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# Screening and greenness profiling of oxidative-coupling and electrophilic aromatic substitution reactions for determination of three phenolic drugs

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ABSTRACT

In this study, we screened oxidative-coupling and electrophilic aromatic substitution reactions for developing simple, economic, and environment-friendly spectrophotometric methods for three widely prescribed phenolic drugs; salbutamol sulfate (SLB), terbutaline sulfate (TBT), and thymol (THY). Methods IA and IB rely on the oxidative-coupling reaction of SLB and TBT with *p*-phenylenediamine in the presence of an oxidizing agent in carbonate buffer while Method II depends on electrophilic aromatic substitution reaction of THY with sodium nitroprusside/hydroxylamine hydrochloride in phosphate buffer (pH 12.0). Different experimental conditions controlling the derivatization reactions were optimized and the reaction products were quantified at 552, 400, and 700 nm, for SLB, TBT, and THY, respectively. Beer's-Lambert law was obeyed over wide linearity ranges with limits of detection from 6 to 126 ppb for the target analytes. Furthermore, the developed methods were validated as per ICH guidance. In addition, profiling of the methods greenness was conducted by adopting the National Environmental Methods Index and the analytical eco-scale score approaches. The two approaches elucidated the excellent greenness of the developed methods which emphasized their applicability for quality control analysis. Thus, the developed methods were efficaciously used for the determination of the three drugs in different dosage forms with excellent accuracy, where % found ranged from 98.86%–102.77%, and no interference from additives in the formulations.

## 1. Introduction

Phenolic compounds are widely used as therapeutic agents with diverse pharmacological activities. Among the most extensively administered phenolic derivatives, salbutamol sulfate (SLB), terbutaline sulfate (TBT), and thymol (THY) are very recognized. SLB and TBT are $\beta_2$-adrenoreceptor agonists with a bronchodilator action that are used as first-line therapies for the treatment of asthma. Also, they are used for the management of chronic obstructive pulmonary disease. In addition, they have a tocolytic effect, thus they are used for the prevention of premature labor. On the other hand, THY has antiseptic, antibacterial, antifungal, and antioxidant activities. It is used for its antimicrobial as well as deodorant effects in mouth washes and gargles. Also, it is used for the treatment of skin and respiratory disorders [1]. The three compounds are approved by the British Pharmacopoeia (BP) [2] and the United States Pharmacopoeia (USP) [3].

SLB is bis[(1*RS*)-2-[(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanol] sulfate [2]. It is determined by the BP [2] and the USP [3] using non-aqueous titration and HPLC methods for pure state and pharmaceutical formulations, respectively. As well, the analytical literature shows many methods for its determination such as chromatographic methods [4–6], electrochemical methods [7,8], spectrophotometry [9–11], and fluorimetry [12].

TBT is bis[(1*RS*)-1-(3,5-dihydroxyphenyl)-2-[(1,1-dimethylethyl)amino]ethanol] sulfate [1]. The BP described non-aqueous titration and HPLC methods for its pure state and tablets [2], while the USP determined it in pure and dosage forms by HPLC methods [3]. It was determined also by many analytical methods such as chromatography

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[13,14], spectrophotometry [15,16], and voltammetry [17].

THY, 5-methyl-2-(1-methylethyl)-phenol, is determined in the USP by titration with bromine [2]. Additionally, chromatography [18,19], spectrophotometry [20,21], fluorimetry [22], and voltammetry [23] methods were adopted for its determination.

Spectrophotometry is still a widely-used technique in the pharmaceutical analysis by virtue of its fast, accurate and easy analysis ability in addition to high throughput. Thus, in this study, we utilized the phenolic nature of the three drugs in order to develop simple, economic, sensitive, and accurate spectrophotometric methods for their determination in different pharmaceuticals via oxidative coupling (Method I) and aromatic electrophilic substitution reactions (Method II). Method I depends on the oxidation of *p*-phenylenediamine (PPD) to *p*-benzoquinone diimine followed by coupling with the phenolic drugs which exist as phenoxide anions in the alkaline medium. The products are indoaniline dyes with characteristic colors [24]. This method was adopted for the determination of SLB and TBT. Method II depends on electrophilic aromatic substitution reaction of THY with sodium nitroprusside and hydroxylamine hydrochloride in alkaline medium forming 2-isopropyl-4-nitroso-5-methyl phenolate anion with concomitant dimerization of sodium nitroprusside giving a characteristic green-colored product [25,26]. In consequence, sensitive spectrophotometric measurement of these products was carried out for quantitative determination of SLB, TBT, and THY in different pharmaceutical products. We also conducted a profiling study of the greenness of the developed methods by applying two metrics, namely; the National Environmental Method Index (NEMI) and the analytical eco-scale score methods. This step becomes important for the conservation of environmental and human safety by developing and applying only green analytical methods [27].

## 2. Experimental

### 2.1. Apparatuses, chemicals, reagents, and standard solutions

Spectrophotometric measurements were done using Shimadzu UV-210A double-beam spectrophotometer (Kyoto, Japan) with 1 cm quartz cuvettes. Heating was achieved using a thermostatically-controlled water bath from Grant Instruments (Cambridge), and pH measurements were done using Philips PW 9420 pH-meter (India).

Thymol, sodium dihydrogen phosphate ($NaH_2PO_4$), sodium hydroxide (NaOH), sodium carbonate anhydrous ($Na_2CO_3$), and ethanol were supplied by BDH (London, UK). Hydroxylamine hydrochloride, *p*-phenylenediamine (PPD), potassium hexacyanoferrate ($K_3Fe(CN)_6$), hydrochloric acid (HCl, 35%), and sodium metaperiodate ($NaIO_4$) were purchased from Fluka (USA) and sodium nitroprusside was obtained from Riedel-de Häen (Germany). TBT and SLB pure powders were from S.D.I. (Samarra, Iraq). Pharmaceutical preparations were obtained from local pharmacies: Butadin tablets (2 mg SLB/tablet), product of S.D.I., Salbutamol injection (0.5 mg SLB/mL), product of Biological Italia Lab Novate (Milano, Italy), Ventoline respirator solution (100 mg SLB/20 mL), product of Glaxowelcome Operations (Middlesex, UK), samabutaline tablets (5 mg TBT/tablet), product of S.D.I., Ataline tablets (2.5 mg TBT/tablet), product of Medochemie (Cyprus), Mentoral mouth wash (THY 0.063%w/v), product of Hungary Pharmaceutical Industries Co., Ltd., Budapest, Hungary, and Lastarime mouth wash (THY 0.06% w/v), product of Mediotic Labs, Roma, Italy.

PPD solution ($4 \times 10^{-3}$ M) was prepared in absolute ethanol while $NaIO_4$ ($1 \times 10^{-2}$ M), hydroxylamine hydrochloride ($4 \times 10^{-2}$ M), $K_3Fe(CN)_6$ ($1 \times 10^{-3}$ M), and sodium nitroprusside (0.1 M) solutions were prepared in distilled water. Sodium nitroprusside solution was protected from light to maintain its stability for at least 1 month. 0.1 M phosphate buffer (pH 12.0) and 0.05 M. carbonate buffers (pH 9.0 and 9.5) were prepared.

Preparation of individual standard solutions of SLB and TBT (100.0 μg/mL) was performed using distilled water as a solvent, while a standard solution of THY(100.0 μg/mL) was prepared by dissolving 0.01 g in 5 mL of ethanol then dilution to 100 mL with distilled water.

### 2.2. Procedures for plotting the calibration graphs

#### 2.2.1. Method IA and IB

Increasing volumes of SLB and TBT were measured and added to two groups of 25 mL volumetric flasks to obtain final concentrations of 0.8–40.0 and 0.6–40.0 μg/mL, respectively. 3.0 mL of PPD solution ($4 \times 10^{-3}$ M) was added to all flasks followed by 3.0 mL of carbonate buffer (pH 9.0 for SLB and pH 9.5 for TBT). 2.5 mL of $NaIO_4$ was added to the reaction mixture in case of SLB and 2.5 mL of $K_3Fe(CN)_6$ were added in case of TBT. The reactions were allowed to proceed at room temperature for 50 and 30 min for SLB and TBT, respectively. The final volumes were made up by distilled water, mixed well, and their absorbance were measured against reagent blank at 552 and 400 nm for SLB and TBT, respectively. The calibration graphs were obtained by plotting the absorbance against the final drug concentrations (μg/mL) and the regression equations were derived.

#### 2.2.2. Method II

Increasing volumes of THY were measured and added to a series of 25 mL volumetric flasks to cover a final concentration range of 0.1–14.0 μg/mL. 0.5 mL of sodium nitroprusside (0.1 M) and 0.5 mL of hydroxylamine hydrochloride ($4 \times 10^{-2}$ M) were added followed by 3.0 mL phosphate buffer (pH 12.0). After a reaction time of 15 min at room temperature, the solutions were made up to the final volume with distilled water and mixed well. The absorbance was measured at 700 nm against reagent blank. The calibration graph was acquired by plotting the absorbance against the final drug concentrations (μg/mL) and the regression equation was derived.

### 2.3. Procedure for pharmaceutical preparations

For ampoules: the contents of 20 ampoules were collected in a beaker and transferred into 100 mL volumetric flask and the volume was completed with distilled water to prepare 100 μg/mL SLB solution. For respirator solution: 5 mL were withdrawn and put in 100 mL volumetric flask and made up to the final volume with distilled water to obtain 250 μg/mL SLB solution. A 100 μg/mL solution was prepared by proper dilution of this solution with distilled water. As for mouth wash; 20 mL were transferred to 50 mL volumetric flask and diluted to the final volume with distilled water. This solution was properly diluted with distilled water to prepare 100 μg/mL THY solution. Regarding tablets; the weight of 20 tablets (Butadin or Ataline Tablets) or 10 tablets (Samabutaline Tablets) was determined, followed by fine trituration and mixing, then an amount equivalent to 25 mg cited drug was weighed and transferred into 250 mL volumetric flask. Water was added to complete the volume to 250 mL then filtration was done to obtain a 100 μg/mL SLB and TBT solutions. Suitable volumes of these solutions were measured in an accurate way and the procedures of calibration graphs were applied to determine the concentration of the active ingredient in each pharmaceutical preparation adopting the corresponding method.

### 2.4. Procedure for Job's continuous variation method

Different volumes of PPD and SLB solutions (with identical molar concentrations equals $1.73 \times 10^{-4}$ M) were mixed together so as to provide different molar ratios from (0:1) to (1:0) of PPD:SLB, respectively while maintaining the total mole number unchanged. In a similar way, the same experiment was done using different volumes of PPD and TBT solutions having the same molar concentrations ($1.0 \times 10^{-3}$ M). The buffer and oxidizing agent were added and the absorbance was measured after the optimum standing time. A graph of the absorbance versus the fraction of SLB (Method IA) or the fraction of TBT (Method

IB) was plotted. The volume fraction at which the highest absorbance was attained was determined for the two methods.

### 2.5. Procedure for mole ratio method

Increasing volumes of PPD ($1.73 \times 10^{-4}$ M) were added to a constant volume of SLB ($1.73 \times 10^{-4}$ M). As well, the experiment was repeated using increasing volumes of PPD ($1.0 \times 10^{-3}$ M) and a constant volume of TBT ($1.0 \times 10^{-3}$ M). The buffer and oxidizing agent were added and the absorbance was measured after the proper standing time (as described for the preparation of the calibration curves). A graph of the absorbance versus the mole ratio of PPD/SLB or PPD/TBT was plotted. The linear parts were extrapolated and the intersection points to the stoichiometry of the reactions.

## 3. Results and discussion

### 3.1. Method I

#### 3.1.1. Optimization of the reaction conditions

The oxidative coupling of SLB and TBT with PPD leads to the formation of colored indoaniline dyes with maximum absorption at 552 and 400 nm, respectively (Fig. 1). This reaction is simple, sensitive, and utilizes economic reagents. Thus, the quantitative determination of SLB and TBT were accomplished by the development and validation of a spectrophotometric method based on this reaction. Firstly, the selection of the most efficient oxidizing agent was considered by attempting various oxidizing agents as presented in Fig. 2a. For SLB, the best oxidant was $NaIO_4$ while $K_3Fe(CN)_6$ was the optimum in case of TBT. The base of such selection is gaining the highest absorbance and the greatest bathochromic shift. As a consequence, the influence of the volume of the oxidizing agent on the proceeding of the reaction and absorption of the reaction product was studied. As can be seen from Fig. 2b 2.5 mL of $NaIO_4$ ($1 \times 10^{-2}$ M) and 2.5 mL of $K_3Fe(CN)_6$ ($1 \times 10^{-3}$ M) were optimum for the highest absorbance value for Method IA and Method IB, respectively.

Carbonate buffers of different pH values were investigated to examine the impact of the pH on the reaction. The highest absorbance was realized using a carbonate buffer of pH 9.0 and 9.5 for SLB and TBT, respectively. This results agreed with the literature which documented that, the best pH for the quantitative formation of indoaniline dyes is around pH < $pK_a$-1. Since the $pK_a$ of most phenolic compounds ranged from 10.0 to 11.0, this reaction is known to proceed at pH 9.0–10.0 according to the structure of the phenol [24]. As the $pK_a$ values assigned to the phenolic moiety of SLB and TBT are 10.3 and 11.0 [28], the selected pH values for the optimum reactions are sensible and rational.

The influence of PPD volume was also optimized by adding various amounts of $4 \times 10^{-3}$ M solution. For SLB and TBT, 3.0 mL was found optimum for the highest absorbance and robustness (Fig. 2c). Furthermore, the sequence of addition of the reactants was investigated. As observed from the results depicted in Table 1, the addition order of (drug + PPD + buffer + oxidizing agent) yields the highest absorbance for both drugs. The results of this study agree with the results of Corbett [24] who confirmed that the oxidant should be the last reagent to be added for the highest absorbance to be obtained.

Moreover, the influence of heating on the progress of the reaction and stability of the products was inspected over temperature settings ranges from room temperature to 60 °C and time scale up to 120 min. Performing the reaction at room temperature for 50 and 30 min was the ideal for Method IA and Method IB, respectively while heating has a slight adverse effect on the absorbance of the reactions products. Energy saving via carrying out the reaction at room temperature adds extra advantages to the developed method by decreasing the costs and preventing occupational hazards. The reactions products showed high stability at room temperature for at least 2 h (Fig. 2d).

Thus, the oxidative coupling reaction of SLB and TBT was carried out adopting the optimum experimental conditions offering excellent sensitivity.

#### 3.1.2. The stoichiometry and mechanism of the reaction

The Job's method of continuous variation and the mole ratio method [29] were executed to determine the stoichiometry of the reactions between SLB or TBT and PPD. The results of these studies are shown in Fig. 3. Regarding Method IA, both Job's and mole ratio methods indicated 1:1 M reactivity of SLB with PPD. Where the highest absorbance of Job's plot (Fig. 3a) was attained at a volume fraction of 0.5:1 (SLB:SLB + PPD) and the mole ratio method (Fig. 3b) shows 1:1 mol ratio (PPD:SLB). This confirmed that one molecule of SLB reacts with one molecule of PPD. This result is rational by virtue of the presence of a single site available for the coupling reaction at *o*-position to the –OH group (Fig. 4a).

On the other hand, the Job's plot for Method IB (Fig. 3c) exhibited the highest absorbance at volume fraction ratio of 0.34:1 (TBT:TBT + PPD) while the mole ratio method (Fig. 3d) shows 2:1 (PPD:TBT) mole ratio. These results indicated that one molecule of TBT reacts with two molecules of PPD. This is justified by the presence of three sites free for coupling in the TBT structure (Fig. 4a).

The reaction mechanisms are deduced as illustrated in Fig. 4 [24,30]. In alkaline medium, PPD is oxidized yielding *p*-benzoquinone diimine which reacts via coupling with SLB and TBT those exist as phenoxide anions in the alkaline condition. As stated earlier, there is

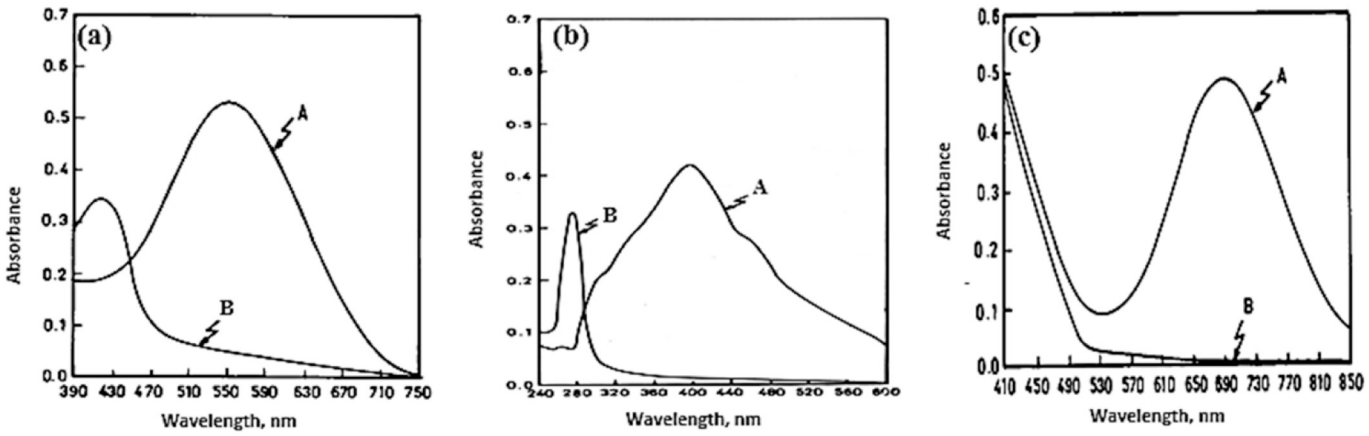



**Fig. 1.** Absorption spectra of (a) SLB (12.0 μg/mL) with PPD, (b) TBT (12.0 μg/mL) with PPD, and (c) THY (4.0 μg/mL) with sodium nitroprusside/hydroxylamine hydrochloride, where; A: reaction product and B: reagent blank.

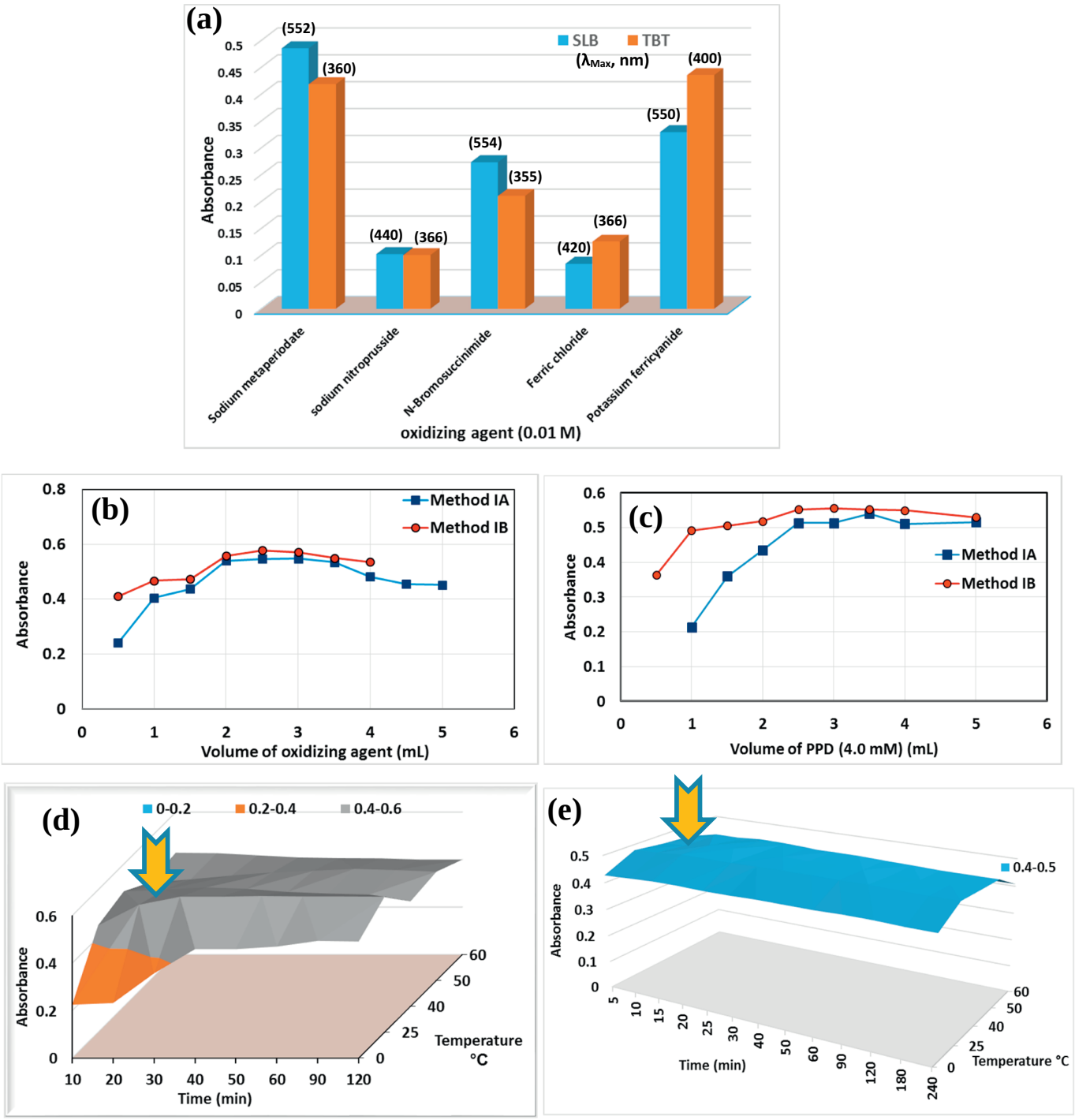



**Fig. 2.** Effects of (a) type of oxidizing agent, (b) volume of oxidizing agent ($1 \times 10^{-2}$ M $NaIO_4$ for Method IA and $1 \times 10^{-3}$ M $K_3Fe(CN)_6$ for Method IB), (c) volume of PPD ($4 \times 10^{-3}$ M) on the absorbance of the reaction products in Methods IA and IB, (d) time and temperature on the development and stability of the reaction product in Method IB, and (e) time and temperature on the development and stability of the reaction product in Method II. Concentration of the drugs in these experiments are: SLB 12.0 μg/mL (Method IA), TBT 12.0 μg/mL (Method IB), and THY 4.0 μg/mL(Method II).

one vacant site for coupling in the case of SLB (*o*-position). On the other hand, three sites are free for coupling in case of TBT: two are activated by being in a *p*-position to one –OH group and *o*-position to the other, and the third is activated by being in the *o*-position to both –OH groups. By analogy to resorcinol, which has the *m*-benzenediol moiety like TBT, the reaction occurs by coupling of two oxidized PPD molecules with one TBT molecule at the two free *p*-positions as illustrated in Fig. 4a [30]. The products are indoaniline dyes with characteristic violet and greenish-yellow colors, for SLB and TBT-derived dyes, respectively. The difference in the colors ($\lambda_{max}$) of the produced dyes is attributed to the variation of the degree of conjugation in the two dyes [30]. The extended conjugation in case of SLB-derived dye is greater than that in case of TBT-derived dye (Fig. 4a), thus the former appeared violet ($\lambda_{max}$ 552 nm) and the later appeared greenish-yellow ($\lambda_{max}$ 400 nm).

The stability constants ($K_{st}$) of the formed indoaniline dyes were calculated. In case of SLB, $K_{st} = 2.16 \times 10^6$ L·mol$^{-1}$ while

**Table 1**
Effect of the order of addition of reactants on the absorbance of the reactions' products for the three studied drugs.

| Drug | Experiment no. | Order of addition | Absorbance |
|---|---|---|---|
| TBT (12.0 μg/mL) | 1 | TBT + PPD + buffer + O | 0.574 |
| | 2 | TBT + PPD + O + buffer | 0.515 |
| | 3 | O + TBT + PPD + buffer | 0.469 |
| | 4 | PPD + O + buffer + TBT | 0.253 |
| | 5 | TBT + O + buffer + PPD | 0.231 |
| SLB (12.0 μg/mL) | 1 | SLB+ PPD + buffer + O | 0.546 |
| | 2 | SLB + PPD + O + buffer | 0.501 |
| | 3 | O + SLB + PPD + buffer | 0.469 |
| | 4 | PPD + O + buffer + SLB | 0.212 |
| | 5 | SLB + O + buffer + PPD | 0.201 |
| THY (4.0 μg/mL) | 1 | THY + R1 + R2 + buffer | 0.495 |
| | 2 | THY + R2 + R1 + buffer | 0.472 |
| | 3 | THY + buffer + R1 + R2 | 0.474 |
| | 4 | R1 + R2 + buffer + THY | 0.212 |

O: oxidizing agent, R1: sodium nitroprusside, R2: hydroxylamine hydrochloride.

$K_{st} = 3.86 \times 10^{10}\ L^2 \cdot mol^{-2}$ in case of TBT. These values confirmed the high stability of the formed indoaniline dyes. In addition, the molar absorptivity (ε) for the reaction products of SLB and TBT were $2.34 \times 10^4$ and $3.31 \times 10^4\ L \cdot mol^{-1} \cdot cm^{-1}$ and their Sandell's sensitivities were 0.0247 and 0.0166 μg/cm$^2$, respectively. These results are indicatives of the high sensitivity of the developed methods.

A comparison of the performance of the developed method and the reported spectrophotometric methods for the two cited drugs, SLB and TBT, is included in Table 2 with a main emphasis on those methods adopting a principle similar to this study. Method IA was found 3–97 times more sensitive for the determination of SLB as compared to the reported spectrophotometric methods [9–11]. Meanwhile, Method IB shows a better or comparable sensitivity and/or molar absorptivity to the reported literature for determination of TBT [15,16].

### 3.2. Method II

#### 3.2.1. Optimization of the reaction conditions

The reaction suggested by Kang and co-workers [25] for determination of phenol has been adopted in Method II for the determination of THY utilizing its phenolic nature. THY reacts with sodium nitroprusside and hydroxylamine hydrochloride in alkaline medium yielding a green-colored product with an absorption maximum at 700 nm (Fig. 1c). Different factors were adjusted to achieve the most favorable conditions for the reaction. The selection of the pH of phosphate buffer was optimized and pH 12.0 was the optimum in accordance with the previous studies on phenol [25,26]. The volume of sodium nitroprusside (0.1 M) was studied using 0.1–4.0 mL, and it was found that 0.5 mL was adequate for the highest absorbance. As well, the volume of hydroxylamine hydrochloride ($4 \times 10^{-2}$ M) was investigated using 0.1–3.0 mL and the experiments show that 0.5 mL was the optimum volume. Next, different volumes of phosphate buffer (pH 12.0) were attempted and 3.0 mL was found the optimum volume. Furthermore, the order of addition of the reactants was studied, and the obtained results showed that using the following addition order (THY + sodium nitroprusside + hydroxylamine hydrochloride + buffer) gives the highest absorbance and the best sensitivity (Table 1). This result agreed well with the previous literature [25].

Lastly, the influence of temperature and time was concomitantly studied (Fig. 2e). It was found that room temperature is optimum for the reaction proceeding and maximum stability of the reaction product. 15 min was satisfactory time for the maximum reaction yield and the highest absorbance value with high product stability (for more than 3 h) while heating caused a slight decrease in the absorbance of the reaction product which may be attributed to the decomposition of the reaction product at elevated temperatures. Thus, performing the reaction at room temperature is the best choice which also minimized the costs and work-related hazards.

After this study, the reaction was carried out under the optimized experimental conditions for the accomplishment of the best analytical

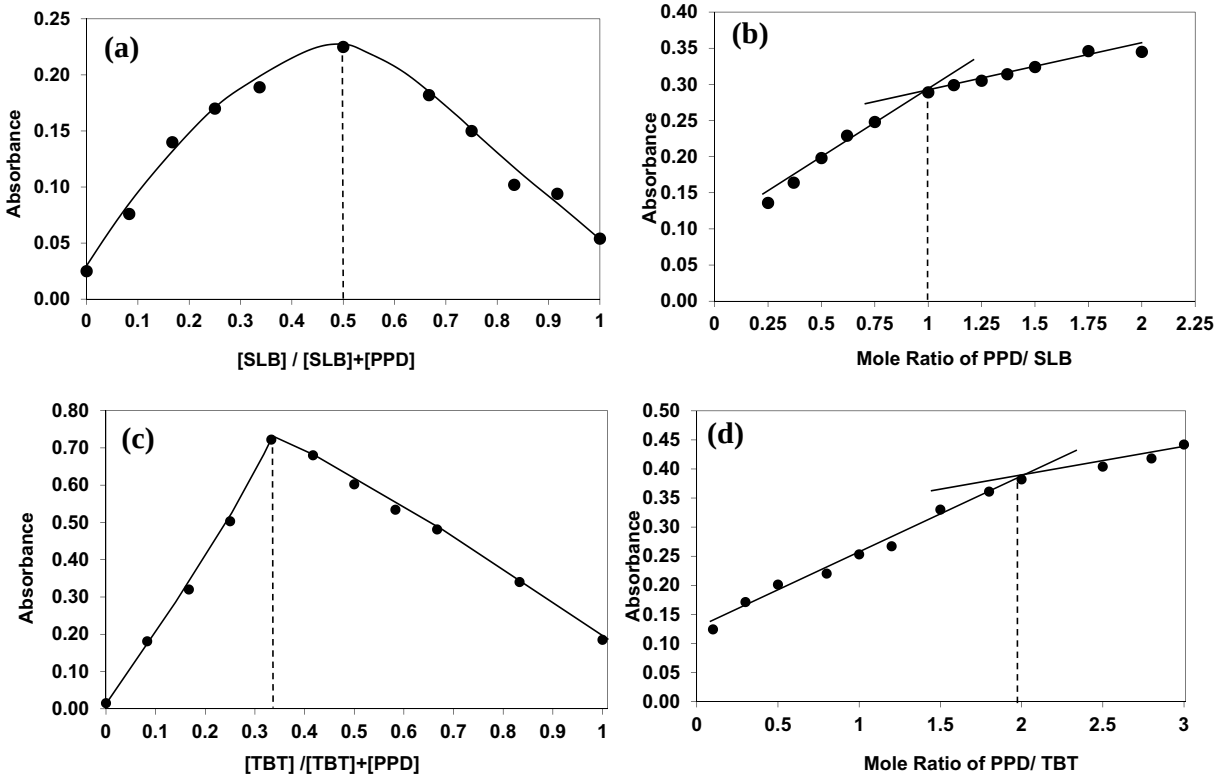
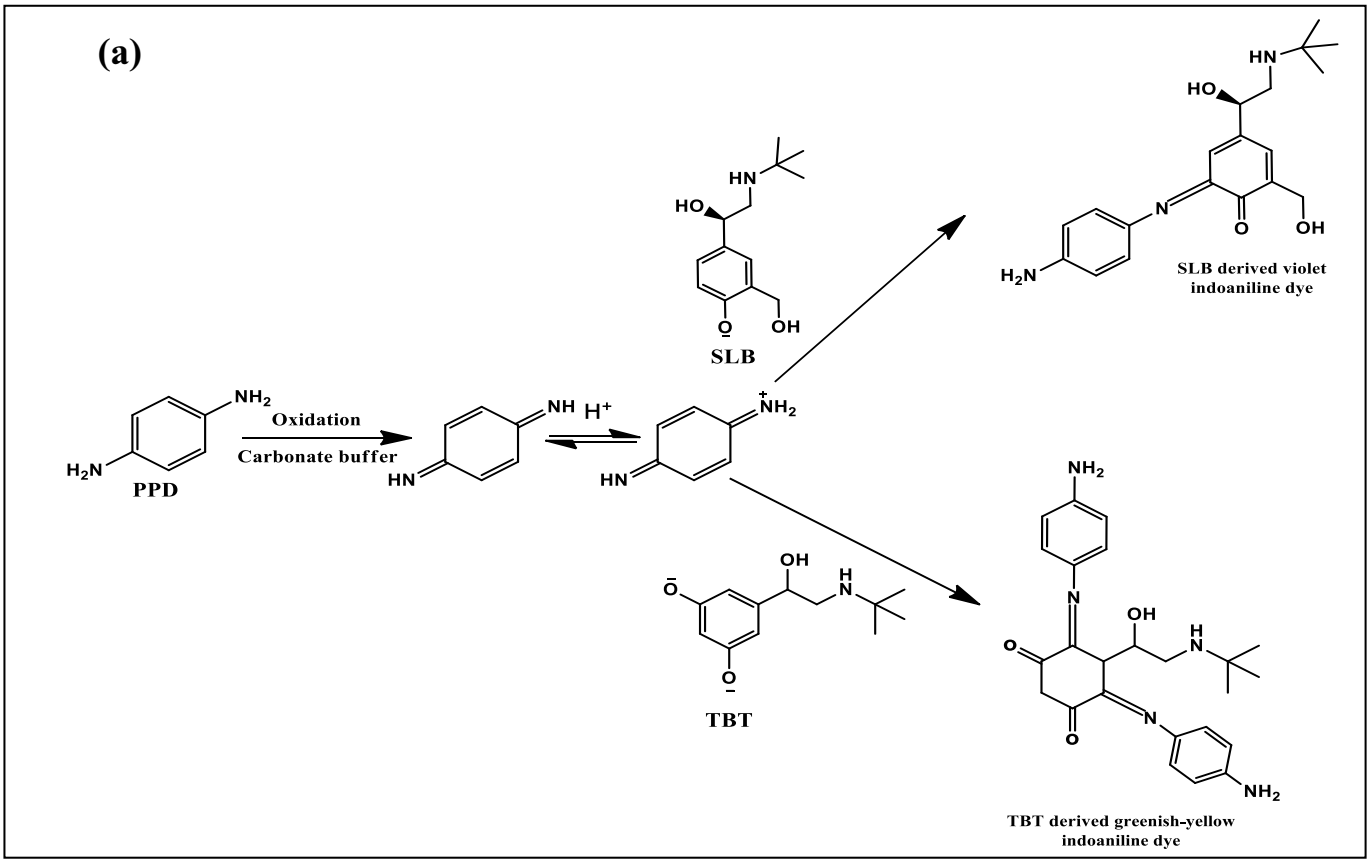



**Fig. 3.** (a) Job's plot for Method IA, (b) mole ratio plot for Method IA, (c) Job's plot for Method IB, and (d) mole ratio plot for Method IB.



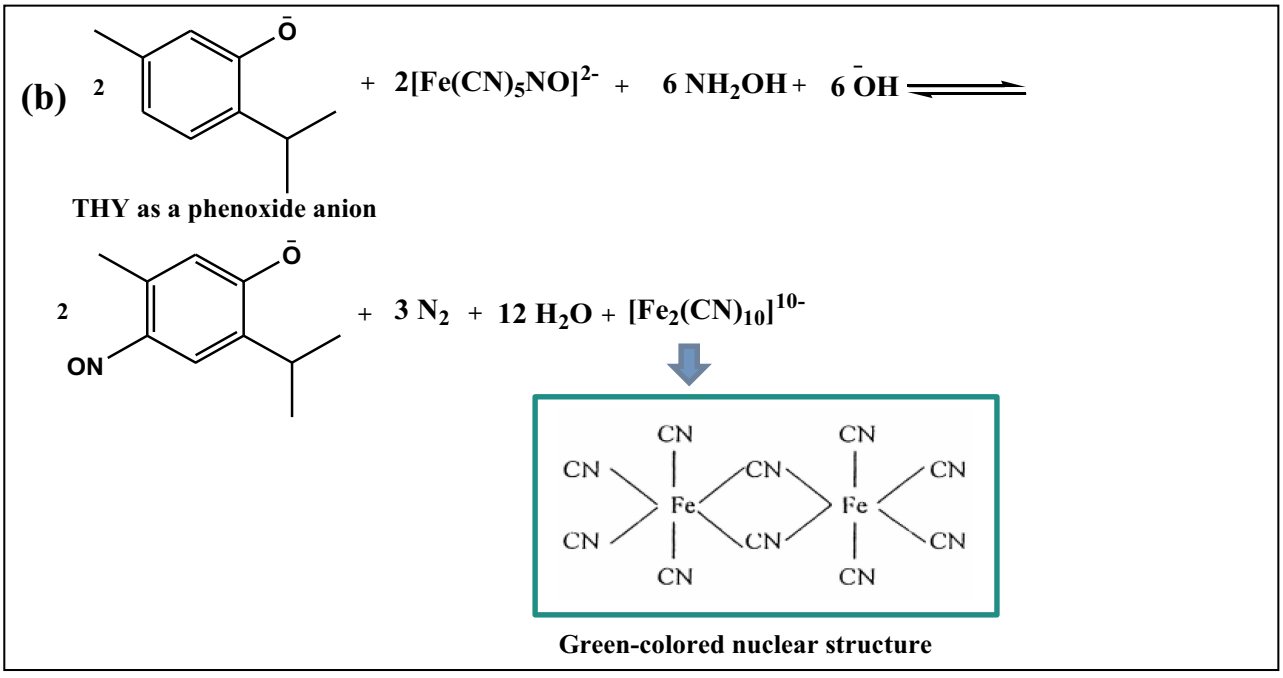




**Fig. 4.** (a) Mechanism of the oxidative coupling reactions of SLB and TBT with PPD and (b) mechanism of the reaction of THY with sodium nitroprusside and hydroxylamine hydrochloride and the nuclear structure of the colored product.

response for the quantitative determination of THY.

### 3.2.2. Mechanism of the reaction

THY exists as a phenoxide anion in the alkaline medium of the reaction. The negative charge on the oxygen strongly repels the electrons causing activation of the benzene ring to become a strong electron donor. Thus, the benzene ring is easily attacked by the nitroso ($^{+}$NO) as an electrophile. The electrophilic aromatic substitution reaction takes place preferentially at the *p*-position since there is less hindrance at this position relative to the *o*-position. The green-colored product has been

**Table 2**
Comparison of the developed methods with the published spectrophotometric methods for the three studied drugs.

| Drug | Reagent | $\lambda_{max}$ (nm) | Temperature (°C)/time (min) of the reaction | LOD (μg/mL) | Molar absorptivity (ε) ($L{\cdot}mol^{-1}{\cdot}cm^{-1}$) | Ref. no. |
|---|---|---|---|---|---|---|
| SLB | 4-Amino-5-isopropyl-1-methyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one/$KIO_3$ | 499 | Room temperature/5 | 0.4 | $9.165 \times 10^3$ | 9 |
| | 2,4-Dinitrophenylhydrazine/$NaIO_3$ | 525 | Room temperature/15 | 0.58 (batch method)<br>12.21 (FIA) | $3.61 \times 10^4$ | 10 |
| | *p*-Aminobenzoic acid/N-chlorosuccinimide | 622 | 45 °C/20 | 0.46 | $1.828 \times 10^4$ | 11 |
| | *p*-Phenylenediamine/$NaIO_4$ | 552 | Room temperature/50 | 0.126 | $2.34 \times 10^4$ | Present method |
| TBT | Amino antipyrine/$K_3Fe(CN)_6$ | 550 | Room temperature/3 | 0.0811 | $1.1905 \times 10^4$ | 15 |
| | Phenanthrol[9,10-*d*]imidazole-2-N-chloroimide | 525 | Room temperature/5 | – | $7.38 \times 10^4$ | 16 |
| | *p*-phenylenediamine/$K_3Fe(CN)_6$ | 400 | Room temperature/30 | 0.074 | $3.31 \times 10^4$ | Present method |
| THY | 4-Aminoantipyrine/$NaNO_2$/triton X-100 | 454 | Room temperature/10 | – | $1.966 \times 10^4$ | 20 |
| | *p*-Nitroaniline | 513 | Room temperature/immediate | – | $2.48 \times 10^4$ | 21 |
| | Sodium nitroprusside/hydroxylamine hydrochloride | 700 | Room temperature/15 | $6.0 \times 10^{-3}$ | $2.78 \times 10^4$ | Present method |

identified as $Na_{10}[Fe_2(CN)_{10}]$ [25,26]. The Mössbauer spectrum of this product revealed a central ferronuclear positioning thus its nuclear structure is realized by Kang et al. as illustrated in Fig. 4b [25].

The molar absorptivity and the Sandell's sensitivity of the colored product were calculated and found to be $2.78 \times 10^4$ $L{\cdot}mol^{-1}{\cdot}cm^{-1}$ and 0.0054 $\mu g/cm^2$, respectively, which clearly indicate the high sensitivity of the developed method. A comparison of the performance of the developed method and the reported spectrophotometric literature for THY [20,21] (Table 2) revealed its high sensitivity (LOD = $6.0 \times 10^{-3}$ μg/mL) and greater bathochromic shift relative to the published methods, which eliminate any potential interference, in addition to its simplicity and cost-effective by virtue of using cheap reagents available in all chemistry laboratories.

### 3.3. Validation of the analytical methods

The guidelines of the International Conference on Harmonization (ICH) [31] were obeyed for validation of the developed methods concerning linearity, range, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, and robustness.

Linear calibration graphs for Methods IA, IB, and II were obtained and the linearity data, LODs, and LOQs are shown in Table 3. Beer's-Lambert law was obeyed over wide concentration ranges of 0.8–40.0, 0.6–40.0, and 0.1–14.0 μg/mL, respectively. The accuracy and precision of the developed methods were also estimated (Table 4). The high percentage found and small values of %relative standard deviation (% RSD) indicated the high accuracy and precision of the developed methods, respectively.

As well, the robustness of the developed methods was evidenced by the significantly un-changed absorbance with the slight variations of experimental factors such as volume of PPD (3.0 ± 0.5 mL), volume of oxidizing agent (2.5 ± 0.5 mL $K_3Fe(CN)_6$ ($1 \times 10^{-3}$ M) or 2.5 ± 0.5 mL $NaIO_4$ ($1 \times 10^{-2}$ M)), reaction time (50 ± 5 or 30 ± 5 min) for Methods IA and IB, respectively. Also, Method II showed excellent robustness regarding minor changes in the volume of sodium nitroprusside (0.5 ± 0.2 mL), volume of hydroxylamine hydrochloride (0.5 ± 0.2 mL), and the reaction time (15 ± 2 min).

### 3.4. Greenness profiling of the developed methods

Greening of the analytical methods developed in chemistry laboratories becomes a major concern in the current days. This is justified by the importance of protection of the environment and the workers from potential hazards and adverse effects aroused from using harmful chemicals and solvents. Thus, it was compulsory to evaluate the status of the developed methods with regard to their agreement with the rules of green analytical chemistry. In this perspective, we applied the NEMI and the analytical eco-scale score methods for inspection of the greenness of the developed methods [27].

The NEMI method is represented by a pictogram comprising a circle divided into four slices, each one represent a criterion to be gratified by the developed methods. The four criteria are: (1) the chemicals used are not comprised in the persistent, bioaccumulative and toxic chemicals list (PBT), (2) the chemicals used are not comprised in the hazardous wastes lists, (3) using a non-corrosive pH during analysis (ranged from 2 to 12), and (4) the generated waste during the analysis is not exceeding 50 g or mL. Adopting these measures, the developed methods, IA, IB, and II, satisfied the four necessities and their pictograms show completely green-colored circles (Table 5).

As noted, the NEMI method is completely qualitative, giving no significance to the quantity of chemicals, or consumption of energy. So, we also applied the more quantitative method; analytical eco-scale score, which depends on the calculation of a score for each method represents its greenness, where the perfectly green method scored 100. A score is given to every analytical method by subtracting penalty points from 100. Such penalty points are calculated according to the

**Table 3**
Analytical performance data for the developed methods.

| Parameter | Method IA (SLB) | Method IB (TBT) | Method II (THY) |
|---|---|---|---|
| Linearity range (μg/mL)[a] | 0.8–40 | 0.6–40 | 0.1–14 |
| Regression equation[b] | X = 0.034 + 0.04 C | X = 0.069 + 0.04 C | X = 0.049 + 0.11 C |
| Correlation coefficient (r) | 0.9987 | 0.9987 | 0.9998 |
| Limit of detection (μg/mL) | 0.126 | 0.074 | $6.0 \times 10^{-3}$ |
| Limit of quantification (μg/mL) | 0.42 | 0.25 | 0.02 |
| Sandell's sensitivity (μg/cm$^2$)[c] | 0.0247 | 0.0166 | 0.0054 |
| Molar absorptivity (ε) (L·mol$^{-1}$·cm$^{-1}$) | $2.34 \times 10^4$ | $3.31 \times 10^4$ | $2.78 \times 10^4$ |

[a] Number of experiments = 10, 11, and 9 for Method IA, IB, and II, respectively.
[b] C is the concentration (μg/mL), X is the absorbance.
[c] Sandell's sensitivity is the drug concentration having an absorbance = 0.001.

**Table 4**
Accuracy and precision data for the three studied drugs in raw material by the developed methods.

| Concentration (μg/mL) | | Found (%), mean ± SD | RSD (%) | Error (%) |
|---|---|---|---|---|
| Taken | Found[a] | | | |
| SLB | | | | |
| 2.0 | 1.91 | 95.50 ± 3.62 | 3.79 | 1.48 |
| 10.0 | 10.39 | 103.90 ± 2.16 | 2.08 | 0.88 |
| 20.0 | 20.03 | 100.15 ± 2.66 | 2.66 | 1.09 |
| TBT | | | | |
| 8.0 | 7.74 | 96.75 ± 0.28 | 0.29 | 0.11 |
| 16.0 | 16.63 | 103.94 ± 0.14 | 0.13 | 0.06 |
| 24.0 | 23.61 | 98.38 ± 0.14 | 0.14 | 0.06 |
| THY | | | | |
| 1.0 | 1.03 | 103.00 ± 0.80 | 0.78 | 0.33 |
| 4.0 | 4.03 | 100.75 ± 0.90 | 0.89 | 0.37 |
| 14.0 | 13.87 | 99.07 ± 0.25 | 0.25 | 0.10 |

[a] Each result is the mean of six individual determinations.

**Table 5**
Results of evaluation of the greenness of the developed methods by the NEMI and analytical eco-scale score tools.

| **Parameter** | **Method IA** | **Method IB** | **Method II** |
|---|---|---|---|
| **1–NEMI pictogram[a]** | |  | |
| **2–Analytical eco–scale score parameters** | | **Penalty points** | |
| **A–Reagents/word sign/no of pictograms** | **Method IA** | **Method IB** | **Method II** |
| $NaIO_4$/danger/3 | 6 | – | – |
| $K_3Fe(CN)_6$/warning/1 | – | 1 | – |
| PPD/Danger/3 | 6 | 6 | – |
| Hydroxylamine HCl/warning/4 | – | – | 4 |
| Sodium nitroprusside/danger/1 | – | – | 2 |
| NaOH/danger/1 | – | – | 2 |
| $NaH_2PO_4$/warning/1 | – | – | 1 |
| HCl/danger/2 | 6 | 6 | – |
| $Na_2CO_3$/warning/1 | 1 | 1 | – |
| **B–Instruments** | 0 | 0 | 0 |
| **C–Occupational hazards** | 0 | 0 | 0 |
| **D–Waste** | 5 | 5 | 5 |
| **Total penalty points** | Σ24 | Σ19 | Σ14 |
| **Analytical eco–scale score** | 76 | 81 | 86 |



[a]W = waste, H = hazardous, PBT = persistent, bioaccumulative, and toxic chemicals, and C = corrosive.

amounts and dangers of the chemicals used, the consumption of energy, the occupational effect, and the quantity of the produced waste. If the score of the method is greater than 75, it is considered excellent green analysis, if the score is within 50–75, the method is considered acceptably green, while inadequate greenness is supposed if the score is less than 50 [27]. For the three developed methods, the scores are >75 (Table 5). The three methods can be ordered in a descending way according to the analytical eco-scale score approach as follows: Method II > Method IB > Method IA. This indicates the excellent greenness of the developed methods which offers them a further advantage for application in quality control laboratories. To the best of our knowledge, our methods are the first ones to be evaluated for their safety and greenness for the analysis of the three quoted drugs.

### 3.5. Pharmaceutical applications

The simplicity, convenience, economy, in addition to sensitivity, accuracy, greenness, and safety of the developed spectrophotometric methods encouraged their application for the determination of the three studied drugs in various pharmaceutical dosage forms including tablets, ampoules, respirator solution, and mouthwashes. Excellent percentage recoveries were obtained for the studied drugs in all of the considered dosage forms indicating the high selectivity and specificity of the developed methods since no interferences aroused from additives, excipients, or other components (Table 6). A statistical comparison study was carried out for comparing the results of the developed methods with those obtained by applying comparison methods or the standard addition method. Student *t*-test and Variance ratio F-test were used and the calculated values were < the theoretical values for all dosage forms [32]. This outcome confirmed that no significant difference exists between the developed methods and the comparison methods and pointing to their agreement regarding accuracy and precision.

## 4. Conclusions

Simple, sensitive, convenient, and green spectrophotometric methods were developed and validated for the determination of SLB, TBT, and THY in pure states and in various pharmaceutical formulations. These methods adopt oxidative coupling and electrophilic aromatic substitution reactions with PPD/oxidizing agent and sodium nitroprusside/hydroxylamine systems, respectively. Wide linearity ranges with excellent sensitivity were successfully accomplished for the three compounds. Pharmaceutical formulations including tablets, injections, respirator solution, and mouthwash were effectively assayed by the developed methods with good accuracy and precision. In addition, a greenness profiling study of the developed methods was accomplished by applying the NEMI and the analytical eco-scale score tactics. These two tactics confirmed the excellent greenness of the developed methods. In conclusion, the superb greenness, as well as the good

**Table 6**
Determination of the three studied drugs in different pharmaceutical preparations by the developed methods and comparison or standard addition methods.

| Pharmaceutical formulations | Proposed methods (n = 3) | | Comparison or standard addition method (n = 3) | t[d] | F[d] |
|---|---|---|---|---|---|
| | Average drug content ± SD | Average %found ± SD | Average %found ± SD | | |
| Butadin tablets (2 mg SLB/tablet) | 2.03 ± 0.04 mg SLB/tablet | 101.71 ± 2.03 | 101.30 ± 1.79[a] | 0.262 | 1.286 |
| Salbutamol injection (0.5 mg SLB/mL) | 0.51 ± 0.01 mg SLB/mL | 102.77 ± 1.45 | 101.61 ± 2.08[b] | 0.792 | 2.058 |
| Ventoline respirator solution (5 mg SLB/mL) | 5.10 ± 0.11 mg SLB/mL | 102.17 ± 2.28 | 101.98 ± 1.67[b] | 0.116 | 1.864 |
| Samabutaline tablets (5 mg TBT/tablet) | 5.10 ± 0.02 mg TBT/tablet | 101.98 ± 0.45 | 102.80 ± 1.40[b] | 0.966 | 9.679 |
| Ataline tablets (2.5 mg TBT/tablet) | 2.51 ± 0.06 mg TBT/tablet | 100.44 ± 2.34 | 98.4 ± 2.00[b] | 1.148 | 1.369 |
| Mentoral mouth wash (0.063%w/v THY) | 0.062 ± 0.001%w/v THY | 98.75 ± 1.92 | 98.48 ± 1.28[c] | 0.203 | 2.25 |
| Lastarine mouth wash (0.06%w/v THY) | 0.061 ± 0.001%w/v THY | 101.83 ± 0.94 | 100.86 ± 0.64[c] | 1.477 | 2.157 |

[a] These results were obtained using the manufacturer method (S.D.I., Samarra, Iraq).
[b] These results were obtained using the standard addition method.
[c] These results were obtained using a comparison method [20].
[d] Tabulated *t* and *F* values are 2.776 and 19.00, respectively, at $P = 0.05$ [32].

validation parameters in addition to the simplicity and cost-effectiveness of the developed methods, qualified them for quality control of the three drugs.

## Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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