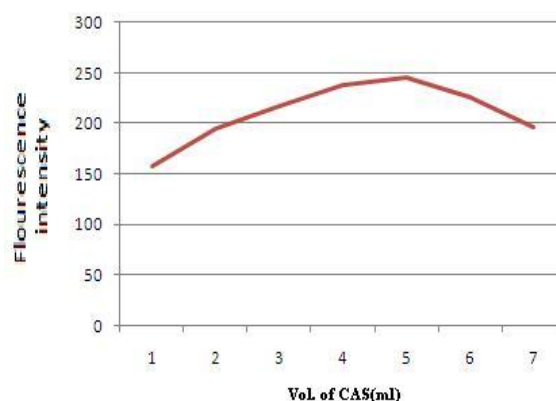
**Fig 1: Excitation and Emission Wavelength of THIO in Presence of Ce (III)**



A series of investigations were carried out to establish the optimum experimental conditions for the oxidation. The optimized parameters included the Ce(IV) volume and concentration, type of acid, strength of sulphuric acid and reaction time. Instrumental variables like excitation and emission band width as well as response time were also optimized. The nature and concentration of the acid used in the reaction have a very significant influence on the THIO emission intensity. Therefore, several kinds of acids, such as HCl, H<sub>2</sub>SO<sub>4</sub> and HNO<sub>3</sub> were added to the Ce(IV) solutions to test the effect of acid on the THIO intensity. The highest emission was observed from sulphuric acid treated Ce(IV) solutions, and the signal was stable. Hence, sulphuric acid was chosen for further study. The concentration of sulphuric acid in Ce(IV) solutions was subsequently optimized. These responses were plotted against different strength of the sulphuric acid (Fig.2). The fluorescence intensities of Ce(III) obtained by thiocolchicoside increased till 0.05 M and remained approximately constant at higher concentrations. Hence, the concentration of acid was fixed as 0.05M for thiocolchicoside in order to carry out the following work.

**Fig 2: Effect of Concentration of Sulphuric Acid on FI of THIO**

The effect of Ce(IV) concentration on the fluorescence intensity was assessed in the range  $2.5 \times 10^{-4}$  -  $3 \times 10^{-3}$  M. In fig. 3, it was shown that Ce(IV) at concentrations of  $5 \times 10^{-4}$  M leads to the saturation signals in the case of thiocolchicoside. At concentrations lower than this range, the fluorescence intensity dropped due to insufficient Ce(IV) for oxidation. On the other hand, higher concentrations of Ce(IV) was reported to probably quench the fluorescence thus decreasing the detected intensity. Optimized volume of Ce(IV) was 5 ml. Fig.4 shows the effect of volume of Ce(IV) on intensity of fluorescence. The effect of dilution of Ce(III) (which is the fluorescence emitter produced in the reaction) might be one of the factors causing the quenching. Hence, various diluting solvents were tried, such as water, acetonitrile, methanol, and ethanol. It was found that maximum fluorescence intensity was obtained when water was used as solvent for dilution.

**Fig 3: Effect of Concentration of CAS on FI of THIO****Fig 4: Effect of Volume of CAS on FI of THIO**

Keeping the other parameters constant when the excitation/ emission band width alone was increased fluorescence was found to increase but on the other hand, the smoothness of the spectrum was lost. When the excitation and emission band width were kept as 5 and 10 nm, a smooth spectrum was obtained. After fixing the excitation and emission band width as 5 and 10 nm, the response time was found from 0.01 sec to 4 sec. The response time was fixed as 0.05sec which would be sufficient to give higher fluorescence intensity. A good linearity was found in the concentration range of 1-10 $\mu$ g/ml. Precision was confirmed by replicate analysis of two working standards of thiocolchicoside for repeatability (intraday precision) and intermediate precision (interday precision). Results of the assay are shown in table 1. It was found that RSD values for intra and interday precision were found to be below 2%. This showed the method was highly precise.

The accuracy was carried out at 50 % and 100 % level from the amount determined. % recovery ranged from 99.40–99.78 % for the method. The results are shown in table 2. The % RSD was found to be within the acceptable range. Stability studies indicated that the samples were stable when kept at room temperature for 2 hours and under refrigeration temperature for 24 hours. It was evident from the assay of formulation that the percentage content was in good agreement with the labeled claim. Results are shown in table 3.

**Table 1: Precision data of THIO by spectrofluorimetric method**

Actual conc.( $\mu\text{g/ml}$ )	Mean Concentration ( $\mu\text{g/ml}$ ) $\pm$ % RSD*	
	Intraday precision	Interday precision
4	4.006 $\pm$ 0.1352	4.0017 $\pm$ 0.1486
5	5.004 $\pm$ 0.0920	5.024 $\pm$ 0.0747
6	6.013 $\pm$ 0.0718	6.005 $\pm$ 0.2011

**Table 2: Recovery Data of THIO**

Conc. of drug taken ( $\mu\text{g/ml}$ )	Conc. of standard added ( $\mu\text{g/ml}$ )	%Mean conc. $\pm$ %RSD*	Mean $\pm$ %RSD*
4.005	2.00	5.99 $\pm$ 0.0751	99.42 $\pm$ 0.227
4.005	4.00	7.99 $\pm$ 0.0520	99.70 $\pm$ 0.044

\*n=6

**Table 3: Assay of formulation for THIO by spectrofluorimetric method**

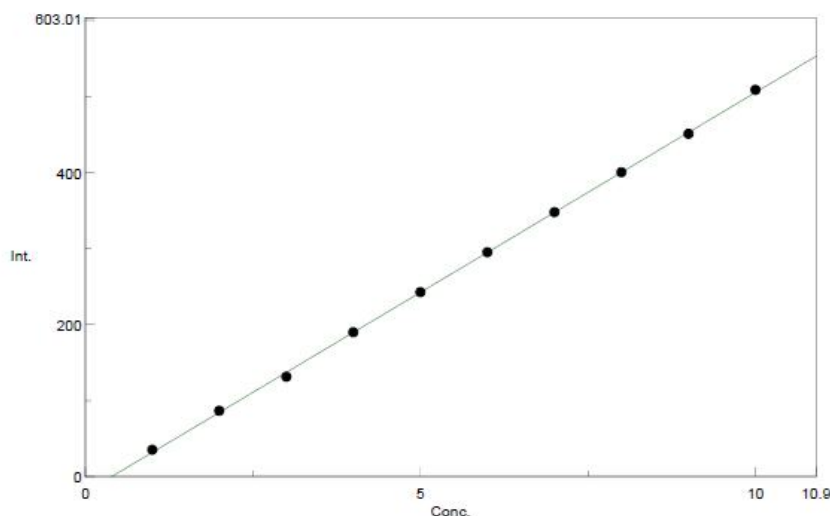
Labeled amount (mg/amp)	Estimated amount (mg/amp)	% Label claim $\pm$ RSD*
4	4.003 $\pm$ 0.1523	100.08 $\pm$ 0.2059

\*Average of 6 determinations

Luminescent spectroscopy is the most sensitive method for the quantitative estimation of trace level analyte. Ce(IV) serves as an oxidizing agent for the estimation of drugs by examining the fluorescence of the reduced Ce(III)<sup>6-8</sup>. Ce(III) usually produces more fluorescence than the oxidized product of the drugs and therefore, measurement of its fluorescence intensity can be utilized as a sensitive method for the quantitative estimation of certain drugs. In the present study, the method involved oxidation of THIO by Ce(IV) and subsequent monitoring of the fluorescence of Ce(III) at 366 nm after excitation at 289 nm. The spectrofluorometric properties of the reaction product as well as the different experimental parameters affecting the fluorophore development and its stability were carefully studied and optimized. Such factors were changed individually while the others were kept constant. These factors included the Ce(IV) concentration, type of acid, and its concentration, heating time, temperature, and diluting solvents.

The oxidation reaction of Ce(IV) had to be performed in acid medium to prevent precipitation

of Ce(OH)<sub>3</sub>, different acids such as sulfuric acid, hydrochloric acid, and nitric acid were tested to determine the most suitable acid for the reaction. Nitric acid is not preferred owing to the inhibitory effect of nitrate ions on the fluorescence of Ce(III). In the presence of hydrochloric acid and sulphuric acid the reaction rate and the fluorescence of Ce(III) were found to be high. However, hydrochloric acid gave high blank readings, so sulfuric acid was selected for the study. The effect of sulfuric acid concentration on the fluorescence intensity was studied using concentrations ranging from 0.01 to 0.07M of sulfuric acid. It was found that the relative fluorescence intensity increased by increasing sulfuric acid concentration up to 0.05M. So, this was used as the optimum concentration of sulfuric acid throughout the study. The study was carried out at room temperature and the effect of equilibration time on fluorescence intensity was also examined. The results showed that an equilibration time of 30 min was adequate to obtain the maximum fluorescence intensity.

**Fig 5: Calibration Graph of THIO****Table 4: Statistical comparison of THIO for precision studies (n=6)**

Parameters	4µg/ml		5µg/ml		6µg/ml	
Mean	4.006	4.0017	5.004	5.024	6.013	6.005
SD	0.1352	0.1486	0.092	0.0747	0.0718	0.2011
SEM	0.0552	0.06067	0.03756	0.03050	0.02931	0.08210
Lower 95% CI	3.864	3.846	4.907	4.946	5.938	5.794
Upper 95% CI	4.148	4.158	5.10	5.102	6.088	6.216
p	0.9592		0.6880		0.9287	
t	0.05243		0.4134		0.09177	

### **Conclusion**

This report describes a validated spectrofluorimetric method for the estimation of thiocolchicoside without interference of common excipients. This may be recommended as a method for thiocolchicoside testing either in bulk or the corresponding dosage form in routine quality control. The precision of the method was statistically evaluated. The p and t values were acceptable within the limit showed that the developed method was more precise (Table 4). In addition, the LOD and LOQ were found to be

0.1943 and 0.589 µg/ml. From the economic point of view, the proposed method is simple, selective, specific, rapid and inexpensive, and thus seems a good alternative to previously reported methods.

### **Acknowledgements**

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