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Simultaneous determination of azithromycin and levofloxacin in pharmaceuticals by charge transfer complexation with alizarin red S using an absorption-factor method

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Abstract: A new approach based on a selective charge transfer reaction with alizarin red S is described for the simultaneous determination of azithromycin and levofloxacin in their binary mixtures using an absorption-factor spectrophotometric method. Native absorbance was used for the determination for levofloxacin, while a charge transfer complexation of alizarin red S served as the basis for the quantitation of azithromycin. The reaction of azithromycin with alizarin red S was possible in methanol under neutral conditions within 15 min at 25 °C. The conditions were suitably optimized for selective complexation of azithromycin with minimal interference from levofloxacin. The calibration curve was linear over the concentration range of 4.0–20 μg mL^{-1} for azithromycin and levofloxacin and the correlation coefficients of the regression equations were consistently greater than 0.9970 for both the drugs. The limit of detection was 1.07 μg mL^{-1} for azithromycin and 0.84 μg mL^{-1} for levofloxacin. The developed method was highly reproducible with precision (RSD%) values in the range of 0.54%–0.95% for both the drugs. The method was successfully applied for the simultaneous determination of these antibacterial drugs from their synthetic mixtures and pharmaceutical formulations with no interference from excipients.

Key words: Absorption-factor method, azithromycin, levofloxacin, alizarin red S, charge transfer complexation

1. Introduction

Azithromycin (AZT) (Figure 1a) is an azalide derived from erythromycin, a subclass of macrolide antibiotics.[1] Its antimicrobial spectrum is similar to that of erythromycin, but is more effective against certain bacteria,[2,3] and is one of the world's best-selling antibiotics.[4] AZT is primarily used to treat or prevent certain bacterial infections, most often those causing middle ear infections, strep throat, pneumonia, typhoid, bronchitis, and sinusitis.[5] AZT prevents the spread of bacterial infection by blocking bacterial protein synthesis so that the bacteria cannot produce proteins that are needed for growth. This action stops the growth of the bacteria and relieves symptoms of the bacterial infection, which include inflammation and pain.[6]

Levofloxacin (LFX) (Figure 1b) is a broad spectrum antibiotic of the fluoroquinolone drug class. LFX is the L-isomer of the racemate ofloxacin, a quinolone antimicrobial agent, and is well tolerated and more active than the racemate.[7] It is used to treat several infections including respiratory tract infections, cellulitis, urinary

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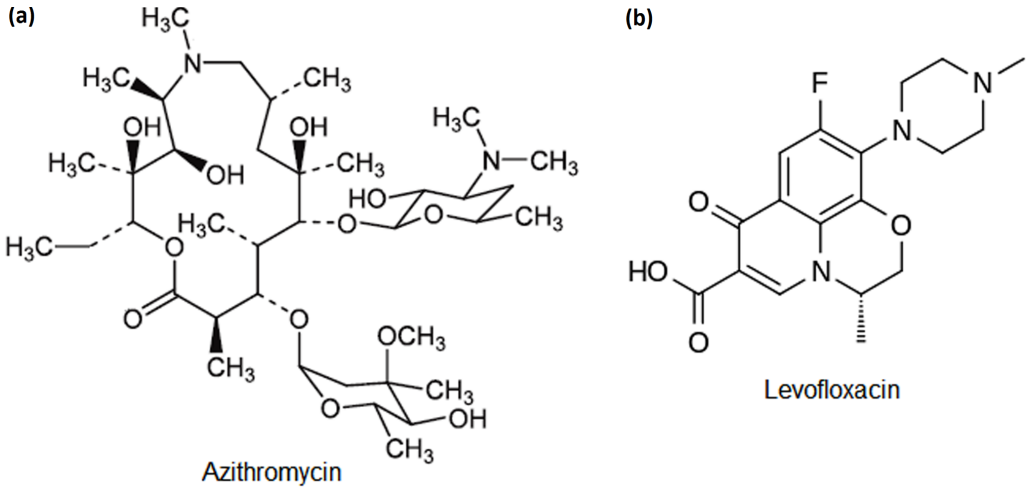

Figure 1. Chemical structures of (a) azithromycin and (b) levofloxacin.

tract infections, prostatitis, anthrax, endocarditis, meningitis, pelvic inflammatory disease, traveler's diarrhea, tuberculosis, and plague.[8] The mechanism of action of LFX involves inhibition of bacterial topoisomerase IV and DNA gyrase enzymes required for DNA replication, transcription, repair, and recombination.[7] LFX is available in tablet form, injectable form, and oral solution, and is also an active ingredient in eye and ear drops. Fixed dose generic combinations of AZT with LFX are available in 250 mg and 500 mg dose strength respectively and used for the treatment of variety of bacterial driven infections.

Several analytical methods have been reported for the individual determination of AZT in pharmaceuticals using chemiluminescence,[9] amperometry,[10] HPLC,[11] HPTLC,[12] and spectrophotometry.[13–16] Similarly, LFX has been analyzed by spectrofluorometry,[17] spectrophotometry,[18,19] HPTLC,[20] and HPLC.[21] Although some of these methods are sensitive, they involve extensive sample preparation, are tedious, and are not cost effective. Simultaneous determination of AZT and LFX has been a subject of very few reports. Three reversed-phase HPLC methods have been described for their simultaneous determination in pharmaceutical formulations.[22–24] Besides HPLC methods, there is only one report based on spectrophotometry for the simultaneous determination of AZT and LFX in bulk drugs and marketed formulations.[25] The method evaluated simultaneous equations (Vierodt's method) for their determination in the concentration range of 50–250 μg mL^{-1} and 2–10 μg mL^{-1} for AZT and LFX, respectively.

Alizarin red S (ARS) has been a popular chromogenic reagent for the spectrophotometric determination of several drugs having primary and secondary amino groups.[26,27] It reacts rapidly with these drugs at room temperature to give a colored product that can be easily quantified using a variety of spectrophotometric methods. Although ARS has been used for the estimation of AZT as a single analyte, there are no reports on the simultaneous determination of AZT and LFZ using ARS.[28] The combination of AZT and LFZ has proved to be effective in the treatment of rheumatoid arthritis and is available commercially as a fixed dose combination tablet. Thus, in the present work an absorption-factor method (AFM) is described for the selective and simultaneous spectrophotometric determination of AZT and LFX based on selective charge transfer reaction with ARS. The developed method is simple and highly reproducible for rapid determination of these drugs in pharmaceutical preparations.

1.1. Absorbance-factor method

The only requirement for AFM is that for a binary mixture containing analytes X and Y the component Y can have some interference at λ_{max} of X (λ_1), while X must not show any signal at the λ_{max} of Y (λ_2), i.e. absorbance of mixture at λ_2 should entirely correspond to the absorbance due to component Y.[29]

To provide a brief explanation of the method, the absorbance of a mixture at λ_1 and λ_2 can be expressed as[30]

$$A_{m1} = A_{X1} + A_{Y1} \tag{1}$$

$$A_{m2} = A_{X2} + A_{Y2} \tag{2}$$

Now, dividing Eq. (1) by A_{Y1} and Eq. (2) by A_{Y2} we get the following expression:

$$\frac{A_{m1}}{A_{Y1}} = \frac{A_{X1}}{A_{Y1}} + 1 \tag{3}$$

$$\frac{A_{m2}}{A_{Y2}} = \frac{A_{X2}}{A_{Y2}} + 1 \tag{4}$$

Upon subtraction and rearrangement of Eqs. (3) and (4) we get

$$\frac{A_{m1}}{A_{Y1}} - \frac{A_{m2}}{A_{Y2}} = \frac{A_{X1}}{A_{Y1}} + 1 - \left(\frac{A_{X2}}{A_{Y2}} + 1\right)$$

$$\frac{A_{m1}}{A_{Y1}} - \frac{A_{X1}}{A_{Y1}} = \frac{A_{m2}}{A_{Y2}} - \frac{A_{X2}}{A_{Y2}}$$

$$\frac{A_{m1} - A_{X1}}{A_{Y1}} = \frac{A_{m2} - A_{X2}}{A_{Y2}} \tag{5}$$

However, as noted earlier component X does not show any response (A_{X2}) at λ_2; hence Eq. (5) can be rearranged as

$$\frac{A_{m1} - A_{X1}}{A_{Y1}} = \frac{A_{m2}}{A_{Y2}}$$

$$A_{m1} - A_{X1} = \frac{A_{Y1}}{A_{Y2}} \times A_{m2}$$

$$A_{X1} = A_{m1} - \frac{A_{Y1}}{A_{Y2}} \times A_{m2}, \tag{6}$$

where A_{X1} is the absorbance due to X in the mixture at λ_1; A_{m1} and A_{m2} are the absorbance of the mixture at λ_1 and λ_2, respectively; A_{Y1}/A_{Y2} is called the absorption factor and it is a constant term for pure Y.

Using Eq. (6), it is possible to determine the contribution of component X at its λ_{max} (λ_1) by simply measuring the absorbance values of the mixture at both the wavelengths λ_1 and λ_2, and previously determined absorbance ratio, i.e. A_{Y1}/A_{Y2}, from pure standard solutions of Y. The concentration of X is determined by substituting A_{X1} values into the regression equation obtained from the plot of absorbance values of the zero order curves of X at its λ_{max} against corresponding concentrations. On the other hand, the concentration of Y may be easily obtained using the absorbance value at its λ_{max} without interference from X.

2. Results and discussion

2.1. Optimization of the experimental conditions

During method optimization, several experimental conditions such as solvent selection, the amount of ARS, reaction time, and temperature were suitably optimized to get a reproducible and adequate response for the simultaneous determination of AZT and LFX. The choice of the optimum concentration range depends on the spectral characteristics of the compounds, their absorptivity, and relative amounts in the mixture. As evident from the overlain spectra (Figures 2a and 2b), it was difficult to determine the drugs simultaneously and establish a similar linear range for both. Moreover, the quantitation of AZT was not feasible at 205 nm (absorption maxima), which is identical to the cut-off wavelength of methanol (205 nm). To overcome this predicament, a different approach based on a charge transfer (n?π) complex reaction with ARS was examined for the quantification of AZT.[28] However, this warranted the need to check the effect of ARS on LFX absorption spectra during their simultaneous determination. For the study with ARS, several organic diluents like ethanol, methanol, acetonitrile, and dichloromethane were tested for optimum reaction conditions. Methanol was selected as the solvent because of its capacity to form stable hydrogen bonds with the formed radical anion[31] and to circumvent the solubility issues with AZT in aqueous solutions. Moreover, the response in methanol was greater as compared to other solvents. The resulting charge transfer complex between AZT and ARS gave a shift in the absorption to 536 nm and at the same time altered the absorption behavior of LFX. This could be due to nonselective charge transfer complexation with both AZT and LFX at higher amounts of ARS (5.0 mL, 0.1%). Nevertheless, it was observed that at lower concentration of ARS the absorbance of LFX at this wavelength remained practically unaffected. Noticeably, there was a small decrease in the absorbance of AZT-ARS complex due to decreasing ARS concentration. Hence, in order to establish a similar linearity range for both the drugs, it was imperative to optimize the reagent concentration.

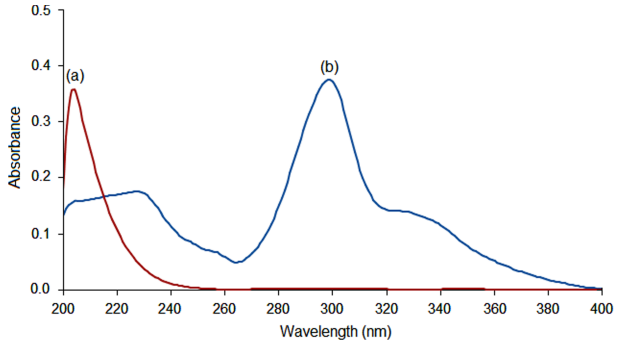

Figure 2. Zero order absorbance spectra of (a) azithromycin (8.0 μg mL^{-1}) and (b) levofloxacin (8.0 μg mL^{-1}) solution using methanol as a blank.

For this purpose, an experiment was performed to study the effect of ARS concentration on the absorption behavior of the drugs. Two different set of solutions were prepared in a series of 10 mL volumetric flasks, with different volumes (0.5–5.0 mL) of 0.1% (w/v) ARS solution keeping fixed amounts of the drugs (80 μg mL^{-1}). The results in Figure 3a indicate that 1.25 mL of ARS solution was adequate to develop a red colored complex, albeit with a lower intensity for AZT. On the other hand, there was negligible influence on the LFX spectra (Figure 3b). Nevertheless, this resulted in reduction in the response of LFX and the formed AZT-ARS complex. This allowed establishing the same linearity range for LFX and AZT as they are available in equal proportion in marketed tablets. The effect of temperature and the reaction time for AZT-ARS complex formation was also evaluated at different temperatures (25, 30, 35, and 40 °C) by monitoring the absorbance at 536 nm. There

was practically no change in the absorbance value up to 35 °C; however, a minor decrease in absorbance was noted at 40 °C. Thus, 25 °C was selected as the preferred temperature for complex formation. Further, the optimum reaction time at 25 °C was set at 15 min as there was no further increase in the absorbance values beyond this time period.

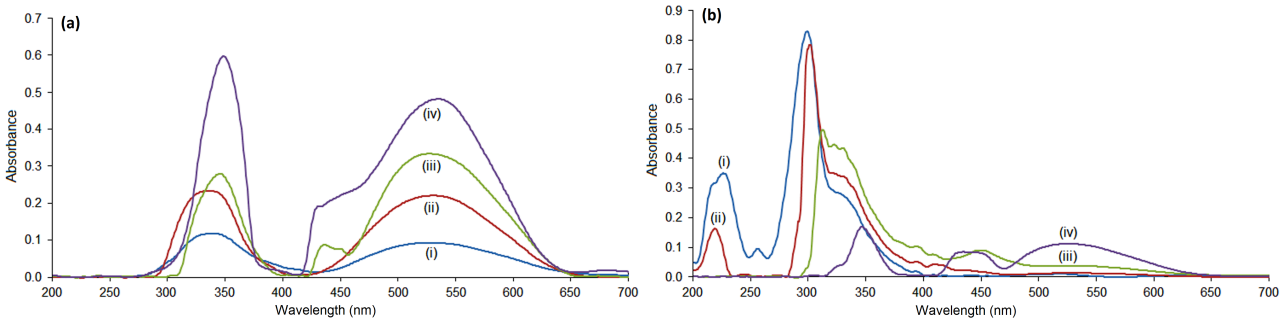

Figure 3. Representative absorbance spectra of (a) azithromycin (8.0 μg mL^{-1}) and (b) levofloxacin (8.0 μg mL^{-1}) in presence of (i) 0.50 mL (ii) 1.25 mL (iii) 2.50 mL and (iv) 5.00 mL of 0.1% (w/v) alizarin red S solution.

The zero-order absorption spectra of AZT (Figure 4a) and LFX (Figure 4b) along with added amount of ARS showed no absorbance contribution due to LFX above 450 nm, which allows the simple analysis of AZT at 536 nm without the interference of LFX. On the other hand, the response due to the formed complex prevented the single-step quantitation of LFX in the presence of AZT at 301 nm. Therefore, the next phase of the study was to develop a simple and accurate method for the simultaneous determination of LFX and AZT in pure powder form, laboratory-prepared mixtures, and tablet dosage forms using the absorption factor method.

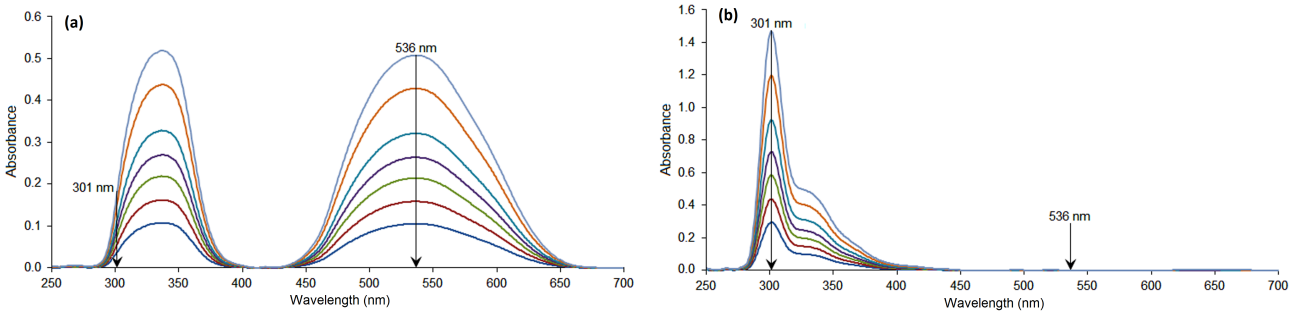

Figure 4. Calibration spectra of (a) azithromycin (4.0-20 μg mL^{-1}) and (b) levofloxacin (4.0–20 μg mL^{-1}) in methanol using 1.25 mL of 0.1% (w/v) alizarin red S solution.

To determine the absorption factor for AZT, the zero order absorption spectra were recorded in the wavelength range of 200–700 nm for standard solutions of AZT under the optimized experimental conditions with ARS within the linearity range. The value of the absorption factor was found to be 0.3427 (the ratio between the absorbance of AZT-ARS complex at 301 nm and 536 nm). The concentration of AZT was calculated from the corresponding regression equation obtained by plotting the absorption values of AZT-ARS complex at 536 nm against the corresponding concentrations. On the other hand, the estimation of LFX in synthetic mixtures and tablet formulations with AZT was based on subtraction of the absorption due to AZT at λ_{max} of LFX using the following Eq. (7),

$$A_{LFX\ at\ 301\ nm} = A_{mix\ at\ 301\ nm} - \frac{A_{AZT\ at\ 301\ nm}}{A_{AZT\ at\ 536\ nm}} \times A_{mix\ at\ 536\ nm} \tag{7}$$

The stoichiometry of AZT-ARS complex was determined by Job's method of continuous variation and mole-ratio method in the neutral medium.[32] The plots in Figure 5a and 5b indicate formation of 1:1 and 1:2 AZT:ARS complexes using both the methods. A similar observation was reported for the complexation of AZT with 1,2-naphthoquinone-4-sulfonate in alkaline conditions.[14] In the mole ratio plot, there was a constant increase in absorbance beyond 1:2 [ARS]/[AZT] ratio without any inflection point. Thus, the probable reaction mechanism involves transfer of electrons from the nitrogen atoms of the dimethylamine group and lactone ring in AZT to the electron deficient site of two ARS moieties as shown in Figure 6. The overall formation constants of the formed complexes were evaluated by Harvey and Manning's method[33] using Eq. (8),

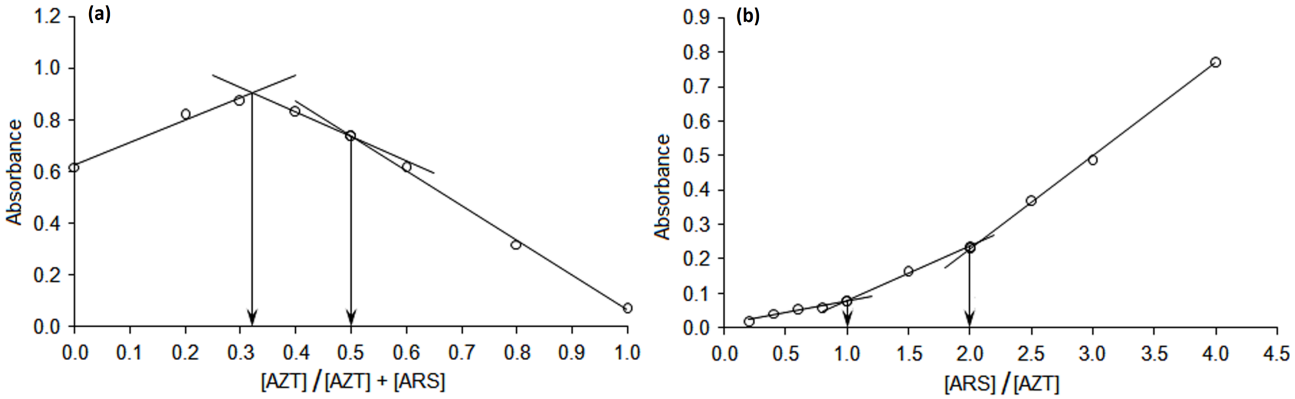

Figure 5. (a) Job's plot of absorbance versus mole fraction of azithromycin for the complexation reaction between azithromycin (4.0×10^{-5} mol L^{-1}) and alizarin red S (4.0×10^{-5} mol L^{-1}) and (b) mole ratio plots of absorbance versus [ARS]/[AZT].

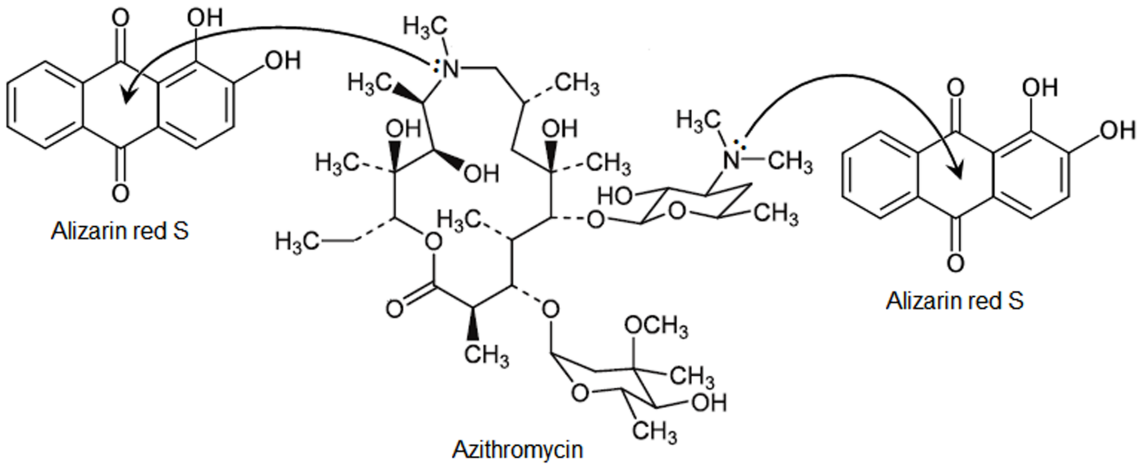

Figure 6. Probable reaction between azithromycin and alizarin red S under neutral conditions.

$$\beta_n = \frac{{}^{A}/_{A_{max}}}{\left[1 - \left({}^{A}/_{A_{max}}\right)\right]^{n+1} \cdot C_{ARS}^{n} \cdot n^2}, \tag{8}$$

where A represents the absorbance of the formed complex in the presence of ARS concentration, C_{ARS}; A_{max} is the maximum absorbance value in the presence of excess of ARS concentration; and n is the complex stoi-

chiometry (AZT:ARS). The formation constant ($\log K_f$) values for 1:1 and 1:2 were 4.79 and 9.60, respectively (Table 1).

Table 1. Optical properties and regression parameters for azithromycin and levofloxacin by the proposed method ($n = 3$).

Parameter	Azithromycin	Levofloxacin
Wavelength (nm)	536	301
Reaction time (min)	15	NA
Stability (h)	24	NA
Complex stoichiometry (drug:dye)	1:1 1:2	NA
Logarithmic formation constant ($\log K_f$)	4.79 9.60	NA
Molar absorptivity (L mol^{-1} cm^{-1})	19743	55369
Beer's law limit (μg mL^{-1})	4.0-20	4.0-20
Sandell's Sensitivity (μg cm^{-1})	0.039	0.013
Regression equation[a] (A = mC +b)		
Slope (m)	0.0255	0.0741
Intercept (b)	0.0078	-0.0010
Correlation coefficient (r^2)	0.9973	0.9983
Limit of detection, LOD = 3.3 s/m (μg mL^{-1})	1.07	0.84
Limit of quantitation, LOQ = 10 s/m (μg mL^{-1})	3.21	2.52

NA: not applicable; A: absorbance; C: drug concentration in μg cm^{-1}

s: standard deviation of the intercept; m: slope of the calibration curve.

2.2. Validation of the proposed method

The developed absorption factor method was validated according to the current ICH guidelines.[34]

2.2.1. Linear range, limit of detection, and limit of quantitation

Under the optimized experimental conditions described, the linearity was checked by analyzing standard solutions that obeyed Beer's law over the concentration range of 4.0–20.0 μg mL^{-1} for LFX and AZT, respectively. All measurements were recorded against the reagent blank. Three calibration curves were constructed and the linear regression equations were computed by the method of least squares. The values of regression parameters like slope, intercept, and correlation coefficients are summarized in Table 1. The correlation coefficients (r^2) of the linear plots were $\geq$0.9973 for both the drugs. The limit of detection (LOD) and limit of quantitation (LOQ) for the drugs were estimated according to ICH guidelines. The optical characteristics like wavelength of maximum absorbance, molar absorptivity, and Sandell's sensitivity are also presented.

2.2.2. Intra- and interbatch accuracy and precision

The precision of the proposed method was expressed in terms of percent relative standard deviation (% RSD). Three different concentrations of LFX and AZT were analyzed in three replicates on the same day (intraday precision) and on three consecutive days (interday precision). The results of this study are summarized in Table 2. The % RSD values were 0.56%–0.91% (intraday) and 0.63%–0.95% (interday) for both the drugs, indicating

high precision of the method. Similarly, the accuracy of the proposed methods, expressed as recovery, was within the acceptable limits.

Table 2. Summary of accuracy and precision results for azithromycin and levofloxacin in bulk form using the proposed method ($n = 3$).

Amount added (μg mL^{-1})	Intraday[a]			Interday[b]		
	Amount found (μg mL^{-1})	Recovery (%)	% RSD	Amount found (μg mL^{-1})	Recovery (%)	% RSD
Azithromycin						
6.0	5.98	99.64	0.56	6.02	100.33	0.63
7.5	7.48	99.67	0.74	7.53	100.38	0.82
9.0	8.97	99.70	0.77	8.99	99.90	0.93
Levofloxacin						
6.0	6.01	100.02	0.84	5.96	99.36	0.95
7.5	7.49	99.77	0.91	7.52	100.29	0.89
9.0	8.99	99.85	0.57	9.06	100.62	0.75

[a] Samples analyzed on the same day; [b] Samples analyzed on three consecutive days;
RSD: relative standard deviation

2.2.3. Selectivity, ruggedness, and stability of solutions

The selectivity of the proposed procedures was assessed by the analysis of laboratory/synthetically prepared mixtures containing different ratios of the two drugs (both above and below their normal strengths in tablets) together with typical tablet inactive ingredients like calcium phosphate, cellulose, magnesium stearate, lactose monohydrate, and starch. Acceptable recoveries and consistent values of precision obtained for different AZT-LFX mixtures (Table 3) confirm the accuracy and precision of the method and demonstrate its analytical power to resolve and quantify the investigated drugs when present in different proportions. The ruggedness of the method was checked by using different pairs of cuvettes and different UV-visible spectrophotometers, and also by employing three different analysts to carry out the same procedure. The results showed no significant difference under all three conditions, with precision being consistently less than 2.0%.

Table 3. Results for the analysis of laboratory prepared mixtures containing azithromycin and levofloxacin ($n = 3$).

Concentration taken (μg mL^{-1})		Recovery (% ± standard deviation)	
Azithromycin	Levofloxacin	Azithromycin	Levofloxacin
6.0	6.0	99.70 ± 0.56	100.39 ± 0.59
6.0	7.5	100.38 ± 0.63	100.59 ± 0.93
6.0	9.0	99.48 ± 0.52	100.32 ± 0.85
7.5	6.0	99.55 ± 1.02	99.72 ± 0.94
7.5	7.5	100.19 ± 0.62	99.83 ± 0.72
7.5	9.0	99.98 ± 0.77	100.22 ± 1.03
9.0	6.0	99.79 ± 0.67	99.64 ± 0.61
9.0	7.5	99.55 ± 0.81	100.48 ± 0.60
9.0	9.0	99.65 ± 0.87	100.53 ± 0.66
Mean recovery (%)		99.81 ± 0.72	100.19 ± 0.77

Stock solutions of the studied drugs were found stable for a minimum storage period of 1 week at 4 °C. The stability of the prepared standard solutions in methanol was also assessed by keeping them at room temperature for 4.0 h; however, there was no significant change in the absorbance values for any solution.

2.3. Application of the proposed method for pharmaceutical formulations

The proposed method was successfully applied to the analysis of both the drugs in five pharmaceutical preparations. The results obtained were precise and in good agreement with the labeled claim as evident from the satisfactory values of recovery (%) and precision (% RSD) summarized in Table 4. The performance of the proposed method was evaluated by comparison with a reference HPLC method for AZT and LFX.[23] The results obtained were compared by applying Student's t-test for accuracy and the F-test for precision with the reference method. The values of t-test and F-tests were estimated at 95% confidence level at four degrees of freedom and were well within the tabulated/theoretical values, suggesting no significant difference between the methods. Quantitative recoveries obtained indicate that there was no interference from the co-formulated inactive ingredients.

Table 4. Determination of azithromycin (AZT) and levofloxacin (LFX) in pharmaceutical formulations by the proposed method.

Tablet formulation	Drug component	Claimed value (mg)	Mean values found[a]± SD	RSD (%)	Recovery (%)[a]	Mean values found[b]± SD	t-value[c]	F-value[c]
Levomac AZ 250	AZT	250	252.39 ± 2.71	1.08	100.96	248.96 ± 2.12	1.730	1.632
	LFX	250	248.76 ± 1.88	0.75	99.50	248.39 ± 1.42	0.467	1.745
Zithrox LX 250	AZT	250	249.49 ± 2.26	0.90	99.80	251.64 ± 2.74	1.071	1.480
	LFX	250	246.53 ± 3.01	1.21	98.61	248.76 ± 2.17	1.136	1.929
Loxof AZ 250	AZT	250	251.27 ± 2.21	0.88	100.51	252.31 ± 1.77	0.949	1.564
	LFX	250	248.64 ± 2.53	1.01	99.46	249.16 ± 1.55	0.309	2.662
Azifine-L 500	AZT	500	501.26 ± 3.11	0.62	100.25	505.47 ± 5.35	3.256	2.958
	LFX	500	494.20 ± 5.92	1.18	98.84	499.05 ± 2.93	1.491	4.083
Levomac AZ 500	AZT	500	497.36 ± 5.33	1.06	99.47	502.28 ± 1.93	2.013	2.347
	LFX	500	495.34 ± 4.76	0.95	99.07	494.67 ± 5.04	0.220	1.120

[a] Mean values for five replicate measurements; [b] Mean results from reference 23; [c] Tabulated values for t and F-values at four degree of freedom and 95% confidence level are $t = 2.776$ and $F = 6.388$; RSD: relative standard deviation

Furthermore, standard addition was also applied by adding the pure drugs to aliquots of the tablet extracts, and the recovery of each drug was then calculated by comparing the drug response with the increment response obtained after addition of the standard. Recovery values obtained by application of the standard addition technique are shown in Table 5. It is evident from these results that the proposed method is applicable to the assay of selected drug combination in their fixed dosage forms with minimum sample preparation and satisfactory levels of accuracy and precision.

2.4. Comparison with reported methods

The developed method offers a major advantage over reported reversed-phase HPLC[22–24] and one spectrophotometric method[25] in that it can be used to analyze any commercial formulation consisting of AZT and LFX, unlike existing methods, which were applied to study only one formulation (Table 6). This aspect of the present method can be useful in content uniformity testing and quality control laboratories for routine analysis.

Table 5. Results obtained by the standard addition technique ($n = 3$).

Amount taken (μg mL^{-1})	Amount added (μg mL^{-1})	Mean amount found (μg mL$^{-1}\pm$ SD)	RSD (%)	Recovery (%)
Azithromycin				
7.5	6.0	6.03 ± 0.03	0.46	100.54
7.5	7.5	7.52 ± 0.04	0.58	100.29
7.5	9.0	8.98 ± 0.07	0.83	99.80
Levofloxacin				
7.5	6.0	5.98 ± 0.06	0.96	99.64
7.5	7.5	7.48 ± 0.03	0.46	99.77
7.5	9.0	9.02 ± 0.06	0.62	100.26

SD: standard deviation; RSD: relative standard deviation

Moreover, all fixed dose combination tablets contain identical dose strength of AZT and LFX and therefore preferably the established linear range should be similar. This can be a limitation of the existing spectrophotometric method,[25] which has a very different calibration range for AZT (50–250 μg mL^{-1} and LFZ (2–10 μg mL^{-1}. Furthermore, compared to reversed-phase HPLC, which affords higher cost per analysis, the spectrophotometer offers simplicity and ease of analysis and is readily available in any chemical or quality control laboratory. Thus, the present method provides an optimum combination of sensitivity without any need for complex and expensive instrumentation.

3. Experimental

3.1. Materials and reagents

Reference drug samples of azithromycin dihydrate (purity 99.72%) and levofloxacin hemihydrate (purity 99.18%) were procured from Clearsynth Laboratories Pvt. Ltd., (Mumbai, India). AR grade alizarin red S (ARS) was a product of S. D. Fine Chem. (Mumbai, India) and used without further purification. The methanol used was of spectroscopic grade purchased from E. Merck (Mumbai, India). Deionized water was prepared from the Milli-Q water purification system from Millipore (Bangalore, India). Five pharmaceutical preparations, namely Levomac AZ 250 (Macleods Pharma Ltd.), Zithrox LX 250 (Macleods Pharma Ltd.) and Loxof AZ 250 (Ranbaxy) labeled to contain 250 mg each of LFX and AZT, and Azifine-L 500 (Glenmark Pharma) and Levomac AZ 500 (Macleods Pharma Ltd.) labeled to contain 500 mg each of LFX and AZT were purchased from a local pharmacy.

3.2. Instrument and analysis conditions

A Shimadzu UV-1700 double beam spectrophotometer (Kyoto, Japan) with matched 10-mm quartz cells was used for spectral measurements. The parameters set were wavelength accuracy ±0.5 nm, bandwidth 1.0 nm, and scan speed 400 nm min^{-1}. The obtained spectral data were processed with Shimadzu UV PC software version 2.0. Weighing of samples was performed on a Sartorius GD503 (Bradford, MA, USA) analytical balance, having a readability of 0.0001 g. A Varivol II Micropipette used for accurate and precise transfer of solutions was obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India) with a varying volume capacity of 0.5 to 5.0 mL. Another Jasco V-570 UV-Visible Spectrophotometer (Tokyo, Japan) was also used to evaluate the ruggedness of the method.

Table 6. Comparison of salient features of methods developed for simultaneous determination of azithromycin and levofloxacin.

Sr. no.	Technique	Analyte	LOD (μg mL^{-1})	Sensitivity (slope of calibration equation)	Linear range (μg mL^{-1})	Application	Ref.
1.	RP-HPLC	AZT	6.13	16616	50–150	Analysis of AZT and LFX in combined dosage form	22
		LFX	5.47	19288	50–150		
2.	RP-HPLC	AZT	0.01	12307	20–100	—	23
		LFX	0.001	88178	2.0–10		
3.	RP-HPLC	AZT	--	19288	50–150	Analysis of AZT and LFX in combined dosage form	24
		LFX	--	16616	50–150		
4.	Spectrophotometry (Vierodt's method)	AZT	0.012	--	50–250	Analysis of bulk drugs and marketed formulation	25
		LFX	0.016	--	2.0–10		
5.	Spectrophotometry (Absorption factor method)	AZT	1.07	0.0255	4.0–20	Analysis of laboratory prepared mixtures and pharmaceutical formulations (Levomac AZ 250, Zithrox LX 250, Loxof AZ 250. Azifine-L 500 and Levomac AZ 500)	PM
		LFX	0.84	0.0741	4.0–20		

RP-HPLC: reversed-phase high performance liquid chromatography; AZT: azithromycin; LFX: levofloxacin; PM: present method

3.3. Standard stock solutions

Standard stock solutions of the studied drugs containing 500 μg mL^{-1} were prepared by dissolving the requisite amount of each drug in 20 mL of methanol and were further diluted to 100 mL with the same diluent to obtain the working solutions. The standard solutions were kept in the refrigerator (5 $\pm$ 2 °C). Stock solution of 0.1% (w/v) ARS was prepared by dissolving the appropriate amount of the reagent in 100 mL of methanol:water (80:20, v/v). All solutions were stable for at least 1 week.

3.4. Spectral characteristics of AZT and LFX

The zero-order absorption spectra of AZT (8.0 μg mL^{-1}) and LFX (8.0 μg mL^{-1}) were recorded against methanol as a blank over the wavelength range of 200–700 nm. Separately, zero-order spectra were also recorded using the same solutions (10 mL) containing 1.25 mL of 0.1% (w/v) ARS working solution against reagent blank containing 1.25 mL of 0.1% (w/v) ARS solution over the wavelength range of 200–700 nm.

3.5. Construction of calibration curves

Two separate calibration sets were prepared for LFX and AZT (within the range of 4.0–20 μg mL^{-1} for both the drugs) by transferring appropriate aliquots from their standard working solutions to a series of 10-mL volumetric flasks. An aliquot of 1.25 mL of 0.1% (w/v) ARS solution was added to each flask and the mixture was shaken in order to facilitate the reaction and the volume was completed to the mark with methanol. The absorbance of the resulting solutions was measured at 301 nm for LFX and 536 nm for AZT against a reagent blank prepared simultaneously. The calibration curves were constructed by plotting the absorbance of the zero-order spectra versus the corresponding final concentrations of the drugs and their corresponding regression equations were computed for both the drugs. Additionally, the scanned spectra of AZT were measured at 301 nm and 536 nm to calculate the absorption factor, which is the ratio of the absorbance at these two wavelengths.

3.6. Assay of laboratory-prepared mixtures

Laboratory-prepared mixtures containing different relative amounts of the aforesaid drugs were prepared in methanol, the zero-order spectra of each mixture were recorded, and absorbance values were measured at 301 and 536 nm. The absorbance at 536 nm was directly utilized to determine the amount of AZT from the corresponding regression equation. The absorbance of mixtures at 301 nm was treated as described in Eq. (6), in order to determine the absorbance due to LFX at 301 nm (A_{X1}). The amount of LFX was determined by substitution of A_{X1} in the corresponding regression equation.

3.7. Assay of pharmaceutical formulations

Twenty tablets each of Levomac AZ 250 (250 mg LFX/250 mg AZT), Zithrox LX 250 (250 mg LFX/250 mg AZT), Loxof AZ 250 (250 mg LFX/250 mg AZT), Azifine-L 500 (500 mg LFX/500 mg AZT), and Levomac AZ 500 (500 mg LFX/500 mg AZT) were accurately weighed and ground to a fine powder. An amount of the powder equivalent to 20 mg of LFX and AZT respectively was weighed, dissolved in about 50 mL of methanol, and thoroughly sonicated for about 15 min. The solutions were filtered; the residues were washed three times with 10 mL of methanol and transferred quantitatively into 100-mL volumetric flasks. The volume was then completed to the mark with methanol. Appropriate dilutions were made to obtain a concentration of 10 μg mL^{-1} for

LFX and AZT. The same procedure was followed as described under section 3.6, and the concentrations of LFX and AZT were calculated from their corresponding equations.

4. Conclusion

A simple, precise, and accurate method based on absorption factor has been developed for routine analysis of binary antibiotic mixtures containing AZT and LFX. This is the first report on simultaneous spectrophotometric determination of these drugs using charge transfer complex formation with ARS. Under the optimized conditions, ARS reacted selectively with AZT in the presence of LFX within 15 min under neutral conditions. The method showed adequate accuracy and precision and quantitative recoveries for both the drugs in bulk and laboratory prepared mixtures. Further, the method was fully validated as per the ICH norms for linearity, sensitivity, accuracy, precision, recovery, reproducibility, and solution stability, and the results were found within the acceptance criteria. The method was applied for the analysis of five fixed dose pharmaceutical formulations and the results demonstrated that the proposed method can be recommended for routine analysis of these drugs in quality control labs.

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