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Application of 2-acetylbutyrolactone to spectrofluorimetry: Fluorescence properties of Schiff bases derived from 2-acetylbutyrolactone and spectrofluorimetric determination of primary amine-containing compounds

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Abstract

2-Acetylbutyrolactone (ABL) has been characterized for use as a fluorogenic reagent for the spectrofluorimetric determination of primary amines. The reagent forms strongly fluorescent Schiff bases upon the reaction with primary amines in acid-catalized aqueous solutions or in dimethylformamide (DMF). Sulfamethoxazole (SMX) and ampicillin sodium (AMP Na) were used as model amines of type $ArNH_2$ and RNH_2, respectively. The reaction conditions, fluorescence spectral properties and the stability of the derivatives have been investigated. The chemistry and the pathway of the reaction have been discussed. Calibration data, accuracy, precision, limits of detection, limits of quantification and other aspects of analytical merit were presented in the text. The utility of ABL for the analysis of the model drugs in pharmaceutical preparations was demonstrated. The results indicated that the proposed methods are equally accurate and precise as the official or other reported methods.

Keywords: 2-Acetylbutyrolactone; Schiff bases; Spectrofluorimetry

1. Introduction

2-Acetylbutyrolactone (ABL) (α-acetyl-γ-butyrolactone or 4,5-dihydro-3-acetylfuran-2(3H)-one) is a useful reagent for the synthesis of many *N*-heterocycles [1,2]. The condensation reactions (i.e. Schiff base formation reactions) of ABL with aniline [3] or 4-fluoro aniline [4] to form 2-(1-phenylimino)ethyl-γ-butyrolactone [3] and 3-[1-(4-fluoro-phenylamino)ethylidene]dihydro-2(3H)-furanone [4], respectively have been reported. The reaction was based on the reactivity of carbonyl moiety (α-acetyl) of ABL. Primary amines, compounds of the type RNH_2 or $ArNH_2$, react with the carbonyl group of an aldehyde or ketone to form *N*-alkyl- or *N*-aryl-substituted imine through nucleophilic addition [5]. Evaluation of ABL as an analytical reagent in the area of pharmaceutical analysis through Schiff base formation reaction is the main subject of this article.

In the current work, the inherent fluorescence characteristics of ABL (a furanone derivative) and of Schiff bases derived from ABL reaction with some amines of type RNH_2 or $ArNH_2$ were studied. Based on these results, ABL is proposed as a fluorogenic agent for primary amines. Consequently, the condensation reaction conditions were optimized to be adopted for use in quantitative spectrofluorimetric methods for the determination of primary amines.

Sulfamethoxazole (SMX) and ampicillin sodium (AMP Na) were used in this study as models of aryl and aliphatic amines, respectively. Sulfamethoxazole, a member of the sulfonamides, is an antimicrobial agent used principally in the treatment and prophylaxis of bacterial infections [6]. Ampicillin is a β-lactam antibiotic. It is bactericidal by virtue of its inhibitory action on the synthesis of the bacterial cell wall [6].

Ampicillin and SMX are official in the USP 2004 [7] and BP 2003 [8]. The pharmacopoeias' monographs specify titrimetric, spectrophotometric and high-performance liquid chromatographic assays for their determination.

Different analytical techniques have been reported for the assay of SMX in pharmaceutical dosage forms (single or in combinations). These include high-performance liquid

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chromatography [9], spectrophotometry [10–15], fluorimetry [16–19], flow injection analysis [20,21], capillary electrophoresis [22] and polarography [23].

Ampicillin is a member of α-amino acyl β-lactam antibiotics having *N*-phenyl glycyl moiety and is known to be a very weakly UV absorbing compound. It is unstable in aqueous solutions at both acidic and alkaline pH [24]. Determination of the degradation products has been possible through fluorimetric [25], spectrophotometric [26], polarographic [27] and high-performance liquid chromatographic [28] methods.

Other reported methods for assaying AMP include spectrophotometry [29–32], fluorimetry [33], high-performance liquid chromatography [34,35], spectrodensitometry [36], polarography [37] and capillary electrophoresis [38,39].

The present work describes the optimization of the condensation reaction conditions of the examined reagent, ABL, with the model drugs in aqueous acidic solution (SMX) or in DMF (AMP Na) and adaptation for fluorimetric determination. The applicability of the reaction was assessed through analysis of the cited drugs in their dosage forms.

2. Experimental

2.1. Apparatus

Fluorescence spectra and measurements were taken on a Perkin-Elmer 650-10S spectrofluorimeter, equipped with 1-cm quartz cell and 150 W Xenon arc lamp.

2.2. Reagents and standard/assay solutions

2.2.1. 2-Acetylbutyrolactone solution

Eight percent and 2% (v/v) ABL (EGA Chemie, Germany) solutions were prepared in 0.35 M *o*-phosphoric acid and in DMF, respectively. The reagent solution should be freshly prepared daily.

2.2.2. Preparation of standard solution

A standard solution of SMX containing 200.0 μg ml^{-1} was prepared in ethanol and kept in a refrigerator. The solution was stable at least for two weeks. This stock solution was further diluted with ethanol to obtain the standard working solution, 4.0 μg ml^{-1}.

A standard solution of AMP Na containing 500.0 μg ml^{-1} was prepared in DMF and kept in a refrigerator. The standard solution should be freshly prepared daily. This stock solution was further diluted with DMF to obtain the standard working solution, 50.0 μg ml^{-1}.

2.2.3. Preparation of sulfamethoxazole tablets assay solutions

A total of 20 tablets were weighed and finely powdered. A portion of the powder, equivalent to 20 mg of SMX, was accurately weighed and about 50 ml of ethanol was added. Sulfamethoxazole was dissolved using ultrasonic bath and the mixture was filtered into a 100-ml volumetric flask and diluted to volume with ethanol. Further dilutions were made with ethanol to obtain a solution containing 4.0 μg ml^{-1} SMX.

2.2.4. Preparation of sulfamethoxazole suspensions assay solutions

An accurately measured volume of oral suspension, equivalent to10 mg of SMX, was transferred to a 50-ml volumetric flask with the aid of about 30 ml of ethanol. Sulfamethoxazole was dissolved using ultrasonic bath for about 20 min. The mixture was diluted to volume with ethanol and centrifuged. Further dilutions of the supernatant were made with ethanol to obtain a solution containing 4.0 μg ml^{-1} SMX.

2.2.5. Preparation of ampicillin capsules assay solutions

A quantity of the mixed contents of 20 capsules, equivalent to 1 g of AMP Na, was mixed with sufficient DMF to produce 100 ml, shaken for 30 min and filtered. Dilution of the filtrate was made with DMF to obtain a solution containing 50.0 μg ml^{-1} AMP Na (or equivalent).

2.2.6. Preparation of ampicillin oral suspension and ampicillin/sulbactam vials assay solutions

The content of AMP Na/sulbactam Na-container for injection (or AMP oral suspension) was constituted with 20 ml of DMF (100 ml for AMP oral suspension) and centrifuged. Further dilutions of the supernatant liquid were made with DMF to obtain a solution containing 50.0 μg ml^{-1} AMP Na (or equivalent).

2.3. Analytical procedure for calibration graphs

Into a set of glass tubes, aliquots of standard working/assay solution (to give final concentration ranges specified for fluorimetric measurements, listed in Table 1) were mixed with 0.5 ml portions of ABL, 8% (v/v) solution in 0.35 M *o*-phosphoric acid solution (SMX–ABL reaction) or 0.2 ml portions of ABL, 2% (v/v) solution in DMF (AMP Na–ABL reaction). The reaction mixtures were heated in a boiling water bath for 30 min, allowed to equilibrate to room temperature and then diluted to a final

Table 1
Analytical features for the determination of sulfamethoxazole (SMX) and ampicillin sodium (AMP Na)

Parameter	Drug	
	SMX	AMP Na
Measurement, $\lambda_{ex}/\lambda_{em}$ (nm)	355/435	355/440
Concentration range (μg ml^{-1})	0.04–0.16	0.2–3.2
Regression equations		
Intercept (*a*)	−0.1467	−0.2060
Slope (*b*)	217.33	17.97
Correlation coefficient (*r*)	0.9988	0.9994
S_a	0.7365	0.4193
S_b	7.0553	0.2145
LOD (μg ml^{-1})	0.009	0.03
LOQ (μg ml^{-1})	0.03	0.1

S_a: standard deviation of intercept; S_b: standard deviation of slope; LOD: limit of detection; LOQ: limit of quantification.

volume of 10 ml with methanol (SMX–ABL reaction) or Britton Robinson buffer of pH 10.5 (AMP Na–ABL reaction). The fluorescence intensities were measured at the specified wavelengths (Table 1) using reagent blank.

3. Results and discussion

3.1. Chemistry and fluorescence characteristics

3.1.1. Chemistry of 2-acetylbutyrolactone Schiff bases

The condensation reaction of aniline with ABL (reflux in ethanol for 5 h [3]) involves the primary amino group of aniline and the reactive carbonyl group (α-acetyl) of ABL. The reaction product was identified as 2-(1-phenylimino)ethyl-γ-butyrolactone (Fig. 1A, I), imino compound. The reaction of 4-fluoroaniline with ABL (reflux in toluene for 24 h [4]) resulted in the enamine form, 3-[1-(4-fluoro-phenylamino)ethylidene]dihydro-2(3H)-furanone (Fig. 1A, II).

Some Schiff base formation reactions have been carried out in acid-catalized aqueous/ethanolic solutions [40]. The acid favors the carbonium ion formation (of the reactive carbonyl group) with subsequent attack of amine to form the intermediate carbinolamine.

In the present work, the reaction of ABL with amino compounds was suggested to be carried out in acid-catalized aqueous solution and in organic solvent, DMF.

3.1.2. Fluorescence characteristics of 2-acetylbutyrolactone

Preliminary investigations indicated a very weak inherent fluorescence emission of ABL. Given that ABL–primary amine reaction was suggested to be carried out in aqueous and in organic solvent solutions, the fluorescence spectral characteristics of ABL, similarly treated, were considered accordingly. Table 2 presents the excitation and emission wavelengths, in different diluting solvents (DMF, aqueous acidic solution (0.017 M *o*-phosphoric acid) and Britton Robinson buffer solution of pH 10.5) for two sets of ABL, one being heated in DMF and the other in acid catalized aqueous solution (0.35 M *o*-phosphoric acid) for 30 min. Based on the spectral data, three aspects should be considered. First, for ABL heated in DMF solution, the slight basic character of DMF facilitates the abstraction of the acidic active proton at α-carbon and as a consequence, carbanion formation is favored. The latter attains a stable feature through electron delocalisation, which leads to enol form (Fig. 1B). On the other hand, for ABL heated in aqueous acidic solution, the proton catalizes the formation of the carbonium ion (of β-keto group) (Fig. 1C). Second, for ABL heated in DMF, the excitation wavelengths of spectra in DMF and in alkaline buffer solution are the same. Such a finding agrees with the postulate of carbanion formation and the favored enol form in DMF as in aqueous alkaline solution. Meanwhile, for ABL heated in aqueous acid, spectra in DMF and alkaline solution do not show this feature. Third, the bathochromic shift in the excitation wavelength of ABL in alkaline buffer solution relative to that in acidic solution can be attributed to the favored enol form.

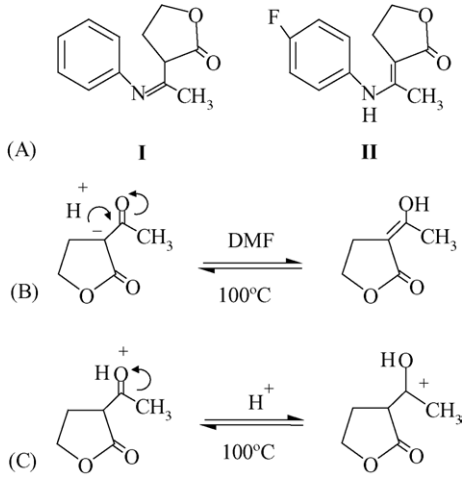


Fig. 1. (A) 2-(1-Phenylimino)ethyl-γ-butyrolactone (I) and 3-[1-(4-fluoro-phenylamino)ethylidene]dihydro-2(3H)-furanone (II). (B and C) Structural proposal of ABL in DMF and in aqueous acidic solution, respectively.

3.1.3. Fluorescence characteristics of 2-acetylbutyrolactone Schiff bases

Pilot experiments were carried out on SMX, as a model of arylamine drug. Two aliquots of 200 μg ml^{-1} SMX standard solution in ethanol were mixed with 0.5 ml of ABL, 8% (v/v) solution in 0.35 M *o*-phosphoric acid. The reaction mixtures were heated in a boiling water bath for 30 min. One reaction mixture was made to volume with methanol and the other with DMF. Similar experiment was carried using 200 μg ml^{-1} SMX standard solution in DMF and 0.2 ml of ABL, 2% (v/v) solution in DMF. The fluorescence spectra were taken for all solutions. The fluorescence properties of 3-[1-(4-fluoro-phenylamino)ethylidene]dihydro-2(3H)-furanone [4] were considered. The study showed the following facts; first, the reactions in aqueous acidic and in DMF solutions resulted in products of different fluorescence excitation and emission wavelengths (Table 2), i.e. non-identical products. Second, the $\lambda_{ex}/\lambda_{em}$ of spectra in DMF coincide exactly with those of 3-[1-(4-fluoro phenylamino)ethylidene]dihydro-2(3H)-furanone, the reference substance. From these observations, it could be concluded that the reaction that occurs in DMF results in a Schiff base product of the enamine type while in acid-catalized aqueous solution, a Schiff base of the imine form is likely to be formed.

The previous study on SMX indicated that the relative fluorescence intensity of SMX-Schiff of imine form (reaction in acid catalized-aqueous solution) is about 14-fold higher than that of the enamine form (reaction in DMF). Accordingly, the reaction of ABL with primary amines in acid-catalized aqueous solutions is the most favorable option. The reactivity of the ABL–amine reaction in DMF is expected to be suppressed by the favored carbanion formation at the α-carbon. However, the reaction in DMF is still the alternative of choice in the case of analytes, which are liable to a significant degradation in acid solution, especially those which concern the amino group.

It has been reported that in aqueous acidic solutions of AMP, kept at 100 °C, the α-amino group of the acyl side chain shares in the internal Schiff base formation with the carbonyl group of

Table 2
Fluorescence properties of 2-acetyl butyrolactone and Schiff bases

Fluorescence properties of ABL			
Condition	$\lambda_{ex}/\lambda_{em}$ (nm)/diluting solvent		
	DMF	Britton Robinson buffer, pH 10.5	Aqueous acidic solution
ABL heated in 0.35 M o-phosphoric acid	360/448	370/460	350/450
ABL heated in DMF	375/450	375/460	350/450
Fluorescence properties of Schiff bases			
Material	$\lambda_{ex}/\lambda_{em}$ (nm)		
	DMF		Methanol
3-[1-(4-Fluoro-phenylamino)ethylidene]dihydro-2(3H)-furanone[a]	375/448		372/448
Reaction in acid-catalized aqueous solution			
SMX	355/425		355/435
Reaction in DMF			
SMX	380/445		375/445

RFI: relative fluorescence intensity; DMF: dimethylformamide.
[a] Reference substance, Ref. [4].

penilloaldehyde [41]. Accordingly, the condensation reaction with ABL was suggested to be carried out in DMF.

3.1.4. Evaluation of 2-acetylbutyrolactone as a fluorogenic reagent

The fluorescence properties of imino compounds formed as a result of the reaction of ABL with some selected amino drugs (in accordance with the procedure described for SMX Section 2.3) in acid-catalized aqueous solutions were recorded (Table 3). The results of this study show to a promising analytical sensitivity. Weakly absorbing aliphatic amines gave weak fluorescence emission. With amantadine and trimethoprim (TMP), no fluorescence was detected up to a concentration level of 10 μg ml^{-1}. It should be noted that the primary amino group of TMP (diaminopyrimidine derivative) is considered to be aliphatic in its reaction [18]. This apparent un-reactivity toward ABL seems to be advantageous in selective determination of sulfa drugs co-formulated with TMP.

3.1.5. Reaction pathways and fluorescence spectra of sulfamethoxazole/2-acetylbutyrolactone and ampicillin sodium/2-acetylbutyrolactone modeles

Scheme 1 displays the reaction pathway of SMX (I) with ABL (II) to form 2-[1-(4-substituted phenylimino)ethyl]-γ-butyrolactone (III), the imine form of the Schiff base. The latter is a yellow fluorescent compound. The fluorophore exhibits maximum excitation and emission wavelengths at 355 and 435 nm, respectively, in methanol (Fig. 2A).

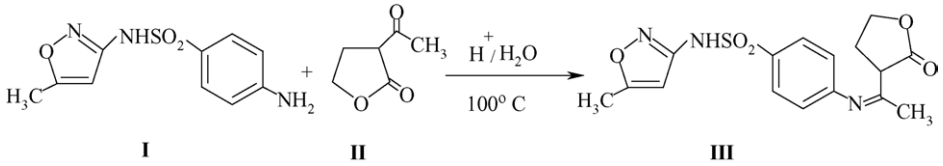
Scheme 2(A) presents the reaction pathway of AMP (I) with ABL (II). The α-amino group of acyl side chain of AMP (I) reacts with the reactive carbonyl group of ABL (II) to form the corresponding Schiff base, of the enamine tautomer type (III). The structural rearrangement to the enamine tautomer can be illustrated by electron delocalization and migration of the active labile proton (at the α-carbon) (B). This occurs as a consequence of the slightly basic character of DMF, which facilitates carbanion formation upon heating. Dilution of the reaction solution with Britton Robinson buffer solution of pH 10.5 is most probably accompanied by β-lactam ring opening (IV) [24]. The latter (IV) is a yellow fluorescent compound, which exhibits maximum excitation and emission wavelengths at 355 and 440 nm, respectively (Fig. 2B).

3.2. Optimization of the reactions conditions

3.2.1. Study of different factors affecting 2-acetylbutyrolactone-primary amine reaction

The influence of some variables on the reaction sequence was studied to establish the most favorable conditions to achieve maximum analytical sensitivity. It should be noted that the first preliminary experiments indicated that 100 °C is just adequate to activate the reaction. Lower temperatures do not meet the rapidity requirement. So, all the studies were carried out at 100 °C.

3.2.1.1. Time course study. A set of reaction solutions was prepared (aliquots of 4.0 μg ml^{-1} SMX standard working solution



Scheme 1.

Table 3
Evaluation of 2-acetylbutyrolactone as fluorogenic reagent

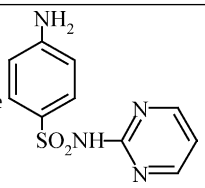
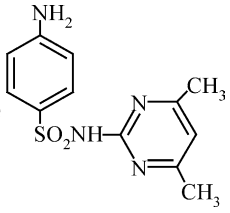
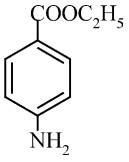
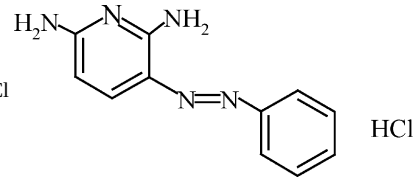
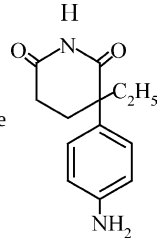
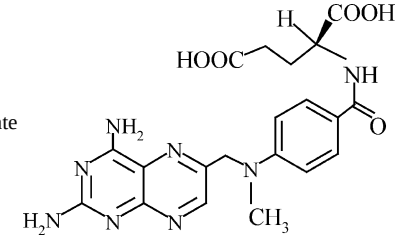
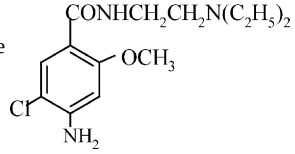
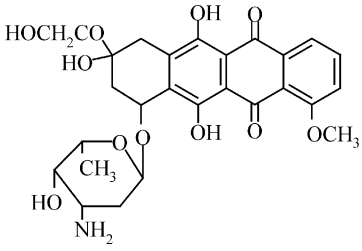
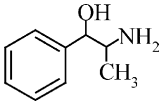
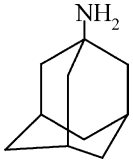
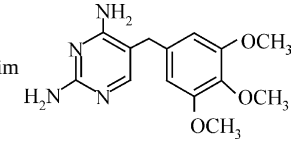
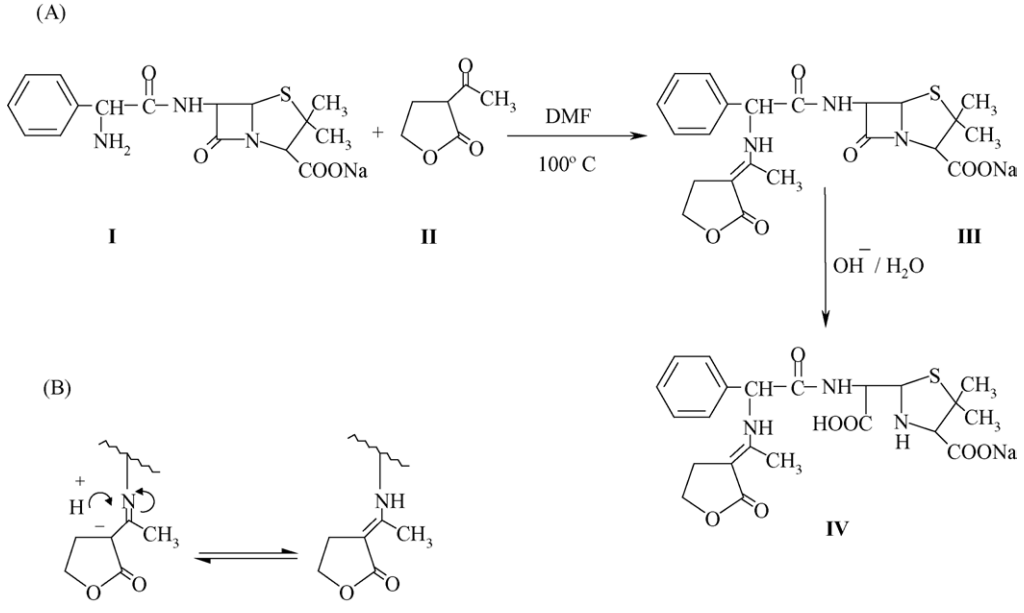
Drug	$\lambda_{ex}/\lambda_{em}$	Diluting solvent	RFI	Concentration examined ($\mu g\,ml^{-1}$)
Sulfadiazine 	355/440	Water	80	2
Sulfadimidine 	355/440	Water	80	2
	355/435	Methanol	160	2
Benzocaine 	355/440	Water	80	2
	355/435	Methanol	240	2
Phenazopyridine HCl 	350/400	Water	100	1
	365/430	Methanol	140	1
Aminoglutethimide 	350/455	Water	18	2
Methotrexate 	350/480	Water	36	2
Metoclopramide 	350/435	Methanol	35	20
	345/440	Water	16	20

Table 3 (*Continued*)

Drug	$\lambda_{ex}/\lambda_{em}$	Diluting solvent	RFI	Concentration examined ($\mu g\,ml^{-1}$)
Doxorubicin 	355/440	Water	100	2
Norpsuedoephedrine 	345/435	Methanol	5	20
Amantadine 	–	Methanol	–	10
Trimethoprim 	–	Methanol	–	10

RFI: relative fluorescence intensity.



Scheme 2.

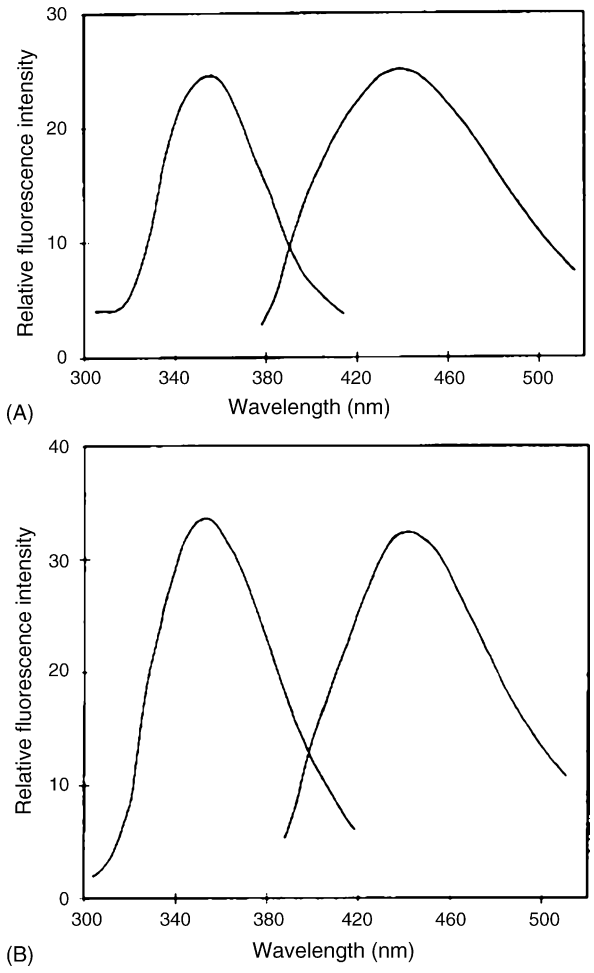


Fig. 2. Excitation and emission spectra of (A) SMX (0.12 μg ml^{-1}) and (B) AMP Na (1.5 μg ml^{-1}) fluorophores in methanol and Britton Robinson buffer of pH 10.5, respectively.

were mixed with 0.5 ml of ABL solution, 4% (v/v) in 1.7 M *o*-phosphoric acid, and aliquots of 50.0 μg ml^{-1} AMP Na standard working solution were mixed with 0.2 ml of ABL solution, 2% (v/v) in DMF). The solutions were heated at 100 °C. At suitable time intervals, a reaction solution was made up to 10 ml with distilled water (SMX reaction) or Britton Robinson buffer of pH 10.5 (AMP Na reaction) and the fluorescence was measured at the specified wavelengths (Table 4). Fig. 3A displays the data in term of relative fluorescence intensity–time profile for SMX (curve 1) and AMP Na (curve 2). In either, the fluorescence values reached a plateau close to 30 min.

3.2.1.2. Effect 2-acetylbutyrolactone concentration. The effect of ABL concentration (0.5 ml taken for reaction solution), prepared in 1.7 M *o*-phosphoric acid within the range from 1 to 12% (v/v), on the resulting SMX reaction was studied (Fig. 3B, curve 1). The maximum fluorescence intensity was obtained using ABL solution within the range, 8–12% (v/v). By analogy, an identical investigation on the AMP Na–ABL reaction (Fig. 3B, curve 2) indicated that a 2–4% (v/v) ABL solution prepared in DMF is adequately appropriate (0.2 ml taken for reaction solution).

3.2.1.3. Effect of o-phosphoric acid strength on 2-acetylbutyrolactone-sulfamethoxazole reaction. Sulfamethoxazole (2 μg ml^{-1}) was allowed to react with 0.5 ml of ABL, 4% (v/v) in *o*-phosphoric acid, varying the strength of the acid from 0.07 to 1.7 M. The fluorescence intensity at 440 nm (λ_{ex} = 355 nm) was measured as a function of *o*-phosphoric acid strength. The results showed that the fluorescence intensity increases gradually as the acid strength decreases, reaching a maximum around 0.17–0.35 M (Fig. 3C).

3.2.2. Factors affecting the fluorescence intensity of the formed fluorophore

3.2.2.1. Effect of pH. A set of reaction solutions of AMP Na–ABL was prepared and the reaction mixtures were made up to 10 ml with Britton Robinson buffer solutions of different pH values. The results are shown in Fig. 4A. The fluorophore attains maximum fluorescence intensity at an alkaline pH of 10–11. On the contrary, SMX's fluorophore exhibits a reverse behavior, as the highest fluorescence intensity was obtained in acidic solution.

3.2.2.2. Effect of organic solvents. To obtain a better fluorescence enhancement, a study of the effects of organic solvents

Table 4
Effect of aqueous and organic solvents on the fluorescence intensity of the investigated Schiff bases

Solvent	SMX–ABL fluorophore[a]		AMP Na–ABL fluorophore[b]	
	$\lambda_{ex}/\lambda_{em}$ (nm)	RFI	$\lambda_{ex}/\lambda_{em}$ (nm)	RFI
Aqueous	355/440	80[c]	355/435	35[d]
Ethanol	355/435	140	368/440	20
Methanol	355/430	200	365/440	19
Acetonitrile	355/428	110	370/440	22
DMF	355/425	90	380/445	23
Dimethylsulfoxide	360/425	108	380/445	22
Dioxane	355/430	125	370/435	29

RFI: relative fluorescence intensity.
[a] 2 μg ml^{-1} SMX, 4% (v/v) ABL in 0.35 *o*-phosphoric acid, 30 min reaction time at 100 °C.
[b] 2 μg ml^{-1} AMP Na, 2% (v/v) ABL in DMF, 30 min reaction time at 100 °C.
[c] Fluorescence intensity measured in aqueous acidic solution.
[d] Fluorescence intensity measured in aqueous alkaline solution.

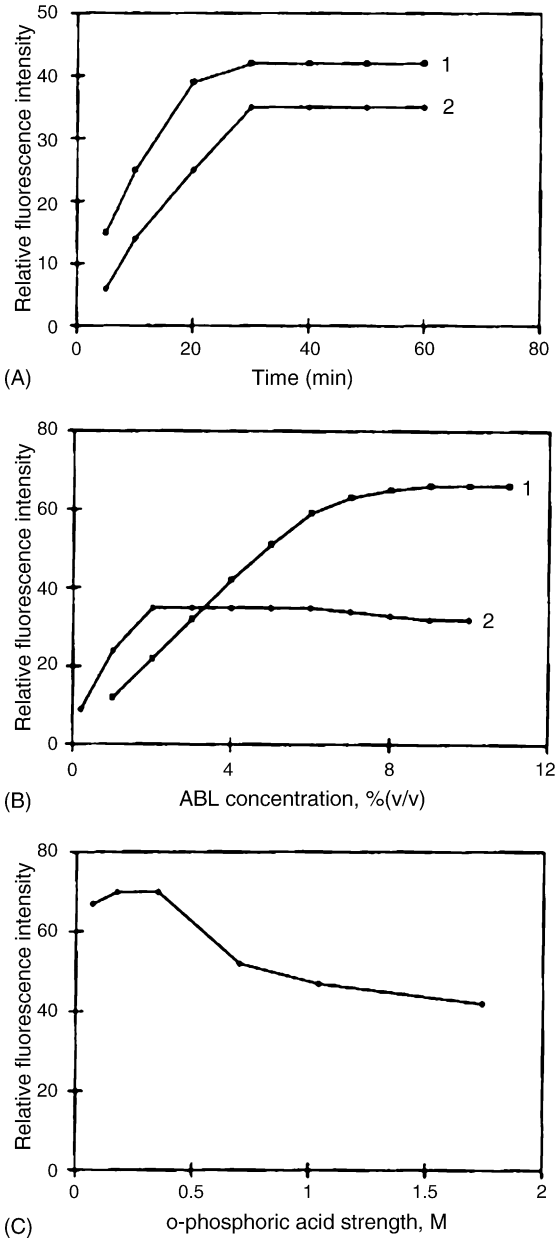


Fig. 3. (A) Time courses of (1) SMX and (2) AMP Na reactions with ABL in acid catalized aqueous solution and in DMF, respectively. (B) Effect of ABL concentration on (1) SMX and (2) AMP Na reactions. (C) Effect of *o*-phosphoric acid strength on SMX reaction. In (A–C), the drug concentration is 2 μg ml^{-1}.

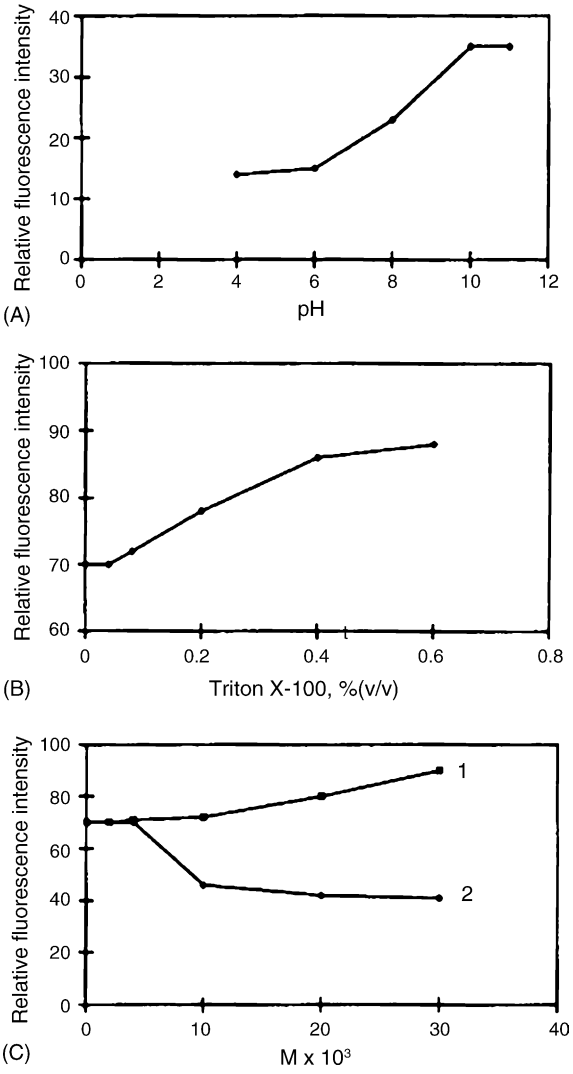


Fig. 4. (A) Effect of pH of Britton Robinson buffer on the fluorescence intensity of AMP Na fluorophore. (B) Effect of Triton X-100 concentration on the fluorescence intensity of SMX fluorophore in aqueous solution. (C) Effect of (1) BDDAB and (2) SDS concentrations on the fluorescence intensity of SMX fluorophore in aqueous solutions. In (A–C), the drug concentration is 2 μg ml^{-1}.

on fluorescence intensity and wavelengths of excitation and emission was performed. The results are presented in Table 4. It seems that no definite pattern could correlate between the polarity of the solvents investigated and the fluorescence properties. However, methanol and dioxane offered maximum fluorescence intensity for SMX and AMP Na fluorophores, respectively.

3.2.2.3. Effect of surfactants. In order to obtain better analytical characteristics for the determination of SMX, a study on the effect of cationic (benzyl dimethyl dodecyl ammonium bromide, BDDAB), anionic (sodium dodecyl sulfate, SDS) and non-ionic (Triton X-100) surfactants on the fluorescence intensity of SMX's Schiff base, in aqueous acidic solution, was carried out. A set of reaction solutions of SMX, to obtain a final concentration of 2 μg ml^{-1} were prepared and increasing volumes of 2% (v/v) Triton X-100, 1×10^{-1} mol l^{-1} SDS or 1×10^{-1} mol l^{-1} BDDAB solutions and distilled water to give a final volume of 10 ml, were added. The fluorescence intensities were measured for all the solutions at 355 nm/440 nm. A concentration of Triton X-100 of 0.6% (v/v) (Fig. 4B) or BDDAB of 3×10^{-2} mol l^{-1} (Fig. 4C, curve 1) provides ~1.3- and 1.2-fold sensitivity enhancement, respectively. About 43% quenching of the fluorescence signal occurred in 2×10^{-2} mol l^{-1} M SDS acidic reaction solution (Fig. 4C, curve 2). At a concentration level lower than 0.1% (v/v) Triton X-100, 1×10^{-2} mol l^{-1} BDDAB or 4×10^{-3} mol l^{-1} SDS no notable effect on the fluorescence intensity was observed. The small improvement in the fluorescence intensity may be a result of shielding of the Schiff base fluorophore from vibrational quenching by increased local

viscosity about the fluorophore–micelle binding sites. However, it seems that this factor is not so effective as the deactivation modes.

A similar study was performed on fluorescence intensity of AMP Na fluorophore in alkaline buffer solution. Within the concentration range of 0.04–0.4% (v/v) of Triton X-100, 2×10^{-3} to 2×10^{-2} mol l^{-1} of BDDAB and 2×10^{-3} to 2×10^{-2} mol l^{-1} of SDS, no effect was observed.

3.3. Specificity and interference study with the determination of sulfamethoxazole and ampicillin sodium

Because of the reactive carbonyl moiety (α-acetyl), ABL reacts specifically with primary amines to form yellow fluorescent Schiff bases. Accordingly, selective determination of the model drugs in presence of co-formulated drugs should be possible. Trimethoprim which is commonly formulated with SMX (co-trimoxazole combinations) was found not to interfere. Although TMP having two primary amino groups, it showed no reactivity towards ABL at least under the reaction conditions specified for the present work. Sulbactam (penicillanic acid sulphone), flucloxacillin and cloxacillin (isoxazolyl penicillins) are commonly formulated with the AMP. Such drugs have no phenyl glycyl moiety and obviously they do not interfere with the AMP Na–ABL reaction, i.e. they show no reactivity towards ABL.

To check for the independence of the measured fluorescence of the intended analyte on the co-formulated drug, five replicate determinations were carried out on synthetic mixtures (Table 5). The precision (in term of R.S.D.%) and accuracy (in term of S.A.E.) of the method were assessed through the statistical analysis of the data (Table 5). The results obtained show the successful application of the proposed methods for the determination of SMX and AMP Na in combined preparations.

The interference of impurities of closely related substances, like sulfanilic acid, sulfanilamide, 6-aminopenicillanic acid, 2-phenylglycine and penicilloic acid which commonly found in commercial samples is however, possible.

It was shown that excipients and diluents such as starch, talc, magnesium stearate and microcrystalline cellulose, which are commonly formulated in tablets and capsules, do not interfere with the proposed methods.

3.4. Stability

The stability of the reactions solutions of SMX and AMP Na fluorophores was followed in methanol and in Britton Robinson buffer of pH 10.5, respectively. The relative fluorescence intensity was measured at 15 min intervals. Up to 3 h, no variation was observed.

3.5. Statistical analysis of results

3.5.1. Concentration ranges and calibration graphs

Using the optimized reactions conditions, the relative fluorescence intensities measured at the specified working wavelengths were found to be linearly correlated to the SMX and AMP Na concentrations. The data recorded in Table 1 summarize the characteristics of the calibration plots. These include linear regression equations, concentration ranges, correlation coefficients (r), and standard deviations of the intercept (S_a) and slope (S_b).

3.5.2. Precision

The within-day precision of the analytical response for SMX and AMP Na determination was assessed using standard reaction solutions of both drugs at concentrations of 0.1 and 1.0 μg ml^{-1}, respectively. The R.S.D. ranged from 1 to 1.6% over a three-day period.

3.5.3. Detection and quantification limits

The limit of detection, LOD, and the limit of quantification, LOQ, were calculated in accordance to the formulas given by the official compendial methods [7], where LOD and LOQ are defined as $3s\,b^{-1}$ and $10s\,b^{-1}$, respectively (s is the standard deviation of replicate blank responses). Data are given in Table 1.

3.6. Analysis of pharmaceutical formulations

The developed fluorimetric methods were applied to the determination of SMX and AMP (as AMP Na, AMP trihydrate or anhydrous AMP) in their commercially available pharmaceutical preparations. Table 6 shows the results of five determinations. The methods gave satisfactory recovery data for both, SMX and AMP. The statistical calculations for the assay results show good precision of the proposed methods. In all cases, the products

Table 5
Determination of sulfamethoxazole (SMX) and ampicillin sodium (AMP Na) in synthetic mixtures with other drugs

Combination (ratio)	Nominal value (μg ml^{-1})	Found ± S.D. (μg ml^{-1})	R.S.D.%	S.A.E.
SMX–trimethoprim (5:1)	0.04	0.038 ± 0.00068	1.79	3.06×10^{-4}
	0.16	0.16 ± 0.0013	0.81	5.79×10^{-4}
AMP Na–sulbactam (2:1)	0.40	0.41 ± 0.0057	1.39	2.57×10^{-3}
	2.0	1.97 ± 0.020	1.02	8.81×10^{-3}
AMP Na–flucloxacillin (1:1)	0.40	0.40 ± 0.0065	1.63	2.89×10^{-3}
	2.0	2.01 ± 0.018	0.90	8.09×10^{-3}
AMP Na–cloxacillin (1:1)	0.40	0.39 ± 0.0046	1.18	2.04×10^{-3}
	2.0	2.00 ± 0.012	0.60	5.36×10^{-3}

S.D.: standard deviation of five determinations; S.A.E.: standard analytical error.

Table 6
Analysis of sulfamethoxazole (SMX) and ampicillin (AMP) in some commercial pharmaceutical preparations

Preparation	Recovery ± S.D.	
	Fluorimetric method	Reference method
SMX preparations[a]		
Septazole tablets	100.5 ± 1.41 $t = -0.95$, $F = 1.98$[b]	101.2 ± 1.00[c]
Sutrim tablets	101.1 ± 0.87 $t = 1.07$, $F = 4.35$[b]	100.7 ± 0.42[c]
Chemotrim forte tablets	98.9 ± 1.11 $t = 0.13$, $F = 2.23$[b]	98.8 ± 0.74[c]
Septazole suspension	100.6 ± 0.93 $t = 1.47$, $F = 2.65$[b]	99.8 ± 0.57[d]
Sutrim suspension	100.6 ± 1.08 $t = 0.13$, $F = 2.23$[b]	100.9 ± 1.24[d]
Septrin suspension	98.6 ± 0.89 $t = 0.18$, $F = 1.87$[b]	98.7 ± 1.22[d]
AMP preparations[e]		
Epicocillin capsules	98.8 ± 0.79 $t = 0.60$, $F = 1.39$[b]	98.5 ± 0.67[f]
Ampicillin capsules	98.7 ± 0.84 $t = -0.44$, $F = 1.83$[b]	98.4 ± 1.14[f]
Ampicolx capsules	99.9 ± 0.56 $t = 0.22$, $F = 5.58$[b]	99.9 ± 0.24[g]
Epicocillin suspension	100.2 ± 1.22 $t = 0.92$, $F = 1.54$[b]	99.6 ± 0.92[f]
Epicocillin vials	100.4 ± 1.16 $t = 0.96$, $F = 1.51$[b]	99.7 ± 0.94[f]
Unasyn vials	100.5 ± 0.56 $t = 1.01$, $F = 1.20$[b]	100.1 ± 0.51[h]

S.D.: Standard deviation of five determinations.

[a] Sulfamethoxazole preparations, Septazole and Sutrim tablets are labeled to contain 400 mg SMX and 80 mg TMP per tablet; Chemotrim forte tablets are labeled to contain 800 mg SMX and 160 mg TMP per tablet; Septazole, Sutrim and Septrin suspensions are labeled to contain 200 mg SMX and 40 mg TMP per 5 ml. Septazole tablets and Septazole suspension are manufactured by Alexandria Pharmaceutical Co., Alexandria, Egypt; Sutrim tablets and Sutrim suspension are manufactured by Memphis Co. for Pharmaceuticals and Chemical industries, Cairo, Egypt; Chemotrim forte tablets are manufactured by Kahira Co. for Pharmaceuticals and Chemical industries, Cairo, Egypt; Septrin suspension is manufactured by Glaxo Wellcome, Cairo, Egypt, under license of Glaxo Wellcome, UK.

[b] Tabulated t-value for $P = 0.05$ and 8 degree of freedom is 2.306, tabulated F-value for $P = 0.05$ and $f_1 = f_2 = 4$ is 6.38.

[c] Ref. [15].

[d] Ref. [8].

[e] Ampicillin preparations, Epicocillin vials are labeled to contain 500 mg AMP Na per vial; Epicocillin capsules are labeled to contain 500 mg AMP trihydrate per capsule; Epicocillin suspension is labeled to contain 125 mg AMP trihydrate per 5 ml. Ampicillin capsules are labeled to contain 250 mg anhydrous AMP per capsule; Ampiclox capsules are labeled to contain 250 mg AMP trihydrate and 250 mg cloxacillin sodium per capsule; Unasyn vials are labeled to contain 250 mg AMP Na and 125 mg sulbactam per vial. Epicocillin preparations are manufactured by E.I.P.I.C.O., Tenth of Ramdan city, Egypt; ampicillin capsules are manufactured by Misr Co. for Pharmaceuticals, Cairo, Egypt; Ampiclox capsules are manufactured by Medical Union Pharmaceuticals Co., Cairo, Egypt, under license of Beecham Pharmaceuticals, England; Unasyn vials are manufactured by Pfizer Egypt, under authority of Pfizer Inc., USA.

[f] Ref. [7].

[g] Ref. [32].

[h] Ref. [31].

tested were confirmed as being compendial quality in terms of the drug content using official procedures [7,8] and other reported methods [15,31,32]. The results obtained were compared statistically by Student's t- and variance ratio F-tests, the calculated values did not exceed the theoretical ones which indicated that there were no significant differences between the methods compared, i.e. the proposed methods are as accurate and precise as the respective reference methods.

4. Scope of the 2-acetylbutyrolactone reaction

From the current study ABL could be used as a fluorogenic agent for both $ArNH_2$ and RNH_2 through the formation of fluorescent Schiff bases. The applicability of ABL was evaluated through spectrofluorimetric analysis of model amino drugs.

The study clarifies some aspects concerning the ABL Schiff base reaction; first, the reaction can be carried out in both aqueous and organic media. Second, it reacts efficiently with primary amines of both aryl and aliphatic types. Third, the Schiff bases exhibit fluorescent properties that allow fluorimetric analysis down to the ng (s) level.

ABL can therefore be used as well as the other, well known, fluorimetric reagents specified for primary amines like fluorescamine [42], o-phthalaldehyde [43], dansyl chloride [44] and naphthalenedialdehyde [45]. The reaction of such reagents with primary amines occurs in aqueous solutions with suitable pH adjustment. Fluorescamine keeps the advantage of very fast reaction, which makes it suitable for post-colum derivatization [46], the matter which is not an option for the ABL reaction.

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