DEVELOPMENT OF A SENSITIVE CLOUD POINT EXTRACTION METHOD FOR THE SPECTROPHOTOMETRIC ASSAY OF CITICOLINE BASED ON MOLYBDATE COMPLEXATION

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Received 25.02.2026
Accepted 15.04.2026

Abstract: *A selective and sensitive method is proposed for the spectrophotometric estimation of Citicoline (CT) in pure pharmaceutical preparations using a cloud point extraction (CPE) procedure. The method involved acidic hydrolysis of CT at 90°C to evolve phosphate ion, which forms a colored complex with molybdate ion after reduction by ascorbic acid, and then the complex was preconcentrated using Triton X-114. The deep blue color formed, separated, and extracted by CPE, and the absorbance was measured at 752 nm. The absorbance value is directly proportional to the amount of CT. The linear calibration graph observed is compatible with Beer's law over the concentration range of 0.5-12 µg/mL with a determination coefficient (R^2 = 0.9989). The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.104 and 0.345 µg/mL, respectively. Molar absorptivity and Sandell's index were calculated and they were 6.0680×104 L/mol.cm and 0.0084 µg/cm2, respectively. The range of relative error percentage of this method is estimated and found to be -0.05% to 0.11%. Precision represented by RSD% was calculated and found in the range 0.101% to 0.050%. The suggested method was successfully applied to estimate CT in its pharmaceuticals.*
Keywords: *Citicoline, Spectrophotometry, Molybdate Complex, Cloud Point Extraction, Ascorbic Acid.*

Introduction

Citicoline sodium (CT) is chemically identified as Cytidine 5'-(trihydrogen diphosphate)-2-(trimethyi ammonio) ethyl ester inner salt [1]. It has an IUPAC name: Sodium [[(2R,3S,4R,5R)-5-(4-amino-2-oxo pyrimidin-1-yl)-3,4- dihydroxy oxolon-2-yl]2-(trimethyl azaniumyl) ethyl phosphate and the molecular weight is 510.36 g/mol. The chemical structure of CT is shown in Fig. 1 [2].

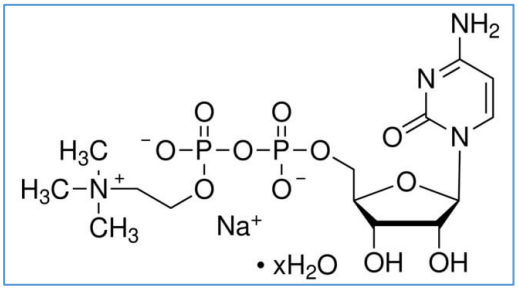

Fig. 1. The chemical structure of CT [2]

CT has the Molecular formula: $C_{14}H_{25}N_4NaO_{11}P_2$ [3] and it appears as a white crystalline substance that readily dissolves in water, yet it does not dissolve in ethanol, acetone, or chloroform [4].

CT is reported to enhance cerebral blood flow and oxygen utilization. It has been utilized in the treatment of cerebrovascular diseases, Parkinson's disease, and brain injuries [5]. Its primary application is in the pharmacological management of neurological conditions such as stroke [6].

CT can be quantified through various analytical methodologies, with UV-visible spectrophotometry being a prominent choice due to its efficiency and accessibility. Several researchers have optimized this approach; for instance, Chaudhary et al. developed a rigorous and cost-effective method for estimating CT in its bulk form. Their findings indicated that CT in an aqueous medium reaches its maximum absorbance at 270 nm, demonstrating a robust linear

relationship within a concentration range of 10-60 µg/mL [7]. Complementing this, Panda et al. introduced a specialized technique for the determination of CT in pharmaceutical tablet formulations. This method is founded on the principle that CT exhibits pH-dependent spectral behavior, manifesting distinct absorption profiles in acidic and basic environments. To exploit this characteristic, the researchers utilized 0.1M HCl and 0.1M NaOH as solvents, allowing for a more nuanced analysis of the drug's stability and concentration across different chemical media [8]. In another study*, Sachan et al.* reported a simple, rapid, precise, and low-cost spectrophotometric method based on absorbance measurement at 272 nm exhibiting satisfactory linearity within the concentration range of 5-50 µg/mL [9]. Among the published spectrophotometric methods in [10], CT was quantitatively estimated through an oxidation reaction with ferric chloride in the presence of chromogenic reagents, namely 1,10-phenanthroline, 2,2'-bipyridyle, and 3-methyl-2-benzothiazolinone hydrazine hydrochloride. The maximum absorption wavelengths were measured at 514 nm, 522 nm, and 625 nm, respectively. The study established that the methods comply with Beer's Law within the concentration ranges of 2-10 µg/mL and 10-60 µg/mL.

CT can be determined by a spectrofluorometric method based on developing a binary complex between CT and Eosin Y in an acidic medium at pH 3.6 (using acetate buffer), followed by measuring the decrease in fluorescence intensity at a wavelength of emission of 540 nm, followed by excitation at 518 nm [11].

A TLC-densitometry technique is applied for the determination of CT beside its impurities. Silica gel plates are used for separation. The spots were detected under UV light at 254 nm [12].

HPLC techniques have been utilized for the quantification of CT [13-15] and RP-HPLC [16-18]. Two liquid-contact ion-selective electrodes were fabricated to facilitate the potentiometric quantification of CT in pharmaceutical preparations [19]. An alternative electrochemical approach was proposed involving the fabrication of three solid-contact potentiometric sensors. These electrodes were successfully applied for the determination of CT in raw materials, dosage forms, and biological fluids (spiked human plasma) [20].

Cloud Point Extraction (CPE) has emerged as a prominent green analytical tool, distinguished by its environmental safety and high operational efficiency. By utilizing minute quantities of non-ionic surfactants in lieu of hazardous organic solvents, CPE aligns with the core principles of green chemistry [21]. This methodology significantly advances innovative analytical frameworks, particularly when integrated with spectrophotometry for the routine quantification of metal ions and organic pollutants [22]. The process is predicated on the Cloud Point Temperature (CPT) at which non-ionic micelles aggregate and separate from the aqueous bulk. This allows for the effective preconcentration of target analytes into a highly condensed, surfactant-rich phase [23]. Furthermore, the use of inexpensive, dilute surfactant solutions minimizes chemical consumption and laboratory waste, offering a sustainable alternative to traditional liquid-liquid extraction [24].

In this study, a sensitive spectrophotometric method was developed for the determination of CT based on its phosphate content. While the phosphomolybdate complexation is a classic approach traditionally reserved for monitoring inorganic phosphates in environmental and agricultural matrices, its diagnostic potential in pharmaceutical analysis remains significantly underutilized. Following the acid hydrolysis of CT to liberate the inorganic phosphate group, a phosphomolybdate complex was formed. This complex was subsequently reduced to produce "molybdenum blue," which exhibits maximum absorption at 752 nm. To enhance the analytical sensitivity and detection limits, the Cloud Point Extraction (CPE) technique was employed. The proposed method was successfully validated and applied for the quantitative estimation of Citicoline in various pharmaceutical formulations with high precision and accuracy.

Experimental part

Equipment. For recording the absorption spectra and measuring absorbance values, a digital double-beam UV-Vis spectrophotometer device (SHIMADZU, UV-1900i, Japan) was used with 1 cm path length silica cells. For measuring pH, a professional bench top pH meter (BP 3001

professional bench top) was employed. In addition, an MSE centrifuge was used to perform the separation.

Drug Sample. The pure CT drug was purchased from Cayman Chemical Co., USA

Materials and Reagents. All chemicals and reagents utilized in this study were of analytical grade and high purity.

Standard Citicoline sodium (CT) solution, 50 µg/mL was prepared by accurately weighing 0.0050 g of the pure drug. The substance was transferred into a 100 mL beaker and dissolved in 50 mL of distilled water. The resulting solution was then quantitatively transferred to a 100 mL volumetric flask, and the volume was adjusted to the mark with distilled water to obtain a final concentration of 50 µg/mL.

Sulfuric acid solution (1M) was obtained by diluting 5.43 mL of concentrated acid (18.4M) to a final volume of 100 mL. Briefly, approximately 50 mL of distilled water was placed in a 100 mL volumetric flask. The calculated volume of the concentrated acid was then added slowly and cautiously down the inner side of the flask to dissipate the heat generated by the exothermic reaction. After the solution had cooled to ambient temperature, the volume was adjusted to the mark with distilled water. The flask was inverted several times to ensure complete homogeneity.

An ammonium molybdate solution (5.54×10^{-3} M) [25] was prepared by dissolving 0.6450 g of ammonium molybdate $(NH_4)_6Mo_7O_{24}$ (M.wt. =1163.65 g/mol) in approximately 40 mL of distilled water, then the solution was transferred to a 100 mL standard flask, and the volume was completed to the mark with the same solvent.

An ascorbic acid solution (2%) was prepared by dissolving 2 g of ascorbic acid (M. wt.=176.12 g/mol) in 20 mL of distilled water, then the solution was transferred to a 100 mL standard flask and the volume was completed to the mark using distilled water.

Triton X-114 (5%) solution. This surfactant solution was prepared by mixing 5 mL of Triton X-114 in about 20 mL of cold distilled water with good stirring. The solution was then transferred to a 100 mL standard flask, and the volume was completed to the mark by the same solvent.

The proposed method includes three steps:

1) Acidic hydrolysis of CT using sulfuric acid solution (1M) with heating in water bath to 90°C for 15 minutes to evolve inorganic phosphate ions [26].
2) Formation of the heteropoly complex between phosphate ion and ammonium molybdate in an acidic environment to form a yellow heteropoly ammonium phosphomolybdate complex, which converts to a molybdenum blue complex after adding ascorbic acid as a reducing agent to convert the phosphomolybdate complex into molybdenum blue, allowing for accurate spectrophotometric quantification.

    a. $PO_4^{-3} + 12(NH_4)_2MoO_4 + 24H^+ \rightarrow (NH_4)_3PO_4\cdot12MoO_3 + 21NH^{4+} + 12H_2O$

    b. $(NH_4)_3PO_4\cdot12MoO_3 + C_6H_8O_6 \rightarrow$ Molybdenum blue [27]

3) CPE step: By adding a suitable amount of 5% Triton X-114 before diluting, after heating, centrifuging, and decantation, the complex was extracted by ethanol, and then the absorbance was measured at 752 nm, which is directly proportional to the amount of CT in the sample.

Results and Discussion

Optimization of Drug Degradation Conditions. To select the most suitable acid for hydrolysis, various acids (including HCl, HNO_3 and CH_3COOH) were evaluated. All exhibited instability or poor color development, except sulfuric acid, which provided acceptable color stability.

The maximum absorbance with acceptable stability was achieved by using 1 mL of 1M sulfuric acid, as illustrated in Fig. 2. The degradation process is complete upon heating at 90°C for 10 minutes, as shown in Fig. 3 (A and B).

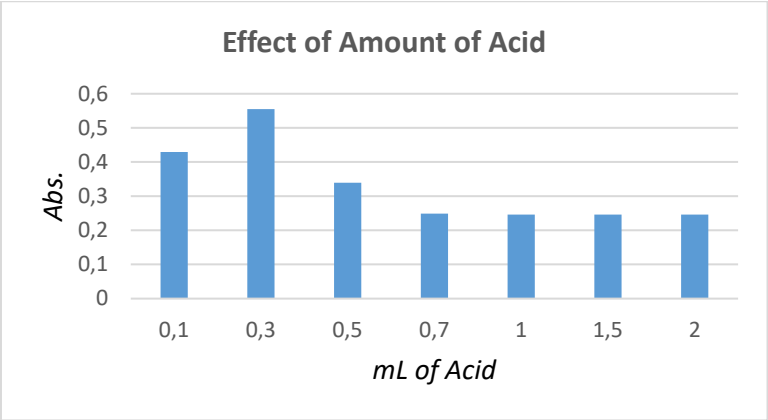

Fig. 2. Effect of sulphuric acid amount

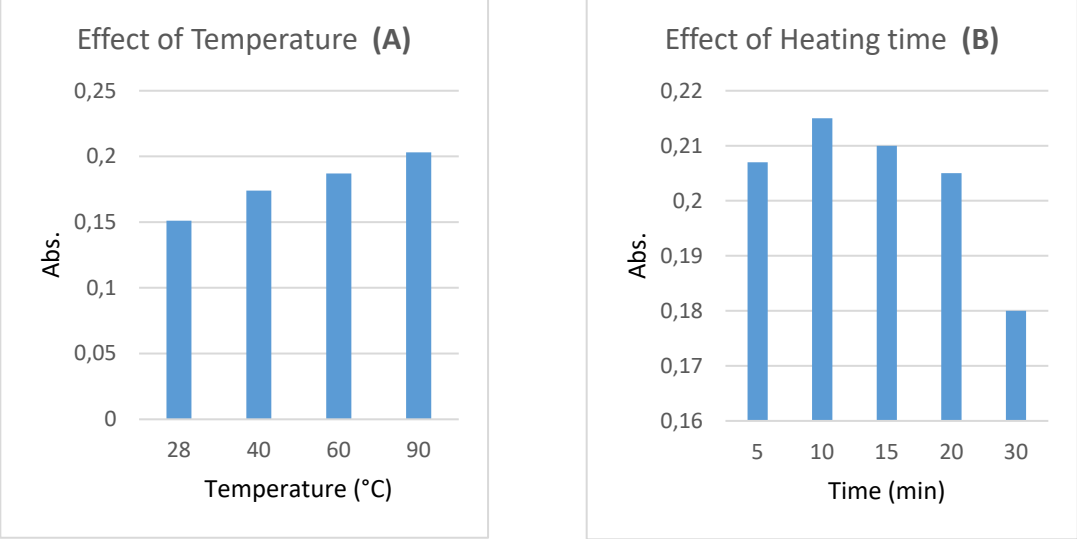

Fig. 3. (A) Effect of temperature and (B) Effect of heating time

Selection of a suitable volume of ammonium molybdate. A study of different volumes of ammonium molybdate solution (5.54×10^{-3}M) with different concentrations of CT shown in Table 1 observed that 1.5 mL of ammonium molybdate gave satisfied absorbance and R^2 values and was used in the next experiments.

Table 1. Optimum amount of ammonium molybdate

mL of 5.54×10^{-3}M Mo^{+7}	Absorbance/µg of CT in mL				
	0.5	1	1.5	2	R^2
0.5	0.088	0.166	0.240	0.360	0.9859
1	0.066	0.165	0.273	0.403	0.9960
1.5	0.157	0.287	0.463	0.585	0.9952
2	0.108	0.227	0.354	0.532	0.9903
2.5	0.086	0.163	0.279	0.422	0.9829

Selection of the suitable reducing agent. A number of reductants were examined for reducing molybdate ions by adding 1 mL of each reductant at a concentration of 0.114 M. Found that ascorbic acid gives the better result and used it in the next experiments as shown in Fig. 4.

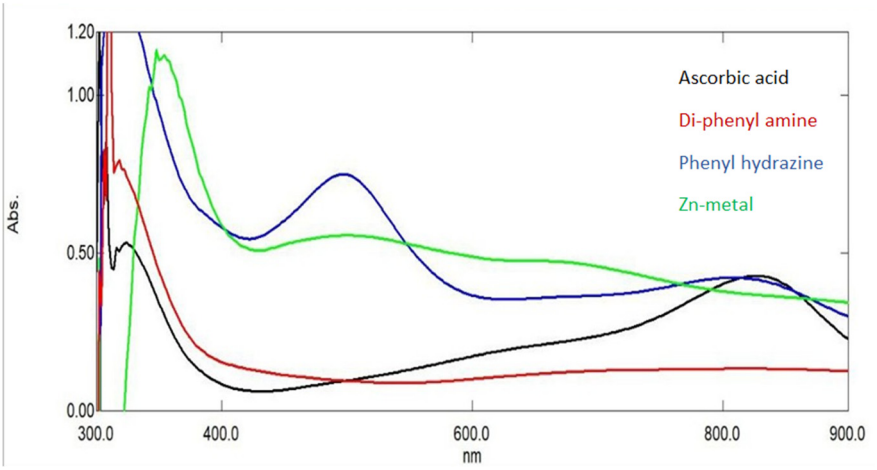

Fig. 4. Selection of the reductant

Fig. 5 illustrates that 2 mL of 0.114 M ascorbic acid gave better reduction of the molybdate ion, and it was used in the next experiments.

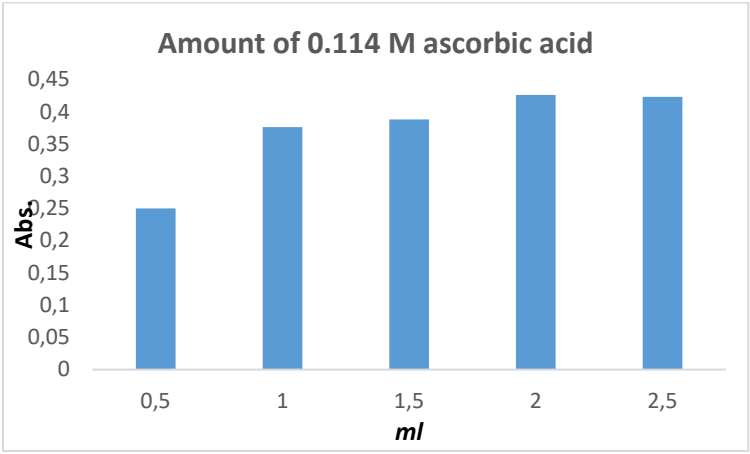

Fig. 5. Selection the amount of 0.114 M Ascorbic acid that gives the best reduction

Selection of the amount of Triton X-114 (5%). For the above procedure, a different amount of Triton X-114 (5%) (as shown in Table 2) was added, then the volume was completed to 10 mL with distilled water, and the extraction method was completed.

Table 2. Selection of the amount of Triton X-114

mL of Triton X-114	Abs.
0.5	0.512
1	0.798
2	0.670

From the above Table 2, it was found that using 2 mL of Triton X-114 gives the best absorption.

Effect of Temperature and Time. After the above additions and dilution by distilled water in a 10 mL standard flask, varied temperature (25-70°C) for 15 minutes to form a cloud point and separated by centrifugation at 5000 rpm for 10 minutes, 2 mL of ethanol was added and measured by UV-Vis at λmax=752 nm. Fig. 6A shows that 50°C is the best temperature for 10 minutes of heating (Fig. 6B).

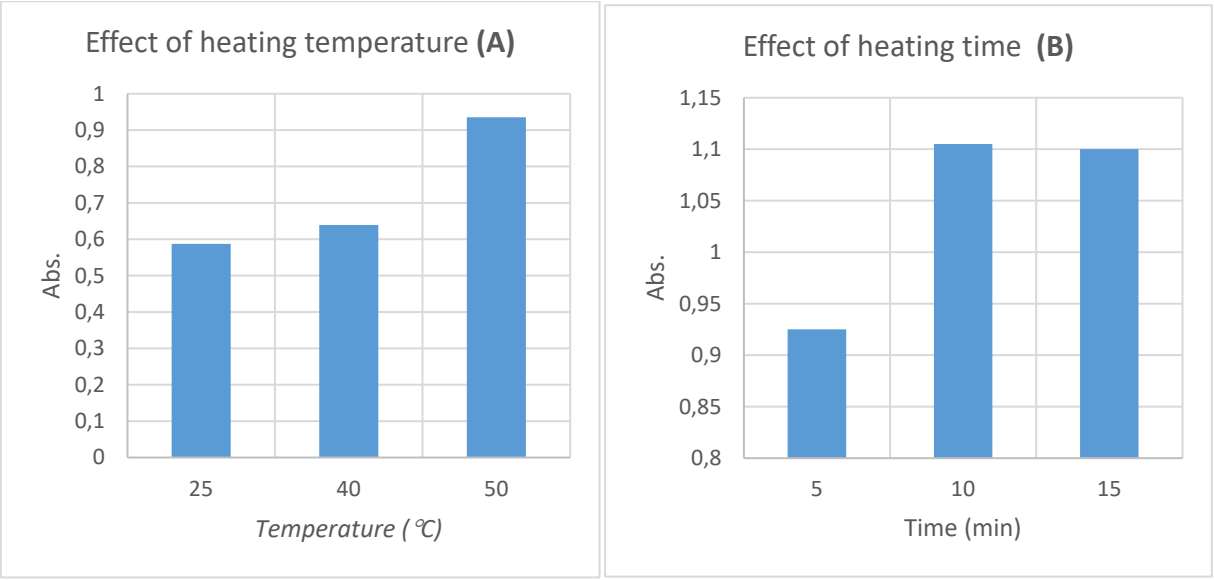

Fig. 6. Selection the best temperature **(A)** and the best time of heating **(B)**

Absorption spectra and calibration curve. An aliquot containing an increasing concentration from 0.5 to 15 µg/mL of standard CT solution was transferred quantitatively to a series of 10 mL standard flasks. To each one, 1 mL of 1 M HCl was added, heating at 90 ºC for 10 minutes for hydrolysis, followed by 1.5 mL of 5.54×10^{-3}M ammonium molybdate and 2 mL of 2% ascorbic acid, then 1 mL of 5% Triton X-114, and the solution was completed to 10 mL with distilled water. Then the solution was heated in a water bath at 50 ºC for 10 minutes, centrifuged at 5000 rpm for 10 minutes, and cooled in ice for degradation; the dye was extracted by adding 2 mL of ethanol. After that, the absorbance was measured at 752 nm versus its corresponding blank. As illustrated in Fig. 7, the product demonstrates a maximum absorbance at 752 nm relative to the corresponding blank.

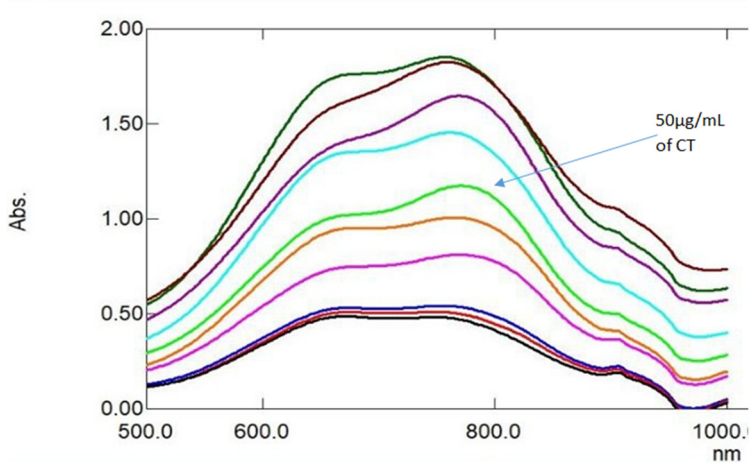

Fig. 7. Absorption spectrum of different concentrations of CT

Under the specified optimum conditions, a linear calibration curve was obtained by plotting the absorbance values against the concentration of CT. The resulting product obeys Beer's law over the concentration range of 0.5-12 µg/mL of CT with an excellent determination coefficient (R^2) of 0.9989, as shown in Fig. 8. The Sandell's sensitivity index and molar absorptivity were calculated and found to be 0.0084 µg/cm^2 and 0.6068×10^5 L·mol^{-1}·cm^{-1}, respectively.

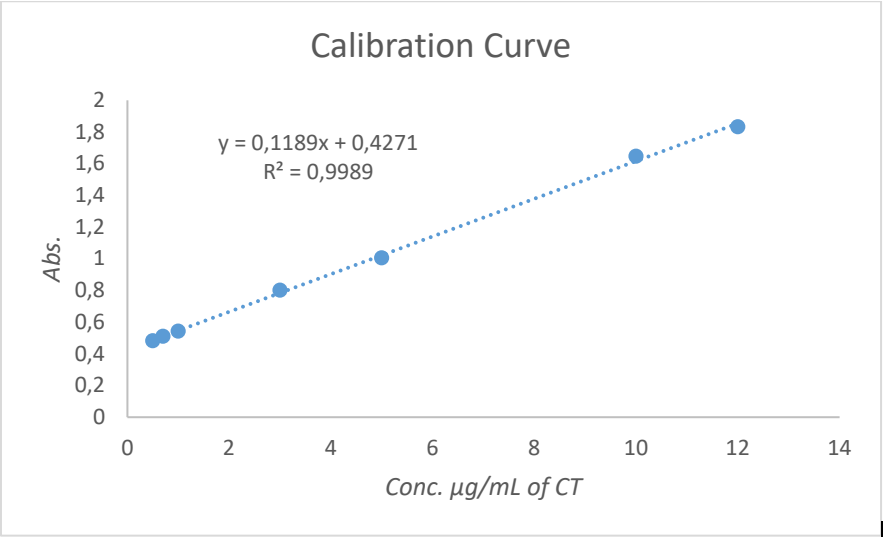

Fig. 8. Calibration curve of CT using cloud point extraction method

Accuracy and Precision of the suggested method. A study of the accuracy and precision of the suggested method was applied by testing two different concentrations (1 and 5 µg/mL) of CT with five replicates.

The results in Table 3 below indicate that the method has excellent accuracy and precision.

Table 3. Accuracy and precision of the suggested method

Amount of CT (µg/mL)	Recovery %*	Relative error %*	Relative standard deviation %*
1	100.11	0.11	4.62
5	99.95	-0.05	4.20

**Average of five replicates*

Optimal Conditions. The parameters in Table 4 show the optimum conditions resulting from previous experiments.

Table 4. The optimum conditions of the previous experiments

Parameters	Proposed method
λ_{max} (nm)	752
Beer's law range (µg/mL)	0.5-12
Sandell's sensitivity (µg/cm^2)	0.0084
Determination coefficient	0.9989
Molar absorptivity (L/mol.cm)	6.0680×10^4
LOD (µg/mL)	0.1040
LOQ (µg/mL)	0.3450

Application. The suggested method was estimated for the determination of CT in pharmaceutical formulations that are commercially available in two concentrations, 1 and 5 µg/mL of CT. Table 5 shows acceptable data that indicates that the proposed method can be applied successfully for the estimation of CT.

Table 5. Analysis of CT in pharmaceutical formulations

	CT conc.	Recovery*%			Drug content (mg)

Sample	Present	Found		R.E %	RSD% N=5	
Citivitae, Galenicum 1000 mg (Spain)	10	10.01	100.10	0.10	0.11	1001.00
	50	49.95	99.92	-0.1	0.085	999.00
Somazina, ferrer 100 mg (Spain)	10	10.012	100.12	0.12	0.11	100.12
	50	50.035	100.07	0.07	0.058	100.07
Meticolin, CPC1HN 500 mg (Vietnam)	10	9.975	99.75	-0.25	0.107	498.80
	50	49.94	99.88	-0.124	0.05	499.40

Average of five replicates

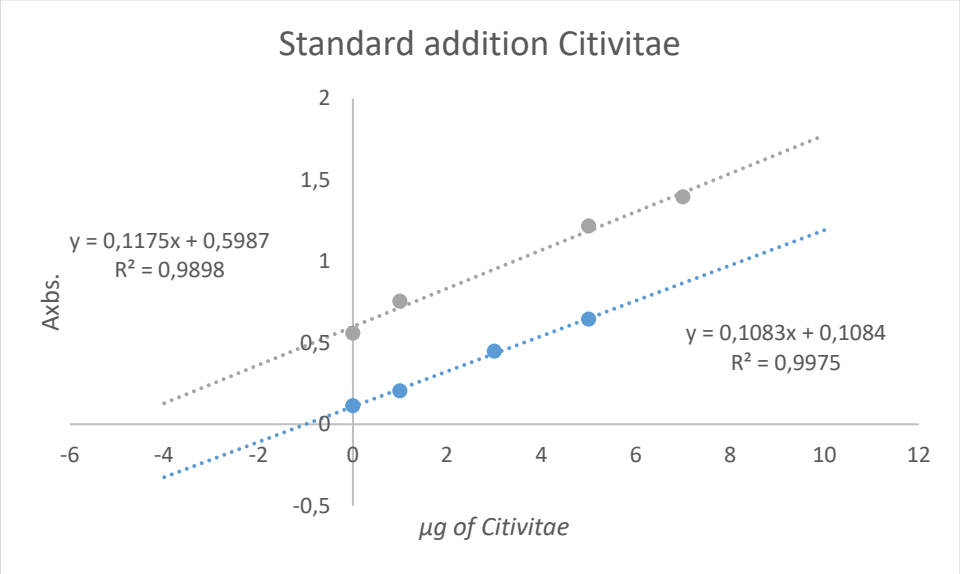

Fig. 9. Standard addition method for estimating Citivitae

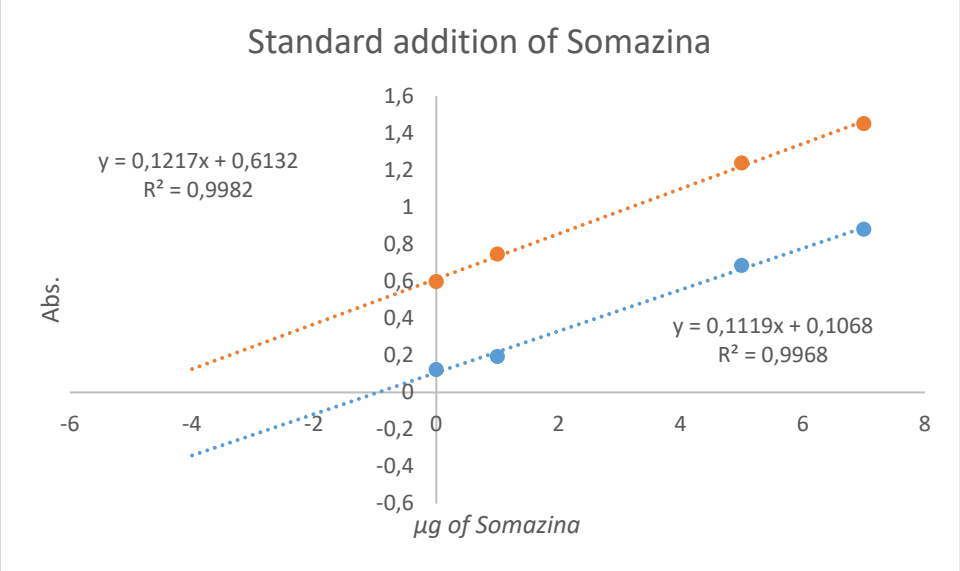

Fig. 10. Standard addition method for estimating Somazina

Validity of the suggested method. The standard addition method was applied to confirm the validity and selectivity of the proposed method. The results demonstrate that the recommended procedure can be successfully applied to the quantification of CT at two distinct concentrations (1 and 5 µg/mL) without interference. The details of the data found in Fig. s 9, 10, and 11 are tabulated in Table 6. From this table, it is confirmed that the results of the standard addition method exhibit close correspondence with the results obtained from the present method, within the permissible limits of experimental error.

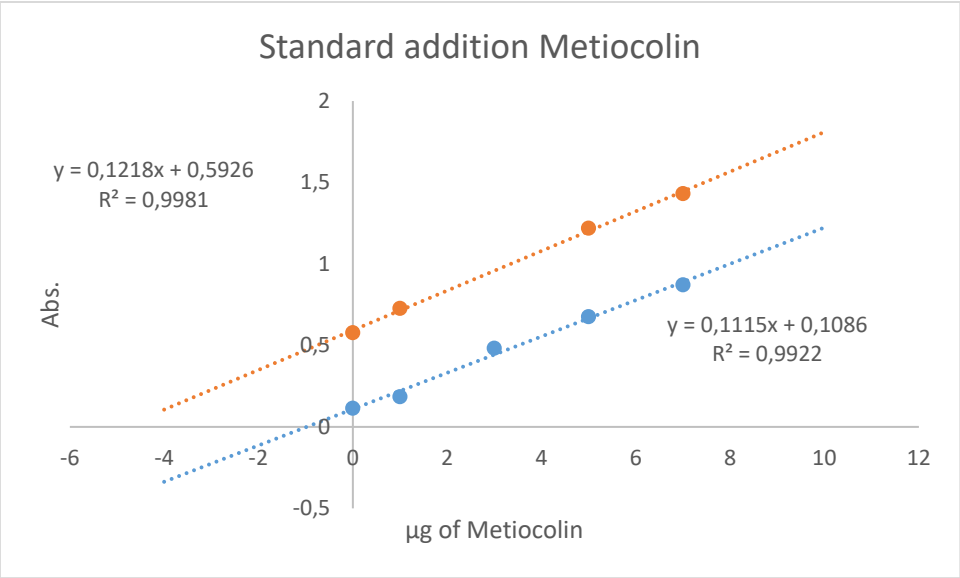

Fig. 11. Standard addition method for estimating Metiocolin

Table 6. Analysis of CT in commercial pharmaceutical formulations by standard addition method

Sample	CT (µg/mL)		Recovery *(%)	R.E (%)	Drug content (mg)
	Present	Found			
Citivitae, Galenicum 1000mg (Spain)	1.0	1.0009	100.37	0.37	1004.0
	5.0	5.0953	101.48	1.48	1014.8
Somazina, ferrer 100 mg (Spain)	1.0	0.9544	100.53	0.53	100.5
	5.0	5.0386	95.36	-4.64	95.36
Metiocolin, CPC 1HN 500mg (Vietnam)	1.0	0.973	96.96	-3.04	485.0
	5.0	4.865	97.15	-2.85	485.8

Comparison of Methods. The performance of the proposed method for the determination of CT was evaluated and compared with a previously reported UV spectrophotometric method [28]. Several analytical parameters obtained for different charge-transfer interactions were assessed, and the results are summarized in Table 7.

Table 7. Comparison of the analytical parameters of the proposed method and the reported UV spectrophotometric method for the determination of CT.

Parameters	Present method	UV method
λ_{max} nm	752	280
Beer's law range µg/mL	0.5-12	10-80
R^2	0.9989	0.9999
Rec.%	99.95-100.11	98.41
RSD%	0.050-0.101	<2

Conclusion

A sensitive, simple, and cost-effective spectrophotometric method coupled with cloud point extraction (CPE) was successfully developed for the determination of CT. The method is based on the formation of a stable phosphomolybdate complex, followed by its preconcentration into a surfactant-rich phase. By adapting the phosphomolybdate complexation approach, which has been widely employed in environmental analysis, to pharmaceutical quality control, this study provides an accessible and efficient alternative for routine drug analysis.

The optimized method exhibited excellent sensitivity, a wide linear dynamic range, and good analytical performance. In addition, it complies with the principles of green analytical chemistry by minimizing the consumption of hazardous organic solvents, thereby reducing environmental impact. The proposed procedure was successfully applied to the analysis of pharmaceutical formulations, yielding high recovery values and demonstrating its accuracy, precision, and suitability for routine quality control applications. Future studies may explore the extension of the molybdate-complexation CPE approach to the determination of other pharmaceutical compounds and its application in more complex biological matrices.

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