Spectrofluorimetric Method for Simultaneous Determination of Trimethoprim and Sulfamethoxazole with *O*-phthaladehyde Reagent by H-point Standard Addition Method

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Abstract: *Simultaneous spectrofluorometric method described for the determination of trimethoprim (TMP) and sulfamethoxazole (SMZ) in pure and pharmaceutical preparations using H-point standard addition method (HPSAM) according to the reaction of nanograms of drugs with* O*-phthaladehyde (OPA) reagent to forms highly fluorescence compounds. The formed fluorophore excitation and emission at 342 and 458 nm, respectively, for OPA-TMP compound, at 424 and 508 nm, respectively, for OPA-SMZ compound under basic condition (pH 9.8) in the presence of 2-mercabtoethanol. A simple and accurate HPSAM is reported to resolve the overlapping in the fluorescence spectrum of these two drugs without prior separation of samples. The linear range was 100–1200 ng/mL for TMP and 100–1100 ng/mL for SMZ. The LOD and LOQ were 16.64 and 36.80 ng/mL, as well as 15.76 and 33.88 ng/mL for TMP and SMZ, respectively. The relative standard deviations and recovery percentages were 0.641% and 101.29% for TMP as well as 0.558% and 100.96% for SMZ, respectively. The procedure has been applied successfully in various pharmaceutical preparations. It was discovered that the experimental F- and t-values at a 95% confidence level were no higher than the theoretical values, showing that the HPSAM method is accurate and valid.*

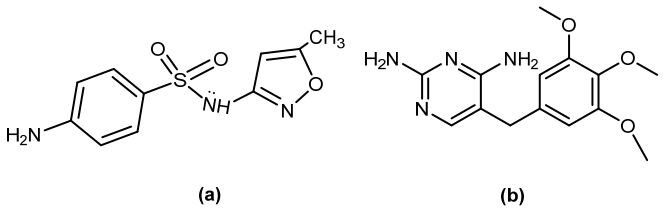
Keywords: *trimethoprim; sulfamethoxazole; standard addition method; spectrofluorometric;* O*-phthaladehyde*

■ INTRODUCTION

Sulfonamides are a class of antibiotics for bacterial infections [1]. These drug classes usually contain broad-spectrum antibiotics that work against a wide range of bacterial species and are used to treat a variety of bacterial infections. Nowadays, many pharmaceutical medications on the market combine sulfonamides with another medication whose goal is to increase the effectiveness of antibiotics [2]. The combination of sulfamethoxazole (SMZ) with trimethoprim (TMP) is an example of these commercial formulations used to treat a wide variety of bacterial infections such as urine, middle ear, intestinal infections, and respiratory infections [3]. It is also used to prevent and treat a certain type of pneumonia [4-5]. Fig. 1 shows the chemical structures of SMZ and TMP.

Spectrofluorometric analysis is a highly sensitive analytical technique for identifying and determining fluorescent substances at the nanogram or lower level. It has extensively been applied to the analysis of multicomponent systems and some applications in the field of pharmaceutical and clinical analysis [6].

H-point standard addition method (HPSAM) was introduced in 1988 by Reig and Falco [6]. It was based on the dual-wavelength spectrophotometry concept and the traditional standard addition method [7]. The biggest benefit of HPSAM is its ability to eliminate bias errors brought on by a blank reagent or interfering substance.



(a) (b)

Fig 1. Chemical structures of (a) SMZ and (b) TMP

However, the approach has the limitation that it can only be used to identify two drugs. In order to do a quantitative analysis of two overlapping analytes without separating them, in the proposed study, the development of HPSAM for the spectrofluorometric determination of TMP and SMZ simultaneously in pharmaceutical formulations has been studied [8-9]. HPSAM was used for simultaneous determination of some drugs such as phenylephrine hydrochloride, chlorpheniramine maleate, paracetamol [10], ciprofloxacin and cephalexin [11], thiamine and pyridoxine [12], captopril and hydrochlorothiazide [13], ascorbic acid and analgine [14]. This study aims to develop spectrofluorometric determination of TMP and SMZ simultaneously in pure and real pharmaceutical preparations by HPSAM.

■ EXPERIMENTAL SECTION

Materials

The pure TMP, SMZ, and *O*-phthaladehyde (OPA) reagents used in this research were obtained from GK biotechnology China. The 2-mercaptoethanol (2ME) and boric acid were obtained from Sigma-Aldrich. Methanol and hydrochloric acid (HCl) were purchased from J.T. Baker. Sodium hydroxide (NaOH) was supplied by BDH. Potassium chloride was obtained by Reachim. Pharmaceutical preparations were purchased by the local market.

Instrumentation

The fluorescence intensity was measured by Shimadzu (RF-5301 PC, Kyoto, Japan) fluorescence spectrophotometer. The materials were weighted using a sensitive electronic balance (BP 3015, Sartorius Germany). A digital pH-meter (in-lab pH 720, Germany) has been used to measure pH. Thermostatically controlled water bath (LCB-22D Daihan Labtech CO, Korea) was used to measure temperature.

Procedure

Solution preparation

A stock solution (TMP and SMZ) was prepared by dissolving 0.01 g of medication in 100 mL of distilled water. The working standard solutions were made by diluting with distilled water to the desired concentration. The OPA reagent (0.1% w/v) was freshly produced by dissolving 0.1 mg of OPA reagent in 100 mL of methanol solvent. 2ME (0.5% v/v) was produced by adding 0.5 mL of 2ME into a 100 mL volumetric flask and making up the volume with methanol. NaOH 0.2 M was made by dissolving 0.8 g of NaOH in distilled water and filling a 100 mL volumetric flask with the same solvent. HCl solution (0.2 M) was prepared by diluting 1.67 mL of concentrated acid with methanol to the mark in a 100 mL volumetric flask. The borate buffer solution was made by weighing 1.237 of boric acid and 1.500 g potassium chloride diluted in 100 mL of distilled water. Drops of NaOH solution were added to the boric acid solution while stirring until a pH of 9.8 was achieved.

Pharmaceutical preparations solutions

Tablets pharmaceutical preparation (400 mg SMZ, 80 mg TMP/tablet). The cited drug solution (100 µg/mL SMZ and 10 µg/mL TMP) was obtained by grinding and thoroughly mixing 5 tablets, weighing the equivalent of 10 mg of SMZ and 1 mg of TMP of pure medicines of pharmaceutical preparations, and then dissolving the mixture in 100 mL of methanol. The other concentrations that were required were prepared with suitable dilution.

Syrup pharmaceutical preparation (200 mg SMZ, 40 mg TMP/5 mL). This solution was prepared by mixing two containers of syrup, and then 2.5 mL of this mixture was transferred to a 100 mL volumetric flask. The volume was made up to the mark with distilled water, gaining 100 µg/mL of SMZ and 20 µg/mL of TMP. The other concentrations that were required were prepared with suitable dilution.

Individually calibration curve

A series of TMP and SMZ standard solutions were accurately transferred into sets of 10 mL volumetric flasks. A micropipette was used to prepare the concentration ranges of 100–1200 and 100–1100 ng/mL for TMP and SMZ, respectively. Then, 2 and 2.5 mL of 0.2 M borate buffer solution (pH = 9.8), as well as 1 and 1.25 mL of 2ME (0.5% v/v) were added to each flask for TMP and SMZ flasks, respectively, and thoroughly mixed. TMP and SMZ reaction mixtures were allowed to stand for 4 and 5 min, respectively. Following that,

2.5 mL of OPA (0.1% w/v) was added to each flask and carefully mixed. TMP and SMZ reaction mixtures were left at room temperature for 40 and 35 min, respectively. The solution in each flask was filled to the mark with methanol solvent. The resulting solution's intensity is measured at 458 nm for TMP and 508 nm for SMZ when excited at 342 and 424 nm, respectively.

HPSAM procedure

Determination of TMP as analyte and SMZ as interferent. A series of 10 mL volumetric flasks were filled with increased concentrations of TMP drug, ranging from 100–1200 ng/mL, by adding the necessary volume of TMP stock solution. Subsequently, 500 ng/mL of TMP was added constantly. Then, a fixed concentration of SMZ (400 ng/mL) was added to all volumetric flasks. For each flask, 2 mL of borate buffer (0.2 M) solution (pH = 9.8) and 1 mL of 2ME (1% v/v) were added and thoroughly mixed. For 4 min, the reaction mixture was left to stand. After that, 2.5 mL of OPA (0.1% w/v) was added and carefully stirred. The mixture was once more allowed to sit at room temperature for 40 min. The solutions were diluted using methanol solvent. The resulting solution's intensity was measured at the fluorescent intensity of the resulting solutions at two wavelengths, 430 and 445 nm, when excited at 424 nm.

Determination of SMZ as analyte and TMP as interferent. A series of 10 mL volumetric flasks were filled with increased concentrations of SMZ drug, ranging from 100–1100 ng/mL by adding the necessary amount of SMZ stock solution. Then a constant amount of SMZ (600 ng/mL) was added. Then a fixed concentration of TMP (500 ng/mL) was added to all volumetric flasks. Then 2.5 mL of borate buffer (0.2 M) solution (pH = 9.8) and 1.25 mL of 2ME (0.5% v/v) were added to the flask and thoroughly mixed. The mixture was left to stand for 5 min. Then, 2 mL of OPA (0.1% w/v) solution was then added and gently mixed. The solutions were left at room temperature for another 35 min. The solution was completed to the mark by methanol solvent. The Fluorescent intensity of the resulting solutions was measured at two wavelengths 520 and 535 nm, when excited at 424 nm.

■ RESULTS AND DISCUSSION

The most widely used fluorogenic agent is OPA, which is used to detect nanograms of primary amine compounds [15], such as TMP and SMZ, which form a highly fluorescent compound when 2ME is present in an alkaline medium. Because of spectra overlap, using standard spectrofluorometric measurements to determine TMP in the presence of SMZ is difficult [16]. A useful technique for resolving this overlapping spectrum is HPSAM (Fig. 2), which allows for simultaneously estimating TMP and SMZ in the same solution. The suggested reaction between drugs with OPA is shown in Fig. 3.

Experimental Conditions

The optimal conditions for the development of OPA-2ME-TMP and OPA-2ME-SMZ fluorophores were examined separately providing an overview of the results obtained. Table 1 shows the optimum conditions for OPA-2ME-TMP and OPA-2ME-SMZ.

HPSAM

If TMP was selected as the analyte, two wavelengths (430 and 445 nm) were selected, which show the same fluorescence intensity for the OPA-2ME-SMZ fluorophore. A sample series with various sets of SMZ

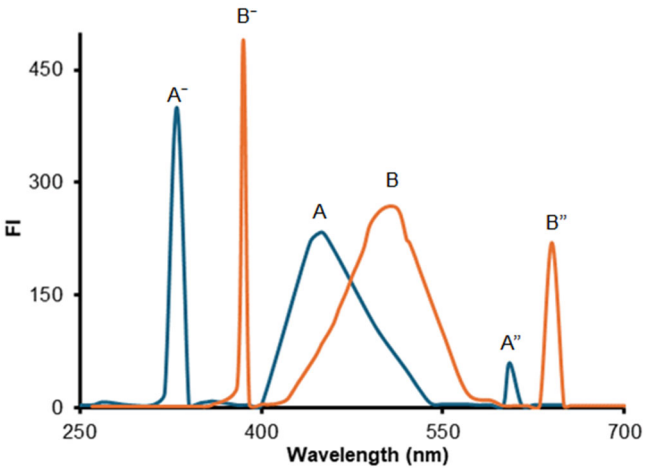


Fig 2. Fluorescence spectra of excitation (A^-, B^-), emission (A, B), and first derivative of excitation spectrum (A", B") of OPA-2ME-TMP and OPA-2ME-SMZ fluorophores, respectively

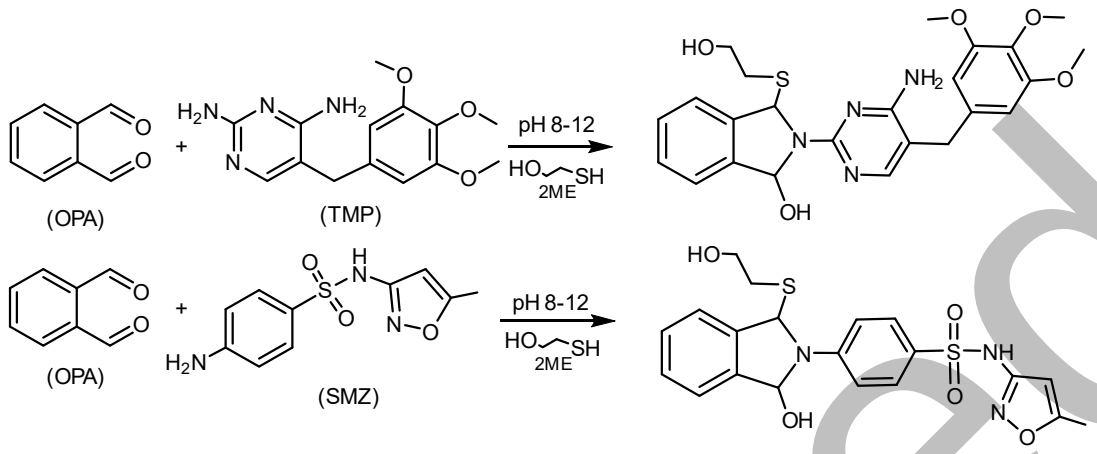


Fig 3. The suggested pathway of reaction between OPA with TMP and SMZ in the presence of 2ME

Table 1. Optimum conditions of OPA-2ME-TMP and OPA-2ME-SMZ fluorophores

Condition	OPA-2ME-TMP fluorophore	OPA-2ME-SMZ fluorophore
OPA reagent volume (mL)	2.50	2.50
Buffer volume (mL)	2.00	2.50
pH	9.8	9.8
2ME volume (mL)	1.00	1.25
2ME time (min)	4	5
Reaction time (min)	40	35

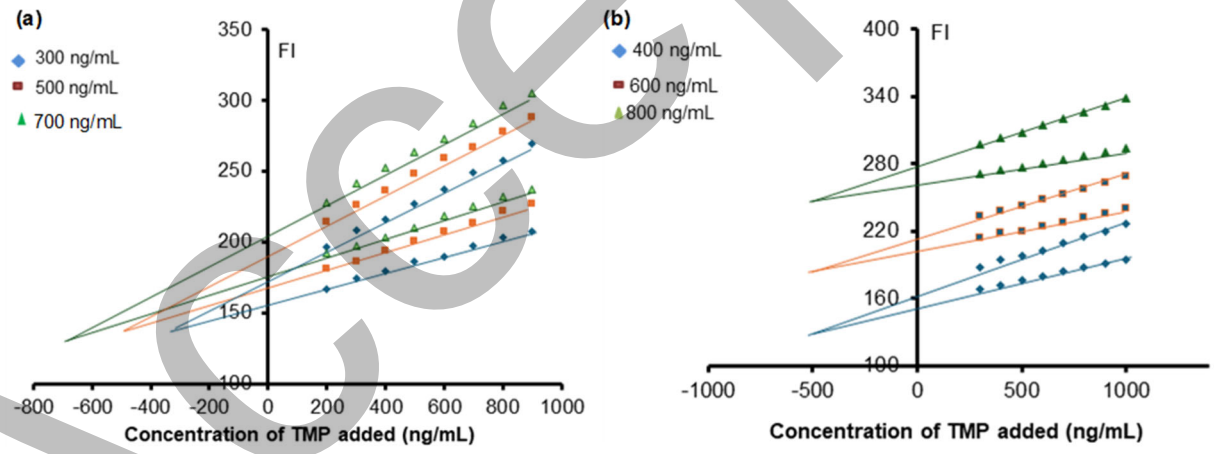


Fig 4. HPSAM for fixed concentration of (a) SMZ (400 ng/mL) and (b) TMP (500 ng/mL) and various amounts of TMP (300, 500, and 700 ng/mL), as well as SMZ (400, 600, and 800 ng/mL)

and standard TMP aliquots (Fig. 4(a)) or fixed TMP amounts with various SMZ sets (Fig. 4(b)) were created by adding TMP standard solutions.

The application of HPSAM in determining TMP and SMZ in a series of solutions containing fixed aliquots of SMZ and different amounts of TMP (Fig. 4(a)) or both fixed aliquots of TMP with various quantities of SMZ (Fig. 4(b)) was tested by performing a standard addition of SMZ. The results demonstrated that this development method successfully identified the TMP and SMZ contents in the samples [17].

$$F_{430} = Y_o + Y + M_{430}C_{TMP} \quad (1)$$

$$F_{445} = V_o + V + M_{445}C_{TMP} \quad (2)$$

$$F_{430} = F_{445} \quad (3)$$

$$C_{TMP} = -C_H \quad (4)$$

The F_{430} and F_{445} represent the analytical signals observed at 430 and 445 nm, as shown in Eq. (1) and (2), respectively. In Eq. (3), the analytical signal of F_{430} is equal to F_{445}. Finally, Eq. (4) shows that C_{TMP} is equal to the negative value of C_H, which is the unknown TMP concentration.

$$Y_o + Y + M_{430}(-C_H) = V_o + V + M_{445}(-C_H) \quad (5)$$

$$-C_H = \frac{\left[(Y_o - V_o) + (Y - V)\right]}{M_{430} - M_{445}} \quad (6)$$

In Eq. (5) and (6), Y_o and V_o are original TMP signals at 430 and 445 nm, respectively. The Y and V are analytical SMZ signals at 430 and 445 nm, respectively. The M_{430} and M_{445} are calibration lines slopes of standard addition at 430 and 445, respectively.

If SMZ concentration is known, and the analytical signal of SMZ (Y and V) at 430 and 445 nm doesn't affected by standard additions of TMP, as shown in Eq. (7).

$$-C_H = \frac{Y_o - V_o}{M_{430} - M_{445}} = -\frac{Y_o}{M_{430}} = -\frac{V_o}{M_{445}} \quad (7)$$

By substitution $-C_H$ value in Eq (1), the analytical signal is calculated using Eq. (8).

$$A_H = Y_o + Y + M_{430}(-C_H) \quad (8)$$

From Eq. (7);

$$Y_o = M_{430}(-C_H) \quad (9)$$

therefore, Eq. (8) becomes Eq. (10).

$$A_H = Y \quad (10)$$

Thus, A_H refers to the SMZ signal at 430 and 445 nm.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were calculated from Eq. (11) and (12).

$$LOD = C_H + 3S_{CH} \quad (11)$$

$$L_{OQ} = CH + 10S_{CH} \quad (12)$$

The mean and standard deviation of five independently measured points of a blank sample using HPSAM are represented by C_H and S_{CH}, respectively. The LOD and LOQ values for SMZ and TMP were 15.76 and 33.88 ng/mL as well as 16.64 and 36.80 ng/mL, respectively.

Accuracy

The results of using the suggested approach to prepare and analyze several mixtures of TMP and SMZ with varying concentration ratios are presented in Table 2. The obtained results showed a high degree of agreement between calculated and true values. Therefore, the suggested method is highly accurate.

Precision

The precision of the proposed method was verified by repeating the experiment of estimation of TMP and SMZ simultaneously in a mixture. The results were summarized at 430 and 445 nm selected wavelengths in Table 3 and at 520 and 535 nm selected wavelengths in Table 4.

Table 2. Estimation of TMP and SMZ in some synthetic mixtures

F vs. C equation	R^2	Added (ng/mL)		Found; Re% (ng/mL)	
		TMP	SMZ	TMP	SMZ
F_{430} = 0.0583x + 155.89	0.9974	300	400	305.50	395.22
F_{445} = 0.1022x + 176.37	0.9985			(101.84)	(98.80)
F_{430} = 0.0672x + 166.92	0.9983	500	400	507.80	404.73
F_{445} = 0.1055x + 194.03	0.9988			(101.56)	(101.18)
F_{430} = 0.0702x + 175.12	0.9981	700	400	693.43	396.42
F_{445} =0.1082x + 208.10	0.9989			(99.06)	(99.10)
F_{520} = 0.0314x + 111.32	0.9985	300	300	304.48	305.62
F_{535} = 0.0938x + 134.02	0.9989			(101.49)	(101.87)
F_{520} = 0.0419x + 121.01	0.9973	300	500	305.50	494.35
F_{535} = 0.1081x + 152.49	0.9980			(101.83)	(98.87)
F_{520} = 0.0548x + 134.65	0.9984	300	700	297.81	707.26
F_{535}= 0.1108x + 175.33	0.9987			(99.27)	(101.03)

Table 3. Precision of the proposed method at 430 and 445 nm selected wavelengths

	Added (ng/mL)		Found (ng/mL)	
	TMP	SMZ	TMP	SMZ
	400	600	404.52	607.63
	400	600	405.02	605.95
	400	600	396.56	607.80
	400	600	397.43	606.73
	400	600	405.45	605.76
Mean			405.196	606.774
SD			2.598	3.387
RSD% (n = 5)			0.641	0.558
Re%			101.29	100.96

Table 4. Precision of the proposed method at 520 and 535 nm selected wavelengths

	Added (ng/mL)		Found (ng/mL)	
	TMP	SMZ	TMP	SMZ
	700	500	704.82	506.86
	700	500	706.29	504.43
	700	500	706.46	506.38
	700	500	705.75	505.54
	700	500	705.47	505.84
Mean			705.758	505.81
SD			2.879	2.905
RSD% (n = 5)			0.407	0.574
Re%			100.82	101.16

Application

To evaluate the application of the proposed method for determining TMP and SMZ in pharmaceutical formulations, the results are compared statistically using percent recovery to those obtained by utilizing the HPLC standard method. The results of determining TMP and SMZ in real pharmaceutical formulations using the proposed method are summarized in Table 5. A comparison of the proposed method with the official method at a 95% confidence level shows that there is a good agreement between them according to t- and F-tests, which were less than the theoretical value (Table 6). The results show that the proposed method can be used successfully to determine TMP and SMZ simultaneously in real pharmaceutical preparations. Several methods in literature have been used for simultaneous estimation of TMP and SMZ can be seen in Table 7, the lowest detection limit was obtained by the proposed method.

Table 5. Determination of TMP and SMZ in different pharmaceutical preparations

Products	Content		Found (mg)		Re%	
	TMP	SMZ	TMP	SMZ	TMP	SMZ
Metheprim tablets	80 mg	400 mg	82.64	406.39	102.05	101.59
Septrin tablets	80 mg	400 mg	79.32	405.20	99.15	101.30
Cortim suspension syrup	200 mg/5 mL	40 mg/5 mL	40.75	202.42	101.87	101.21
Bactrim syrup	200 mg/5 mL	40 mg/5 mL	40.63	203.28	101.57	101.64
Septrin syrup	200 mg/5 mL	40 mg/5 mL	40.54	203.67	101.35	101.83
Metheprim syrup	200 mg/5 mL	40 mg/5 mL	39.42	199.26	99.55	99.63

Table 6. Statistical comparison between the proposed and official method for simultaneous determination of TMP and SMZ

	TMP		SMZ	
	HPSAM	Official method	HPSAM	Official method
Re%	99.39	97.63	101.19	102.01
SD	0.732	1.430	0.046	0.952
N	7	6	7	6
t-test	2.053 (2.571)	-	1.270 (2.364)	-
F-test	1.695 (4.592)	-	0.235 (5.143)	-

Table 7. The most important methods used for simultaneous determination of TMP and SMZ

Method	Linear range		RSD%		Re%		LOD		Ref.
	TMP	SMX	TMP	SMZ	TMP	SMZ	TMP	SMZ	
Spectrophotometric	10–50 µg/mL	2.5–20 µg/mL	1.50	1.42	99.69	98.15	0.0806 µg/mL	0.333 µg/mL	[18]
	14.0–26.0 mg/L	2.8–5.2 mg/L	-	-	99.62	100.05	-	-	[19]
	2.0–30.0 mg/L	2.5–64.0 mg/L	1.109	1.349	99.5	99.9	0.13 mg/L	0.22 mg/L	[20]
HPLC	3.0–9.0 µg/mL	15.0–30.0 µg/mL	0.38	0.68	100.79	101.47	0.060 µg/mL	0.947 µg/mL	[21]
	25–400 µg/L	50–800 µg/L	-	-	105.0	98.0	15.0 µg/L	25.0 µg/L	[22]
RP-HPLC	10–50 µg/mL	20–100 µg/mL	0.38	0.31	99.75	99.96	0.55 µg/mL	0.48 µg/mL	[23]
Electrochemical	0.1–0.7 µmol/L	0.1–0.7 µmol/L	-	-	101.0	98.0	31.0 µmol/L	24.0 µmol/L	[24]
	1–180 µmol/L	1–220 µmol/L	4.70	4.90	95.3	97.0	0.3 µmol/L	0.4 µmol/L	[25]
	1–10 µmol/L	1–10 µmol/L	4.47	4.42	100.14	102.04	0.04 µmol/L	0.09 µmol/L	[26]
	0.1–10 µmol/L	1.0–10 µmol/L	3.10	3.70	96.1	92.3	60 nmol/L	38 nmol/L	[27]
Batch injection analysis	2.0–40 mg/L	4.0–320 mg/L	2.50	1.30	-	-	0.15 mg/L	0.20 mg/L	[28]
Spectrofluorometric (HPSAM)	100–1200 ng/mL	100–1100 ng/mL	0.641	0.574	101.29	101.16	16.64 ng/mL	15.76 ng/mL	This work

■ CONCLUSION

The usefulness of HPSAM is the determination of two analytes in the same solution using a fluorescence or absorbance reagent. This method can measure the concentration of one species from spectral data at two wavelengths where the other species present the same fluorescence relationship. The ability of HPSAM to resolve the overlapping fluorescence spectra of TMP and SMZ has been demonstrated based on the reaction of TMP and SMZ with OPA reagent in basic conditions in the presence of 2ME. The proposed HPSAM method is very suitable for the estimation of unknown concentrations of TMP and SMZ simultaneously in pure and real pharmaceutical formulations with high accuracy and precision according to RSD% and Re% which were 0.641% and 101.29% for TMP as well as 0.558% and 100.96% for SMZ, respectively. The suggested method can be applied to estimate the studied drugs as pharmaceutical preparations in some biological fluids such as blood and urine.

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■ CONFLICT OF INTEREST

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript. We certify that the submission is original work and is not under review at any other publication.

■ AUTHOR CONTRIBUTIONS

Amneen Mohammed Alsayegh and Abbas Noor Alshirifi conducted the experiment and calculations, as well as wrote and revised the manuscript. All authors agreed to the final version of this manuscript.

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