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New validated spectrofluorimetric protocol for colistin assay through condensation with 2,2-dihydroxyindan-1,3-dione: application to content uniformity testing

Tamer Z. Attia, [iD] *[a] Mahmoud A. Abdelmajed,[b] Mahmoud A. Omar,[ac] Sultan S. Al Thagfan[d] and Khalid M. Badr El-Din[a]

A new, cost-effective and sensitive spectroscopic assay for the quantification of Colistin Sulfate (CS) and its prodrug colistimethate sodium (CMS) has been developed and validated. The validated technique depends on the condensation of the studied drug with 2,2-dihydroxyindan-1,3-dione (ninhydrin) and phenylacetaldehyde using Teorell and Stenhagen buffer (pH = 6) to yield a fluorescent product that is estimated at emission wavelength (λ_{em} = 474 nm) after excitation wavelength (λ_{ex} = 390 nm). The reaction's affecting factors were carefully studied and adjusted accurately. Over the following range (0.4–2.4 µg mL^{-1}), the produced calibration plot looked rectilinear, and the estimated limits of detection and quantification (LOD and LOQ) were 0.051 & 0.154 µg mL^{-1} respectively. The recommended approach was utilized to evaluate market products containing the investigated drug. Moreover, content uniformity testing was employed as a new procedure not found in the previously reported fluorimetric technique.

1 Introduction

With the advent of multidrug-resistant (MDR) Gram-negative bacteria and the absence of novel antimicrobial drugs, scientists believe in the necessity of polymyxins. Infections caused by MDR Gram-negative bacteria, particularly *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, have lately increased dramatically, and polymyxins are typically the only active antibiotic available for these bacteria.[1–3] The two types of colistin, Colistin Sulfate (CS) for oral use and its prodrug colistimethate sodium (CMS) for parenteral use, have been the drugs of choice for treating such infections. CS is mainly composed of an amide linkage that binds a cationic cyclic decapeptide to a fatty acid chain (Fig. 1).

Numerous analytical approaches based on microbiological,[4–6] capillary electrophoretic methods,[7] and spectroscopic (spectrophotometric[8] and fluorimetric methods[9]) assays were reported. Moreover, published chromatographic techniques such as liquid chromatography (LC)[10–16] and high-performance liquid chromatography (HPLC)[17–21] were carefully collected for analysis of CS.

The spectrofluorimetry technique had widely applicable nowadays owing to its sensitivity, simplicity, and not requiring a sophisticated instrument or sample pretreatment. On the other hand, separative methods suffer from huge consumption of solvents, high-cost devices, and exhausted extraction processes. Besides, microbiological and spectrophotometric assays suffer from lacking sensitivity. Despite the instrument's simplicity, sensitivity, and availability, there is only one documented fluorimetric technique for the medication under study.

Microbiological and spectrophotometric methods of assay suffer from lacking sensitivity. While, separative methods suffer from huge consumption of solvents, high-cost devices, and exhausted extraction processes. Nowadays, the spectrofluorimetry technique has been widely applicable for determination of several pharmaceutical drugs owing to its sensitivity, simplicity, and not requiring a sophisticated instrument or sample pretreatment. Despite of that, there is only one documented fluorimetric method for determination of the medication under study.[9] This spectrofluorimetric method suffered from some drawbacks such as proceeding in extreme conditions as it requires heating in boiling water (100 °C) for 35 minutes. Furthermore, tests for content homogeneity and quantification of the tested medication in tablet dosage form were not performed.

[a]Analytical Chemistry Department, Faculty of Pharmacy, Minia University, Minia, Egypt. E-mail: Tamer_zekry_a@yahoo.com

[b]Analytical Chemistry Department, Faculty of Pharmacy, Deraya University, New Minia, Egypt

[c]Department of Pharmacognosy and Pharmaceutical Chemistry, College of Pharmacy, Taibah University, Medinah, Saudi Arabia

[d]Department of Clinical and Hospital Pharmacy, College of Pharmacy, Taibah University, Medinah, Saudi Arabia
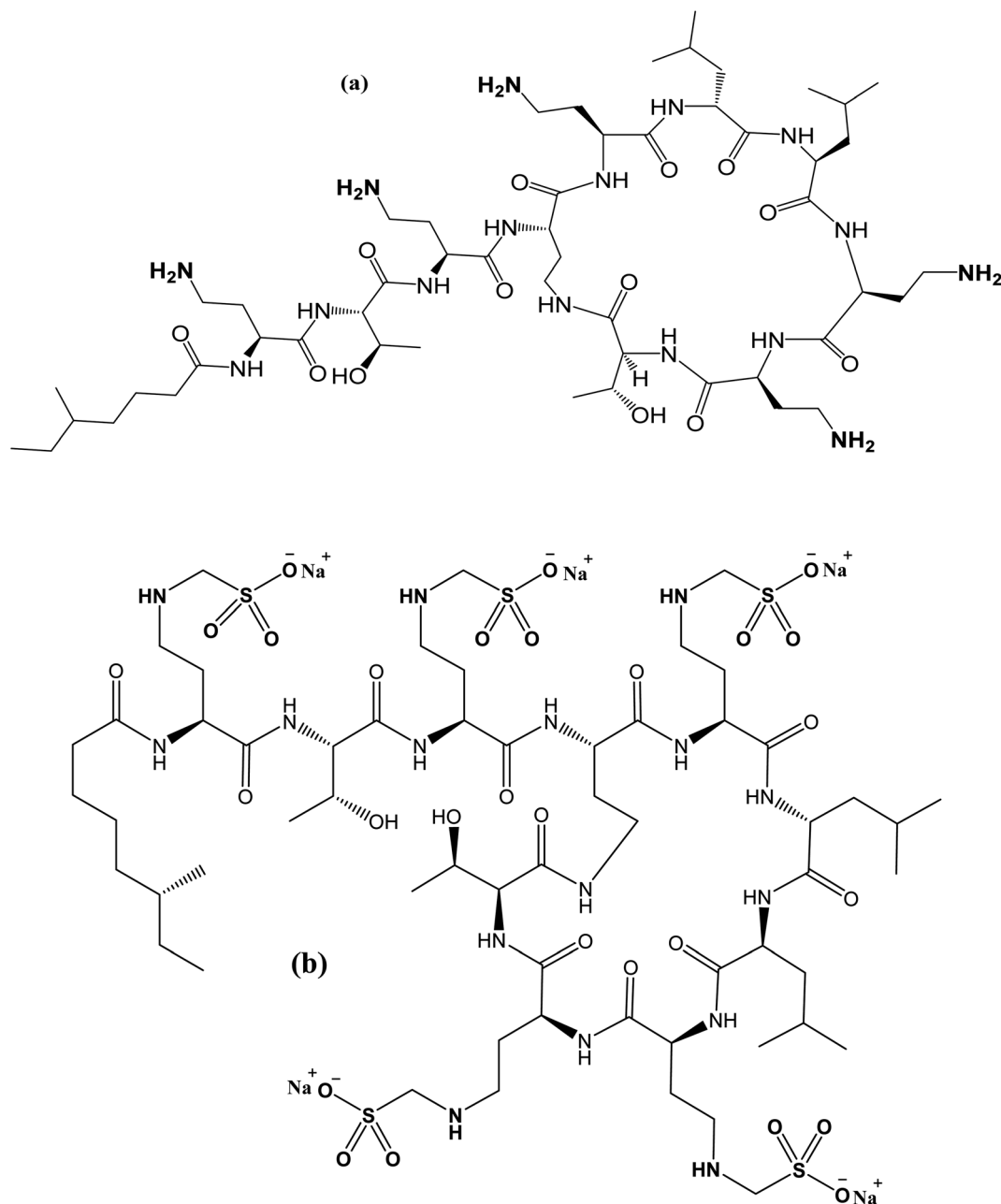
Fig. 1 (a) Chemical structure of colistin (CS) and (b) colistimethate sodium (CMS).

As a consequence, the proposed work aimed to establish a simple, cheap, time-effective, and environmentally friendly spectrofluorimetric approach for the quantification of the investigated drug in its tablet and parenteral dosage forms. Moreover, the application of content uniformity testing for CS tablets was successfully employed.

2 Experimental

2.1 Devices

To obtain the fluorimetric measurements, a 150 watt xenon lamp combined in PerkinElmer LS 45 luminescence spectrometer (United Kingdom) with a 1 cm quartz cell was employed.

Both the emission and excitation monochromators were set to a slit width of 10 nm. The data were collected utilizing the software of FL WINLAB™ on a PC that connected to the fluorimeter. Among the equipment employed were a thermostatically controlled MLW-type water bath (Memmert Gmbh, Schwabach, Germany), digital analytical balance (Mettler Toledo, Glattbrugg, Switzerland), and ADWA 11 pH meter (Romania).

2.2 Pharmaceutical forms and authentic

Standard CS was kindly gifted by the National Organization of Drug Control and Research (NODCAR) and utilized without

further purification. The market product of CMS including Colistin Sulfate® tablets (Sigma Pharmaceutical Co. Cairo, Egypt) and Colomycin® lyophilized powder for injection (Forest Laboratories, United Kingdom) were purchased.

2.3 Chemicals and reagents

2,2-Dihydroxyindan-1,3-dione (ninhydrin; Alpha Chemika, Mumbai, India) was daily prepared as 0.1% w/v in distilled water. Phenylacetaldehyde (Sigma-Aldrich) was weekly prepared as 0.02% v/v in ethanol and still stable when refrigerated at 4 °C. All solvents such as ethanol, methanol, acetone, acetonitrile, and dimethylformamide (DMF) along with HCL, phosphoric acid, citric acid, and NaOH were purchased from El Nasr Chemical Co. (Cairo, Egypt). Mixing suitable volumes of concentration 1 molar of phosphoric, citric acid, NaOH, and adjusting the pH utilizing 0.1 M HCL yield pH range (5 to 9) of Teorell & Stenhagen buffer which is used throughout the investigation.

2.4 Drug stock solution

Using distilled water, CS was set as a stock solution of 100 μg mL^{-1}. One more dilution was employed yielding working solutions. Its stability remained for nearly a double of weeks when it was kept in a refrigerator at 4 °C.

2.5 General methodology

Besides 1 mL of working solutions ranging from 2 to 24 μg mL^{-1}, 1 mL of Teorell & Stenhagen buffer, 1 mL of 0.1% (w/v) ninhydrin, and 1 mL of 0.02% (v/v) phenylacetaldehyde were transferred and well mixed into a set of test tubes. To achieve a complete reaction, all test tubes were heated in a water bath adjusted at 80 °C for 15 minutes after that cooled into a bath of ice. Test tube contents were accurately moved to a 10 mL volumetric flask and diluted to mark utilizing ethanol as diluents. Lastly, the relative fluorescence intensity (RFI) of the yielding solutions was estimated at λ_{em} 474 nm (after λ_{ex} = 390 nm). Simultaneously, a blank solution was performed using the same previously mentioned methodology omitting the investigated drug.

2.6 Quantification of CS in market products

2.6.1 CS in its tablet form. Weighing, grinding and uniformly mixing of twenty tablets Colistin Sulfate® were implemented. An aliquot amount equivalent to 10.0 mg of the drug was dissolved in 30 mL of distilled water followed by sonication for 15 minutes. In a 100 mL volumetric flask, filtration was employed and finally adjusted utilizing the experimental solvent to the mark. Accurate volumes of the filtrate were diluted to yield the working range of concentration and followed the general analytical procedure.

2.6.2 CS in its parenteral form. An exact quantity from the Colomycin® vial corresponding to 10.0 mg of the studied drug was transferred to a 100 mL calibrated flask and completed to the mark with distilled water. 10 mL of this solution was then treated with 5 mL of 0.2 M sulfuric acid and allowed to rest for

10 minutes to ensure full hydrolysis of CMS and conversion to CS.[21] The reaction was then stopped by adding 10 mL of 0.2 M sodium hydroxide, a pH meter was employed to confirm the neutralization. Finally, a working solution was obtained by further dilution to 100.0 mL with the same solvent and a previously general methodology was employed.

2.7 Procedure for content uniformity testing

Ten individually well-crushed Colistin Sulfate® tablets were employed in this assay. Then a specific amount equivalent to 10 mg of the authentic powder was taken from each crushed tablet and filtered with the same solvent to yield 10 μg mL^{-1} working solutions. Content uniformity testing was implemented following USP guidelines.[22] After individually assessing ten pills, the acceptability value (AV) was calculated.

3 Results and discussion

Reviewing the reported HPLC methods employed to quantify the referenced medication, several drawbacks were revealed including frequently requiring enormous quantities of high-analytical grade solvents such as tetrahydrofuran[17,19–21] and acetonitrile[18–21] which are highly expensive and have a significant harmful influence on the environment. Also, extremely expensive columns such as RP-LiChroCART® Purospher Star® C18 column,[18] Cadenza CD-C18 column,[19] or an Agilent Poroshell 120 EC-C18 column[20] were necessarily equipped in HPLC instrument. In addition, detectors like fluorescence detector, which is very expensive and unattainable in normal quality control laboratories, must be employed in the reported methods.[17–21] Moreover, extraction processes such as solid phase extraction using Oasis® HLB cartridges that consumed a large amount of solvent and expensive cartridges were necessary prior to determination.[18]

Therefore, the present study is aimed to develop an environmentally friendly strategy adaptable to routine and rapid screening without being costly, time-consuming, or requiring complicated instrumentation. Generally to assay drugs that have primary amine groups, 2,2-dihydroxyindan-1,3-dione is employed as a derivatizing agent in presence of phenylacetaldehyde.[23–26] The reagent reacts with the main amino moiety in the structure resulting in a highly yellow fluorescent derivative. Precision, repeatability, and lower
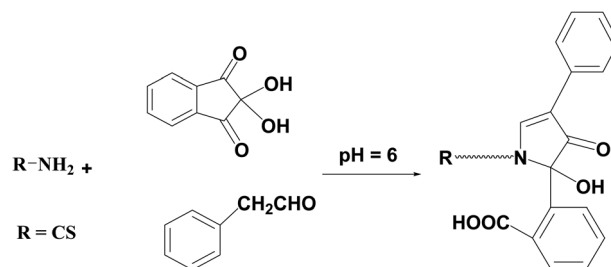

Fig. 2 Suggested pathway of the reaction between the investigated drug and the fluorogenic reagent.
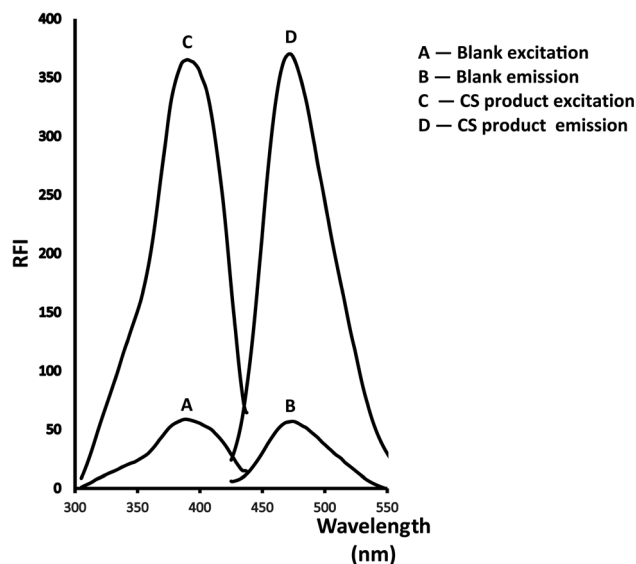
Fig. 3 Excitation and emission spectra of colistin (2 μg mL^{-1}) with fluorogenic reagents.

the fluorescent product was measured at $\lambda_{em} = 474$ nm (after excitation at 390 nm). The suggested reaction and also the spectra of the studied methodology were illustrated in (Fig. 2 and 3) respectively.

3.1 Optimization

Various methodology parameters were changed whilst freezing the others, to identify the most ideal reaction conditions necessary to yield maximum RFI values.

3.1.1 Buffer volume and pH adjustment. As any variation in pH had a significant impact on the methodology's resultant fluorescence, a buffer of Teorell & Steinhagen was implemented to accomplish a pH range of 5 to 9. The RFI was maxima in the pH range (5.8–6.2). Any variation out of this pH range significantly declined RFI levels. The greatest levels of RFI were detected between the quantities (0.8–1.2 mL) during testing the ideal volume of the employed buffer. The product's fluorescence intensity decreased when quantities were below 0.8 mL or over 1.2 mL. As a result, 1 mL of Teorell and Steinhagen buffer (pH 6) was the optimum utilized buffer. All curves were illustrated in (Fig. 4).

3.1.2 Fluorogenic reagents' effect. To show how 2,2-dihydroxyindan-1,3-dione (0.1% w/v) and phenylacetaldehyde (0.02% v/v) influence the methodology, different quantities of
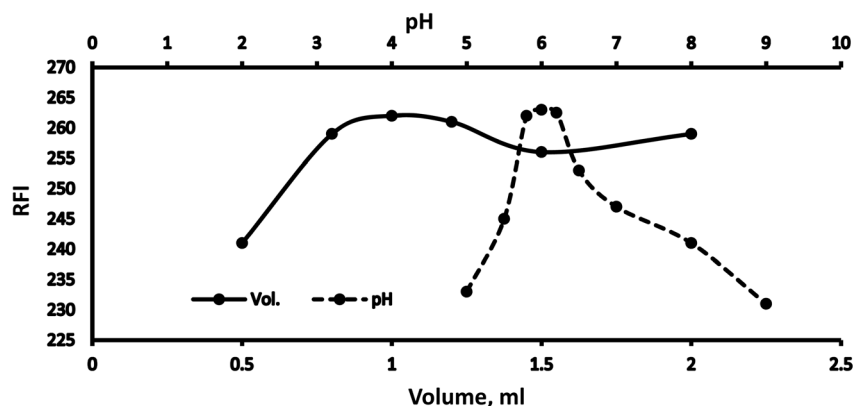
analytical cost are the main key benefits that distinguish this condensation reaction. So in this prescribed study, the derivatizing agents were condensed with the amino moiety of CS and

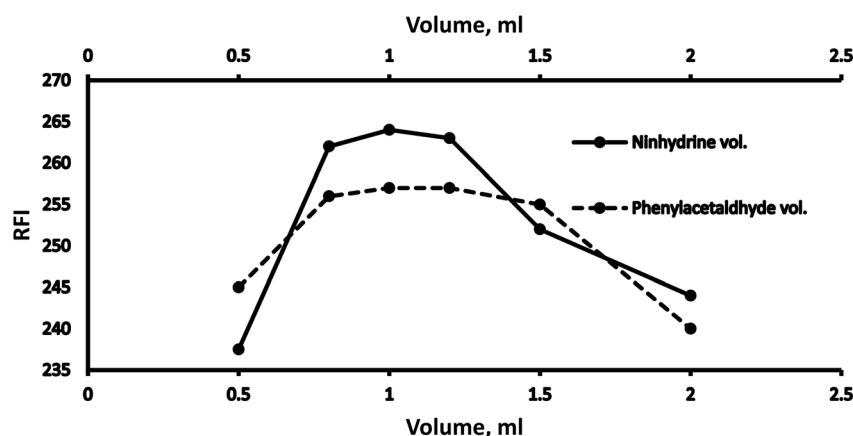**Fig. 4** Effect of the pH and buffer volume on the RFI of the reaction product of colistin (1 μg mL^{-1}).

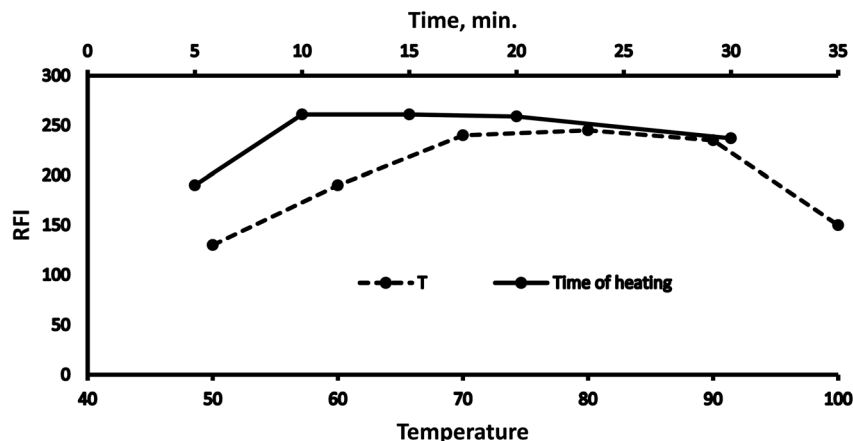**Fig. 5** Effect of fluorogenic reagents volumes on the RFI of the reaction product of colistin (1 μg mL^{-1}).

Fig. 6 Temperature and time effects on the RFI of the reaction product of colistin (1 µg mL^{-1}).
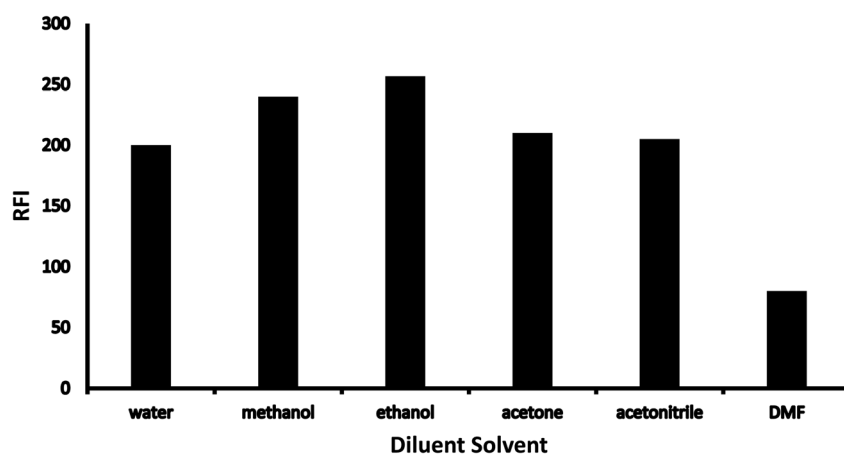

Fig. 7 Diluting solvent type effect on the RFI of the reaction product of colistin (1 µg mL^{-1}).

each in range of (0.5–2.0 mL) were employed. RFI was increased in parallel to increase the quantities of each of them till yielded a plateau at quantities of 0.8 to 1.2 mL. Beyond 1.2 mL a significant decrease was observed in the case of two reagents. As a result, the most optimal volume was found to be 1 mL from each one. All data were obtained in (Fig. 5).

3.1.3 Temperature and heating time. Upon freezing other parameters, a thermostatic water bath was employed to obtain temperatures ranging from 50 to 100 °C. The RFI of the product peaked in the temperature range of 70 to 90 °C. Outside of this range, the fluorescence gradually declined. Furthermore, the prescribed methodology was implemented in various periods utilizing a water bath at 80 °C. As the time intervals were prolonged, the RFI levels were dramatically enhanced until they obtained a constant line at 10 to 20 minutes. Following that RFI declined. As a result, the best heating conditions were discovered to be warming for 15 minutes at 80 °C water bath. All results were gathered in (Fig. 6).

3.1.4 Diluting solvent effect. Distilled water, ethanol, methanol, acetone, acetonitrile, and DMF were employed to dilute the final reaction product whilst other variables are held

constant. The solvent with the highest RFI value was the most suited. The highest RFI values were found in ethyl and methyl alcohols (Fig. 7). Owing to its lower toxicity, ethyl alcohol was selected as the preferred diluent.

3.2 Methodology validation

Considering ICH criteria,[27] the suggested approach was validated by assessing its linearity and range, accuracy, precision, robustness, and sensitivity.

3.2.1 Linearity & range. By graphing the acquired RFI *versus* the matching CS concentration, a calibration curve was estimated utilizing various concentrations of the studied drug. All analytical parameters related to the linear regression equation were obtained in (Table 1). In the range of 0.2–2.4 µg mL^{-1}, the relationship between CS concentration and RF intensities was linear with a correlation coefficient value of 0.9997.

3.2.2 Accuracy. Five different concentrations of the cited drug were examined three times over the calibration range of 0.2, 0.8, 1, 1.6, or 2.4 µg mL^{-1}. The estimated data reflected a close match between the measured and real values,

Table 1 Regression equation and related validation parameters

Parameters	Developed method
λ_{ex} (nm)	390
λ_{em} (nm)	474
Linear range ($\mu g\ mL^{-1}$)	0.2–2.4
Correlation coefficient (r)	0.9997
Determination coefficient (r^2)	0.9994
Intercept $\pm$ SD[a]	2.99 $\pm$ 2.32
Slope $\pm$ SD	151.17 $\pm$ 1.61
LOD[b] ($\mu g\ mL^{-1}$)	0.051
LOQ[c] ($\mu g\ mL^{-1}$)	0.154

[a] SD: standard deviation. [b] LOD: limit of detection. [c] LOQ: limit of quantitation.

Table 2 Evaluation of the accuracy of the proposed spectrofluorimetric method

Sample no.	Taken conc. ($\mu g\ mL^{-1}$)	Found conc.[b] ($\mu g\ mL^{-1}$)	% recovery
1	0.2	0.197	98.50
2	0.8	0.810	101.25
3	1	0.993	99.30
4	1.6	1.609	100.56
5	2.4	2.379	99.13
Mean			99.75
SD			1.124
RSD[a]			1.126

[a] RSD: relative standard deviation. [b] Mean of three replicate measurements.

Table 3 Developed approach intra- and inter-day precisions

Concentration level ($\mu g\ mL^{-1}$)	% recovery[a] $\pm$ SD	
	Intra-day	Inter-day
0.4	100.46 $\pm$ 1.45	99.83 $\pm$ 1.03
1	99.18 $\pm$ 0.66	101.02 $\pm$ 0.59
2	99.71 $\pm$ 0.93	98.99 $\pm$ 1.16

[a] Mean of three determinations.

demonstrating that the suggested approach was accurate. All values were inserted in (Table 2).

3.2.3 Precision. Three concentrations (0.4, 1, and 2 $\mu g\ mL^{-1}$) and three replicates of each concentration were utilized to assess intraday and interday precision. The computed mean relative standard deviation (RSD) was very small (below 2), illustrating that the suggested approach was repeatable and reliable. All data were shown in (Table 3).

3.2.4 Robustness & sensitivity. The procedure's robustness was determined by examining the impact of little changes in methodology conditions such as pH, 2,2-dihydroxyindan-1,3-dione, and phenylacetaldehyde reagent volumes on the suggested approach's procedure. This tool indicates the validity of the described procedure for drug assay in everyday use. One experimental variable was altered while the others remained

Table 4 Robustness for determination of CS (2 $\mu g\ mL^{-1}$) by the developed approach

Method parameters	% recovery[a] $\pm$ SD	RSD
pH		
5.8	100.09 $\pm$ 1.12	1.12
6	99.15 $\pm$ 0.94	0.95
6.2	99.30 $\pm$ 1.60	1.61
2,2-Dihydroxyindan-1,3-dione volume		
0.8	98.82 $\pm$ 0.48	0.49
1	99.76 $\pm$ 1.75	1.75
1.2	101.01 $\pm$ 1.30	1.29
Phenyl acetaldehyde volume		
0.8	99.74 $\pm$ 0.87	0.87
1	98.92 $\pm$ 1.07	1.08
1.2	100.19 $\pm$ 0.53	0.53

[a] Mean of three determinations.

fixed in these studies, and the recovery percentage (% R) was measured each time. Small fluctuations in any of the evaluated variables were shown to have no major impact on the method's outcomes, as obtained RSD did not surpass 2%. This was a proof that the suggested approach will be reliable during routine use. Results were illustrated in (Table 4).

Furthermore, to assess the sensitivity of the studied approach, LOD and LOQ values were estimated. Utilizing the formula "LOD = 3.3 SD/S or LOQ = 10 SD/S," where S is the slope of the calibration graph and SD is the standard deviation of the intercept. LOD and LOQ calculated values were 0.051 and 0.154 $\mu g\ mL^{-1}$, respectively.

3.3 Applications of the described approach

3.3.1 Assay of CS in parenteral dosage form (Colomycin® vial). Through the action of sulfuric acid, CMS was completely hydrolysis to colistin. To statistically compare the outcomes of our study with a previously described approach, we employed the Student's t-test and F-test to assess accuracy and precision. There was no significant difference between the investigated assay and the reported technique. Values were shown in (Table 5).

3.3.2 Assay of CS in oral dosage form. The approach of standard addition was successfully employed in the described technique for commercially Colistin Sulfate® tablets. The suggested method's mean % R of four sets was 99.71% with a RSD of less than 2%, proving its excellent accuracy and precision in determining CS in tablets. All data were inserted in (Table 6).

3.4 Content uniformity test

For content uniformity testing, the CS content within every tablet was estimated according to USP guidelines to assure that each tablet had the specified drug content. The estimated AV was 3.084, which is less than the maximum value permitted of 15, showing that the tablets tested had the acceptable uniformity of CS. All values were observed in (Table 7).

Table 5 Data for estimation of colistin in Colomycin® vial by the developed approach

| Dosage form | Mean of % recovery[a] ± SD | | t-Value[b] | F-Value[b] |
	Developed approach	Comparison approach		
Colomycin® vial	100.26 ± 0.77	99.42 ± 1.34	1.094	3.037

[a] Mean is average of five determinations. [b] Tabulated values at 95% confidence limit are t-value = 2.306, F-value = 6.338.

Table 6 Standard addition technique for estimation of CS in Colistin Sulfate® tablets

Dosage form	Amount analyzed ($\mu g\ mL^{-1}$)	Percentage added (%)	% recovery[a]
Colistin	0.5	0	99.81
Sulfate®	0.5	50	100.07
tablets	0.5	100	99.22
	0.5	150	99.72
Mean			99.71
SD			0.36
RSD			0.36

[a] The value is the average of three determinations.

Table 7 Application of the proposed method to determine content uniformity of CS in tablet form

Tablet number	% recovery[a] of the claimed content Colistin Sulfate® tablet
1	97.88
2	98.75
3	99.61
4	100.89
5	97.47
6	96.99
7	100.53
8	99.59
9	99.74
10	99.16
Mean	99.06
SD	1.29
RSD	1.30
Acceptance value (AV)	3.084
Maximum allowed AV (L1)	15

[a] Mean of three replicate measurements.

4 Conclusion

Various obstacles faced in previously described procedures for the analysis of CS and its prodrug CMS, such as the extraction step or even utilizing costly apparatus, solvents, or reagents, were overcome in the current research work.

Although the simplicity, sensitivity, and availability of its instrument, only one fluorimetric method for determination of the studied drug has been reported. So the prescribed work aimed to establish a new, reproducible, and easy-implementable methodology for the routine analysis of pharmaceutical dosage forms of CS and its prodrug CMS in quality control laboratories. Moreover, to make a unique value to our work, content uniformity testing was highly recommended and successfully applied.

Conflicts of interest

There are no conflicts to declare.

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