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Spectrophotometric Determination of Some Antibiotics Using Bromophenol Blue as Ion Pair Reagent

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Abstract

Objective: Simple and rapid methods have been developed for the determination of azithromycin, sparfloxacin and cephalexin monohydrate in bulk and pharmaceutical formulations.

Method: These methods were based on the formation of bluish-green ion pair complexes of these antibiotics with bromophenol blue (BPB). Acetonitrile was used as a solvent for azithromycin and sparfloxacin whereas methanol-acetonitrile medium for cephalexin monohydrate.

Results: 2:1 complexes were formed between the drug and reagent almost instantaneously with absorption maxima 595 nm, 620 nm, 600 nm for the three drugs respectively. Different parameters such as effect of time, effect of reagent concentration were optimised. Under optimum conditions, calibration curves were found to be linear over the range of 0-50 μg mL^{-1} for azithromycin, 10-80 μg mL^{-1} for sparfloxacin and 10-170 μg mL^{-1} for cephalexin monohydrate respectively. The detection limits were found to be 0.10 μg mL^{-1}, 0.21 μg mL^{-1} and 1.69 μg mL^{-1} with Sandell's sensitivity 0.0559 μg cm^{-2}, 0.1034 μg cm^{-2} and 1.3920 μg cm^{-2} respectively for the three drugs. Stability constant (log K) was found to be 6.19 ± 0.04, 5.00 ± 0.07 and 4.05 ± 0.05 showing high stability of the complexes. Molar absorptivity was found to be 1.369×10^{4} L mol^{-1} cm^{-1}, 3.774×10^{3} L mol^{-1} cm^{-1} and 2.620×10^{2} L mol^{-1} cm^{-1} with Gibb's free energy change -2.725×10^{3} kJ mol^{-1}, -2.393×10^{3} kJ mol^{-1} and -1.938×10^{3} kJ mol^{-1}. These methods were subjected to analytical quality control. Accuracy, precision, recovery and interference studies have been carried out.

Conclusion: The proposed methods were successfully applied to the determination of these drugs in their pharmaceutical formulations and human urine samples.

Keywords: Azithromycin; Sparfloxacin; Cephalexin monohydrate; Bromophenol blue; Ion pair complex; Pharmaceutical formulations; Urine sample

Introduction

Azithromycin is an important member of macrolide class of antibiotics. It is European Pharmacopoeia recommended antibiotic [1]. Macrolides are a group of antibiotics that belongs to the polyketide class of natural products. Their activity stems from the presence of a macrolide ring, a 15-membered macrocyclic lactone ring to which one or more deoxy-sugars may be attached [2]. Sparfloxacin is third generation quinolone antibiotic. It is official in Martindale extra Pharmacopeia [3]. Due to the presence of fluorine atom at C-6 position of quinolone, this clinically useful quinolone is described as fluoroquinolone. It is active against both gram-positive and gram-negative bacteria. It is widely used to treat human and veterinary diseases [4,5]. Cephalexin monohydrate is first generation cephalosporin antibiotic. Mode of action of cephalexin monohydrate is same as that of β- lactam antibiotics; it inhibits the synthesis of peptidoglycan layer of bacterial cell wall [6]. It is antibacterial, used as alternative to penicillin. It is useful for bone joint infections, pneumonia, urinary tract infection and its common side effect is intestinal upset. It is European Pharmacopoeia recommended antibiotic [7].

Bromophenol blue (BPB) is a triphenylmethane dye and is commonly used as indicator and spectrophotometric reagent. The structures of the three antibiotics and bromophenol blue are shown in Figure1.

Several methods have been reported in literature for the analysis of azithromycin, sparfloxacin and cephalexin monohydrate such as HPLC [8], spectrofluorometry [9], capillary electrophoresis [10], voltammetry [11], etc.

Spectrophotometric method is based on the formation of coloured (charge transfer or ion-pair) complex between drug and reagent which can be estimated by visible spectrophotometer. In ion-pair complex, ions of opposite electric charge are held together in solution by Coulomb attraction to form a distinct chemical entity. It behaves as a single unit. Ion pair formation, initially investigated by the physical chemistry has been found extremely interesting for the chemical analysis, including pharmaceutical analysis. Reported spectrophotometric methods of these three antibiotics includes complex formation with quinalizarin [12], eosin Y [13], rose Bengal [14], chloranilic acid and 7,7,8,8-tetracyanoquinodimethane [15], bromocresol purple and bromecresol green [16], ammonium vanadate [17], *p*-nitro phenol [18], Mo(V)-thiocyanate [19], *p*- dimethyl aminobenzaldehyde [20], molybdenum blue [21], Sodium 1,2-napthoquinone-4-sulphonate [22], *p*-benzaquinone [23], iodine, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone and 7,7,8,8-tetracyanoquinodimethane [24].

The proposed methods are based on formation of ion pair complexes with bromophenol blue. Bromophenol blue has been used for the first time with significantly low detection limit, high sensitivity and wider dynamic range. An important feature of these methods is that no extraction is required and it is feasible at room temperature.

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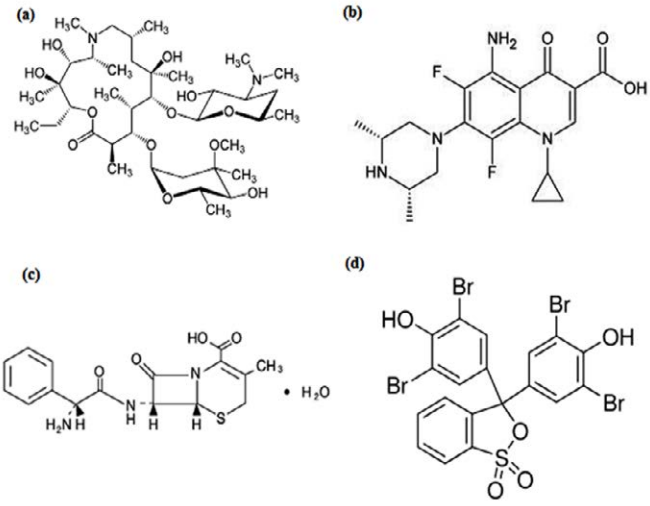

Figure 1: Structures of a) azithromycin, b) sparfloxacin, c) cephalexin monohydrate and d) bromophenol blue (BPB).

These methods could be applied to the analysis of pharmaceutical formulations and urine sample.

Experimental

Apparatus

Spectrophotometric studies were carried out with Spectronic 20D+ (Thermo-Spectronic) visible spectrophotometer. A Mettler balance H 51AR (Ner-Parma instrument Corp. L.C. = 0.01mg) was used for weighing purpose.

Materials and reagents

All chemicals and reagents used were of analytical grade. Azithromycin, sparfloxacin and cephalexin monohydrate were obtained from ZIM laboratories. Bromophenol blue, HPLC grade acetonitrile and methanol were obtained from LOBA Chemie.

Preparation of solutions

Stock solutions of azithromycin or sparfloxacin were prepared as 0.01M in acetonitrile while 0.01M cephalexin monohydrate solution was prepared in methanol whereas 0.01M bromophenol blue solution was prepared in acetonitrile. The solutions were further diluted as per requirement.

Procedure for calibration curve

Suitable aliquots of azithromycin or sparfloxacin solutions in acetonitrile or cephalexin monohydrate in methanol were transferred into 10 ml volumetric flasks. To it, 2 ml of $8x10^{-5}$ M bromophenol blue solution for azithromycin, 5ml of $8x10^{-5}$ M bromophenol blue solution for sparfloxacin and 6ml of 5×10^{-5} M bromophenol blue solution for cephalexin monohydrate was added and volume was made up to 10 ml with respective solvents. This made the final concentration of bromophenol blue to 16 µM, 40 µM and 30 µM respectively. After 15, 10 and 5 minutes, for three drugs respectively, the absorbance of bluish-green solution was measured at 595 nm, 620 nm and 600 nm against the appropriate reagent blank.

Procedure for dosage form

For analysis of tablets or capsules, five tablets or capsules were weighed and average weight of one tablet or capsule was determined. They were powdered and 0.05 g of azithromycin and 0.04 g of cephalexin monohydrate exactly weighed and shaken with 30 ml of acetonitrile and methanol respectively for 30 minutes. These solutions were filtered with Whatmann filter paper no. 40 and made up to 50 ml with respective solvents. The same procedure was applied for oral suspension of azithromycin using 1ml suspension. Suitable aliquots were analysed using general procedure.

Procedure for urine sample

The urine samples were collected from healthy volunteer. In order to analyse urine samples, 10 ml of urine samples were spiked with azithromycin and sparfloxacin separately. The drugs were extracted with dichloromethane and evaporated to dryness. The residue was dissolved in respective solvents and analysed using general procedure.

Results and Discussion

Effect of solvent

Various solvents like methanol, ethanol, acetone, dichloromethane, dichloroethane, dimethylsulphoxide, chloroform and acetonitrile were used to check the solubility, complex formation, to achieve maximum sensitivity and product stability. Acetonitrile for azithromycin, sparfloxacin and bromophenol blue and methanol for cephalexin monohydrate were found to be most suitable solvents.

Absorption spectra

Solutions of azithromycin or sparfloxacin and bromophenol blue in acetonitrile and cephalexin monohydrate in methanol were prepared. Absorption spectra of these solutions were recorded individually. When the drug solutions were mixed with BPB solution, bluish-green complexes were formed with absorption maxima at 595 nm for azithromycin, 620 nm for sparfloxacin and 600 nm for cephalexin monohydrate respectively (Figure 2). Under experimental conditions, the reagent as well as the drug showed negligible absorbance while the complexes showed maximum absorbance at these wavelengths. Hence, it was concluded that the studies for quantitative analysis could be carried out at these wavelengths.

Stoichiometric relationship and stability studies

Composition and stability constants of these complexes were established by applying Job's method of continuous variation. Equimolar solutions of the drug and the reagent were mixed in various proportions and absorbance of each mixture was recorded. The results indicated that the complexes are formed in the ratio of 2:1 (D:R) (Figure 3). Mechanism of formation of such complexes with composition $(DH)^{+}_{2}$ $(R)^{2-}$ has been discussed by Gainza and Konyeaso [25]. The suggested structure of ion pair complex of azithromycin can be shown as in Figure 4. Similar structures can be suggested for sparfloxacin as well as for cephalexin monohydrate. The stability constants (log K) values were found to be 6.19 ± 0.04 for azithromycin, 5.00 ± 0.07 for sparfloxacin and 4.05 ± 0.05 for cephalexin monohydrate respectively showing high stability of the complexes. The large negative values of Gibb's free energy change for complex formation show spontaneity of process (Table 1).

Effect of time

Mixtures of drug and reagent were prepared; the optimum reaction time was determined by recording the absorbance of the formed complexes at different time intervals. The variation has been shown in Figure 5. It was found that the complexes were formed instantaneously at room temperature. The absorbance was found to be steady after

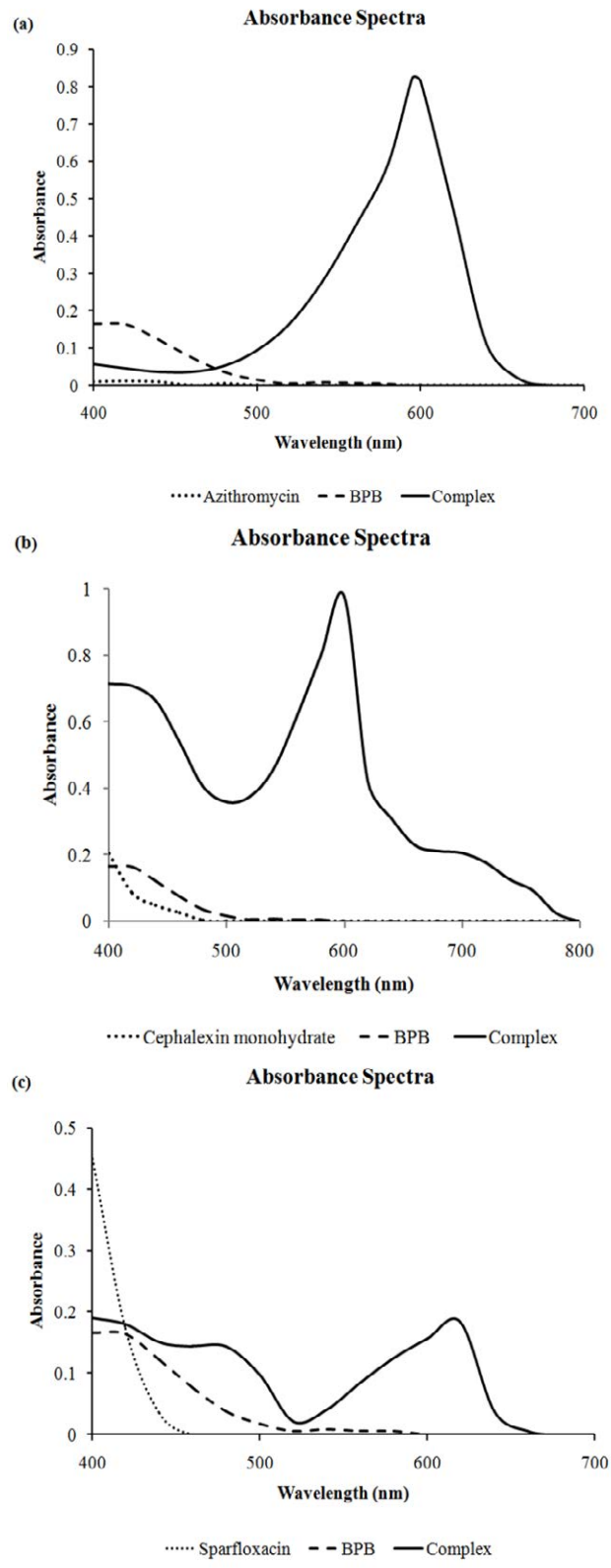

Figure 2: Absorption spectra of drug, reagent and complexes: a) azithromycin (767 µg mL^{-1}), BPB (80 µM) and their complex, b) cephalexin monohydrate (3654 µg mL^{-1}), BPB (80 µM) and their complex and c) sparfloxacin (39 µg mL^{-1}), BPB (80 µM) and their complex.

15, 5 and 20 minutes for azithromycin, sparfloxacin and cephalexin monohydrate respectively. Hence, in order to remove time effect, all observations were made after respective time interval of complex formation. The sample solutions were kept in air tight flasks to avoid any evaporation losses.

Effect of reagent concentration

The optimum concentration of bromophenol blue was determined by adding various concentrations of bromophenol blue to the drugs. The colour intensity was found to increase with addition of bromophenol blue up to a particular concentration and then either decrease or remain steady. The absorbance was found to be maximum at bromophenol blue concentration of 16 µM for azithromycin, 40 µM for sparfloxacin and 30 µM for cephalexin monohydrate. Therefore, these concentrations were used to prepare calibration curve (Figure 6).

All the observations were made in triplicate and mean of the three values have been plotted in each graph.

Analytical parameters

Calibration curves for azithromycin, sparfloxacin and cephalexin monohydrate were plotted between absorbance and

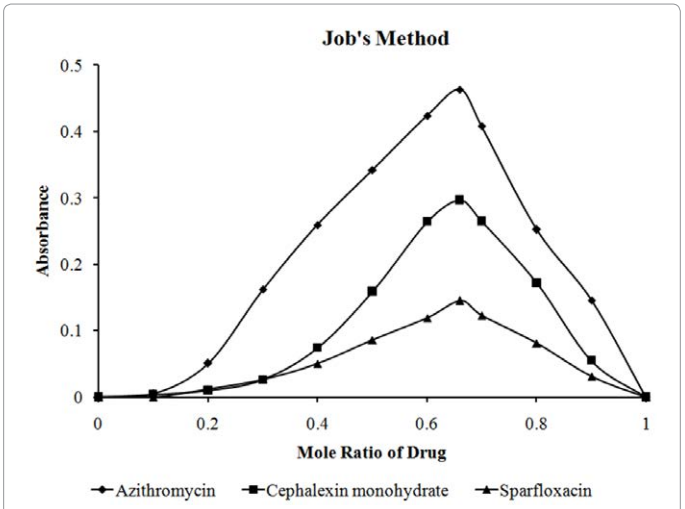

Figure 3: Continuous variation plots for the ion pair complexes of azithromycin (3×10^{-5} M), cephalexin monohydrate (5×10^{-4} M) and sparfloxacin (1×10^{-4} M) with BPB.

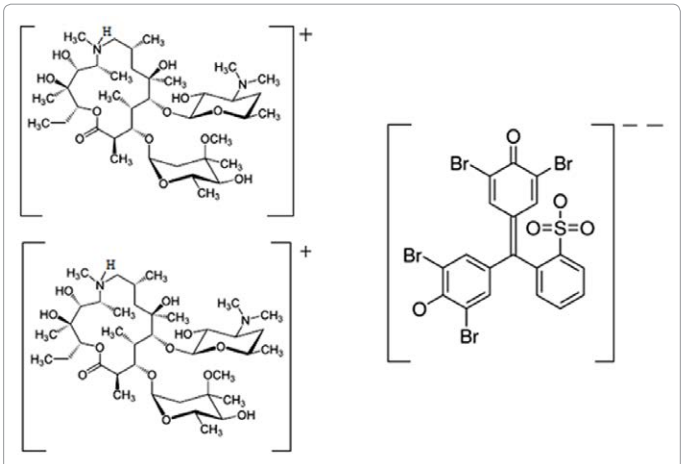

Figure 4: Schematic representation of ion pair complex of azithromycin.

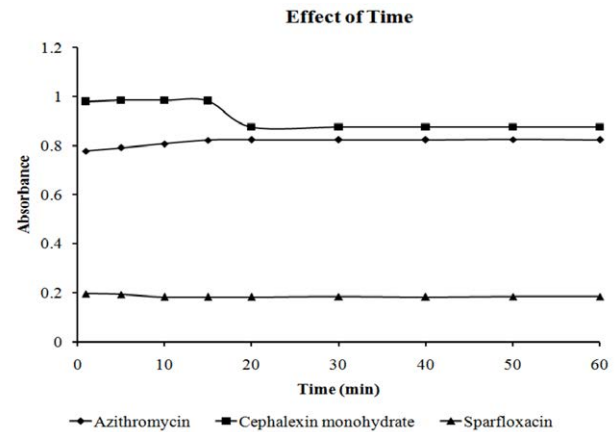

Figure 5: Effect of time on the absorbance of ion pair complexes of azithromycin, cephalexin monohydrate and sparfloxacin with BPB.

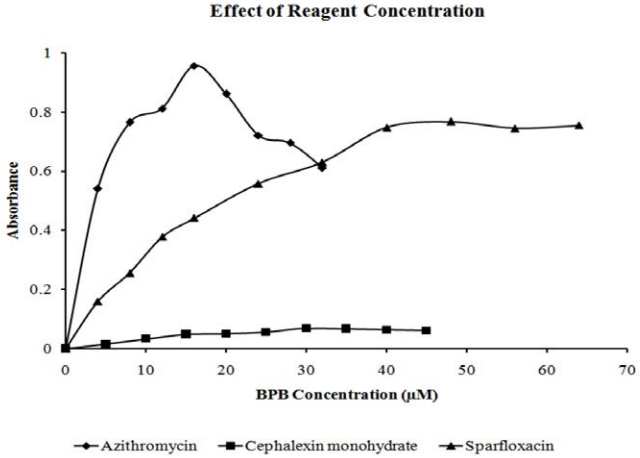

Figure 6: Effect of BPB concentrations on ion pair complexes of azithromycin (100 µg mL^{-1}), cephalexin monohydrate (100 µg mL^{-1}) and sparfloxacin (100 µg mL^{-1}).

concentration. A linear absorbance-concentration correlation was found to be 0-50 µg mL^{-1}, 10-80 µg mL^{-1} and 10-170 µg mL^{-1} with correlation coefficients 0.9871, 0.9967 and 0.9992 respectively for the three drugs. The molar absorptivity values were found to be 1.369×10^4, 3.774×10^3 and 2.620×10^2 L mol^{-1} cm^{-1} respectively. The limit of detection and limit of quantitation were calculated in accordance with equations,

$$LOD = 3\sigma/S$$

$$LOQ = 10\sigma/S$$

The σ is the standard deviation of the response and S is the slope of calibration graph. The detection limits were found to be 0.10, 0.21 and 1.69 µg mL^{-1} respectively with Sandell's sensitivity 0.0559, 0.1034 and 1.3920 µg cm^{-2}, respectively. Under optimum conditions, various analytical parameters were obtained (Table 1). The value of correlation coefficient indicates good linearity for all the three systems. Values of molar absorptivities and Sandell's sensitivities reflect high sensitivity of these methods.

Recovery Studies

Recovery studies were carried out for all the three drugs using calibration curve at three different concentrations over the linear range. The recoveries were found to be in vicinity of 100% (Table 2).

Accuracy and precision

Accuracy expresses the closeness between the reference value and the found value. It was evaluated as percentage relative error between the measured concentrations and taken concentrations of these three drugs. The precision of these methods were calculated in terms of intermediate precision (intra-day and inter-day). Three concentrations of all the three drugs were analysed in three replicates during same day (intra-day precision) and three consecutive days (inter-day precision). RSD (%) values of intra-day and inter-day studies show high degree of precision (Table 3).

Robustness and ruggedness

The robustness of all the three systems were evaluated by making small incremental changes in time (25 ± 5 min) and the effect of the change of absorbance were studied on the ion-pair complex. It was found that the changes had negligible influence on the results and expressed as % RSD values. The ruggedness of these methods were evaluated by performing analysis using two different cuvettes and expressed in % RSD values as shown in Table 4.

Interference Studies

The effect of common excipients and other additives were tested for possible interferences in the assay. Various amount of excipients such as lactose, dextrose, cellulose, magnesium stearate, talc, starch, gelatine were added to known amount of three drugs and were examined using developed procedure. It was found that these compounds have negligible solubility in acetonitrile and methanol therefore; they did not interfere in the determination of these three drugs even when present 100 times in excess. To evaluate the selectivity of the proposed method of analysis in pharmaceutical formulations, placebo blank was compared with synthetic mixture and the results are incorporated as % RSD (Table 5).

Applications

The proposed methods have been successfully applied for the determination of azithromycin and cephalexin monohydrate in their pharmaceutical formulation such as tablets, capsules and oral

Parameters	Azithromycin	Sparfloxacin	Cephalexin monohydrate
λ_{max} (nm)	595	620	600
Molar ratio (D:R)	2:1	2:1	2:1
Linear range (µg mL^{-1})	0-50	10-80	10-170
Slope	0.0178	0.0096	0.0007
Intercept	0.0418	-0.1028	-0.0080
Sandell's sensitivity (µg cm^{-2})	0.0559	0.1034	1.3920
Correlation coefficient	0.9871	0.9967	0.9992
Stability constant (log K)	6.19 ± 0.04	5.00 ± 0.07	4.05 ± 0.05
Molar absorptivity (L mol^{-1} cm^{-1})	1.369×10^4	3.774×10^3	2.620×10^2
LOD[a] (µg mL^{-1})	0.10	0.21	1.69
LOQ[b] (µg mL^{-1})	0.35	0.71	5.63
%RSD[c] (at 10 µg mL^{-1})	0.55	0.33	3.06
ΔG^{od} (kJ mol^{-1})	-2.725×10^3	-2.393×10^3	-1.938×10^3

[a]Limit of detection
[b]Limit of Quantitation
[c]Relative standard deviation
[d]Free energy change

Table 1: Qualitative and statistical parameters for proposed methods.

Drug	Taken (µg mL^{-1})	Found[a] (µg mL^{-1})	%Recovery	Mean ± SD
Azithromycin	10.0	10.5	105.0	103.6 ± 1.3
	20.0	20.5	102.5	
	30.0	31.5	103.3	
Sparfloxacin	25.0	26.0	104.0	100.6 ± 4.4
	45.0	43.0	95.6	
	65.0	66.5	102.3	
Cephalexin monohydrate	55.0	53.0	96.4	98.6 ± 2.3
	95.0	93.5	98.4	
	145.0	146.4	101.0	

[a]Average of three determinations.

Table 2: Recovery studies of the three drugs.

Drug	Taken (µg mL^{-1})	Intra-day (n=3)			Inter-day (n=3)		
		Found[a] (µg mL^{-1})	% RSD	%RE	Found[a] (µg mL^{-1})	% RSD	%RE
Azithromycin	10.0	10.5	0.5	5.0	10.3	0.8	3.0
	20.0	20.5	0.3	2.5	21.0	0.4	5.0
	30.0	31.0	0.1	3.3	31.5	0.2	5.0
Sparfloxacin	25.0	26.0	6.7	4.0	26.0	6.7	4.0
	45.0	43.0	3.8	4.4	43.0	3.8	4.4
	65.0	66.5	3.8	2.3	66.5	3.8	2.3
Cephalexin monohydrate	55.0	53.0	0.3	3.6	54.5	4.0	0.9
	95.0	93.5	1.7	1.6	95.2	2.6	0.2
	145.0	146.4	0.5	1.0	147.0	1.6	1.4

[a]Average of three determinations
% RSD: Relative standard deviation
%RE: Relative error

Table 3: Evaluation of intraday and interday precision and accuracy.

Sample	Taken (µg mL^{-1})	Robustness Reaction time[a] (n=3) % RSD	Ruggedness Inter cuvettes (n=2) % RSD
Azithromycin	10.0	0.4	0.3
	20.0	0.9	0.7
	30.0	0.3	0.5
Sparfloxacin	25.0	0.8	0.4
	45.0	0.2	0.6
	65.0	0.8	0.5
Cephalexin monohydrate	55.0	0.5	0.7
	95.0	0.2	0.6
	145.0	0.6	0.3

[a] Reaction time- 20,25 and 30 min

Table 4: Evaluation of robustness and ruggedness.

Excipients	Amount added (mg)	Azithromycin[a] % Recovery ± SD	Cephalexin monohydrate[b] % Recovery ± SD
Lactose	20	99.3 ± 0.7	98.2 ± 0.3
Dextrose	20	98.4 ± 0.9	99.5 ± 0.7
Cellulose	20	98.5 ± 0.8	101.1 ± 0.6
Magnesium stearate	20	101.2 ± 0.4	99.4 ± 0.4
Talc	20	98.4 ± 0.4	100.2 ± 0.4
Starch	20	99.5 ± 0.2	99.4 ± 0.7
Gelatine	20	100.2 ± 0.5	98.8 ± 0.6

[a]40 (µg m L^{-1}) of azithromycin
[b]100 (µg m L^{-1}) of cephalexin monohydrate

Table 5: Determination of drugs in presence of excipients.

suspension. Calibration curve method and standard addition method were adopted for quantitative analysis (Table 6). Urine samples were successfully analysed with azithromycin and sparfloxacin. Quantitation was carried out using calibration graph (Table 7).

Conclusion

The developed methods were found to be versatile and have many advantages over the previously reported methods. A comparison of these methods with reported methods have been presented in (Table 8). It has been observed that, bromophenol blue methods have wider linear ranges as compared to most of the reported methods [11,12,14-16,20-23]. These proposed methods are more sensitive compared to some of the established method as shown by the molar absorptivity [14]. The detection limits are lower than the reported methods [11,13,18,19,23].

These methods require only dye and solvents which are comparatively cheaper and readily available. These methods are simple as they do not involve adjustment of critical conditions like temperature, pH or tedious sample preparation. These methods have many advantages over other analytical methods due to its simplicity, sensitivity, rapidity, low cost instrumentation, accuracy, free from interference by common additives and excipients. Due to these advantages these methods can be used for quality control and routine analysis.

Acknowledgements

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Pharmaceutical Preparation	Labelled Amount (mg)	Found Amount (mg) [a]	
		Calibration curve Method	Standard addition Method
Lazithro tablets [b]	250	249.7 ± 2.7	247.6 ± 3.4
Azee tablets [c]	250	260.7 ± 6.9	249.3 ± 1.1
Azithral Liquid[d]	20	20.2 ± 1.2	21.1 ± 1.8
Phexin capsules[e]	500	493.1 ± 3.0	499.1 ± 5.9
Ceff tablets[f]	250	247.6 ± 3.0	251.7 ± 1.9
Phexin oral suspension[g]	250	254.5 ± 1.6	245.2 ± 2.7

[a](Avg ± SD) of three determinations
[b]Lesanto laboratories
[c]Cipla Pharmaceuticals
[d]Alembic Pharmaceutical Limited
[e,g] GlaxoSmithKline Limited
[f] Lupin Limited

Table 6: Analysis of pharmaceutical formulations.

Drug	Taken (µg mL^{-1})	Found[a] (µg mL^{-1})	% Recovery	% RSD
Azithromycin	10.0	10.5	105.0	0.7
	15.0	14.5	96.7	0.7
	20.0	19.5	97.5	0.8
	30.0	31.0	103.3	0.4
	35.0	36.0	102.9	0.1
Sparfloxacin	20.0	21.0	105.0	1.4
	30.0	29.5	98.9	0.6
	40.0	38.5	96.3	0.2
	50.0	51.0	102.0	0.1
	60.0	59.0	98.3	0.3

[a]Average of three determinations
%RSD Relative standard deviation

Table 7: Analysis of spiked urine samples.

Drug	Reagent used	Linear range (μgmL^{-1})	LOD (μgmL^{-1})	Molar absorptivity ($L\ mol^{-1}\ cm^{-1}$)	Applications	Ref.
Azithromycin	Quinalizarin	4-20	0.35	--	Tablets	12
	Eosin Y	1-10	--	--	Human urine, plasma	13
	Rose Bengal	4-20	0.31	3.78×10^4	Tablets, capsules, syrup	14
	Chloranilic acid	5-225	--	2.4×10^3	Tablets	15
	7,7,8,8-tetracyanoquinodimethane	0-30	--	2.7×10^4 5.0×10^4	Tablets	15
	Bromophenol blue	0-50	0.10	1.369×10^4	Tablets, syrup, Human Urine	Present work
Sparfloxacin	Bromocresol purple	5-25	--	2.988×10^4	Tablets	16
	Bromocresol green	5-25	--	3.152×10^4	Tablets	16
	Ammonium vanadate	0.8-28	--	--	Tablets, Human Urine	17
	Mo(V)-thiocyanate	10-150	9.00	62×10^3	Tablets	19
	p- dimethyl amino-benzaldehyde	2-80	0.22	4.9×10^3	Tablets, Human urine, Blood serum	20
	Bromophenol blue	10-80	0.21	3.774×10^3	Human Urine	Present Work
Cephalexin monohydrate	Molybdenum blue	0-45	--	--	Capsules	21
	Sodium 1,2-napthoquinone-4-sulphonate	1.5-34	0.49	1.79×10^3	Capsules	22
	p-benzoquinone	10-160	--	1.37×10^3	Capsules	23
	Iodine	6-40	1.37	9.64×10^3	Capsules	24
	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone	40-180	2.76	2.22×10^3	Capsules	24
	7,7,8,8-tetracyanoquinodimethane	4-12	0.23	31.90×10^3	Capsules	24

Table 8: Comparison of present work with reported methods.

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