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Der Pharma Chemica, 2015, 7(12):160-167
(<http://derpharmachemica.com/archive.html>)



ISSN 0975-413X
CODEN (USA): PCHHAX

Quantitative determination of azithromycin by extractive ion-pair spectrophotometry using methyl orange as ion pairing agent

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ABSTRACT

Azithromycin is a macrolide antibiotic used in the treatment of bacterial infections. The present study is aimed at developing and validating a new colorimetric method for its determination in both pure form and in tablet formulation. The developed method utilizes ion-pair spectrophotometry and is employed for the quantitative evaluation of the drug using the acidic dye - methyl orange (MO) as ion-pairing agent at pH 2 (phthalate buffer). The yellow ion-pair complex was extracted with butanone and spectrophotometrically estimated at 422.5 nm. The developed method was validated according to ICH guidelines. The ion-pair complex of Azithromycin and methyl orange obeyed Beer's law in the range of 10 -50 µg/ml with a correlation coefficient (r^2) of 0.971. Recovery by the method was also good, with a relative standard deviation (%RSD) of 0.65 -1.06; precision (inter-day 0.675 - 0.883 and intra-day 0.712 - 1.072) were also within validation limits. The limit of detection (LOD) and limit of quantitation (LOQ) were 1.1 and 5.7 µg/ml respectively. The developed method was successfully applied to determine Azithromycin in tablet formulation and was demonstrated to be accurate, precise and reproducible. It is also sufficiently sensitive and specific for the determination of the drug in bulk and formulation with satisfactory results.

Keywords: Azithromycin, Methyl orange, Ion-pair complexation, method development and validation

INTRODUCTION

Azithromycin is an azalide, a subclass of macrolide antibiotics derived from erythromycin but more potent than it against certain bacterial species [1]. It differs from erythromycin structurally in having a methyl-substituted nitrogen atom incorporated into the lactone ring [2] and this modification increases its acid stability. Chemically, it is (2R, 3S, 4R, 5R, 8R, 10R, 11R, 12S, 13S, 14R) -13-[(2,6-dideoxy-3-c-methyl-3-0-methyl- α -L-ribo-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11-[(3,4,6-trideoxy-3-(dimethylamino)- β -D-xylo-hexopyranosyl)oxy]-1-oxa-6- azacyclopentadecan-15-one [3]. It exerts its antibacterial action by interfering with protein synthesis specifically binding to the 50S ribosomal subunit of the bacteria and ultimately inhibiting RNA-dependent protein synthesis by preventing transpeptidation and translocation reactions [4]. It is effective against a wide variety of bacteria such as *Hemophilus influenza*, *Streptococcus pneumonia*, *Mycoplasma pneumonia*, *Staphylococcus aureus*, *Mycobacterium avium* and many others [5]. Azithromycin is indicated for the treatment of chronic obstructive pulmonary disease, non-eosinophilic asthma, acute bacterial sinusitis, pneumonia tonsillitis, acute pelvic inflammatory diseases and a host of other bacterial infections [6].

The British pharmacopoeia [7] describes a liquid chromatographic method for the analysis of azithromycin. A review of existing literature revealed that a number of unofficial methods also exist for its analysis. Some of these include: Near infra-red (NIR) spectrometry [8], HPLC [9, 10, 11]. Abdelmageed and Osama [12] developed a novel colorimetric method for the drug that involved its condensation with 2, 4-dinitrophenyl hydrazine to form an intensely colored chromogen that was subsequently assayed. Another worker [13] developed an ion pair spectrophotometric method for the drug with eosin-G dye with the resultant complex extracted into chloroform and assayed. Other analytical methods that have been employed for the determination of the drug include: TLC-densitometry [14], Voltametry [15], Mass spectrometry [16], amperometry [17], spectrofluorimetry [18], chemiluminescence [19].

In this study, a simple and cost effective colorimetric method is proposed for the analysis of Azithromycin in pure form and in tablet dosage forms. The method is based on the ion-pair complex formation between Azithromycin and methyl orange dye in acidic medium followed by extraction of the complex into butanone. The method is rapid and easy to perform. In addition, it avoids the need for costly instrumentation required by some other known methods.

MATERIALS AND METHODS

Chemicals and Reagents

Azithromycin Reference Tablets was kindly donated by CHAN Medi-Pharm, Rayfield, Jos, Nigeria. Four (4) different brands of Azithromycin tablets were purchased from a retail Pharmacy outlet in Jos, Nigeria. Methyl orange (Surechem, Needham, England). The following were obtained from Sigma-Aldrich, Germany: Dimethyl formamide, Dichloromethane, Acetonitrile, Chloroform, Potassium hydrogen-phthalate, Butanone (Hopkins and Williams Ltd, England), Hydrochloric acid (May & Baker Ltd. Dagenham, England), Sodium hydroxide (Avondale Laboratory Ltd. England). All other reagents were of analytical reagent grade and used without further purification. Double distilled water was used to prepare all solutions. Freshly prepared solutions were also used for method development and validation.

Instrumentation

pH measurements were carried out using a digital pH meter (GallenKamp, England). A Shimadzu UV-VISIBLE double beam spectrophotometer with 1cm matched quartz cells was used to take all spectral measurements with the aid of the Shimadzu UV probe software.

PREPARATION OF REAGENTS

Preparation of Azithromycin standard solution

The stock solution of 1 mg/ml was prepared by weighing and dissolving 100 mg of Azithromycin reference powder in 10 ml dilute HCl (0.05 M), and making up to 100 ml with HCl in a volumetric flask. Working standards were prepared by appropriate dilution of the standard stock solution.

Preparation of Potassium Hydrogen Phthalate buffer solution (pH 2.0)

A 2.553 g potassium hydrogen phthalate was weighed and dissolved in 0.25 ml of 0.1 M HCl. The volume was then made up to 250 ml with distilled water in a volumetric flask. The pH was then adjusted to 2.0.

Preparation of Methyl orange dye (0.01 % w/v)

A 0.01 g of methyl orange powder was weighed and dissolved in 10 ml methanol and this was made up to 100 ml with distilled water in a volumetric flask. Working solutions were always freshly prepared.

General Procedure

From the 100 µg/ml working standard solution, aliquots were transferred to a series 100 ml separating funnels and 3 ml of phthalate buffer solution (pH 2) and 1 ml of % w/v methyl orange was added to each separating funnel and shaken vigorously and allowed to react for 2 minutes. This was followed by the addition of 5 ml of butanone. The contents were shaken well and kept aside to allow for separation. The Butanone layer was then separated and the extraction was repeated using a fresh 5ml aliquot of butanone. The yellow colored organic phases were combined. All the above was done in triplicates.

Determination of maximum absorbance (λ_{\max}) and Linearity

Absorption spectrum of the yellow Azithromycin-methyl orange ion pair complex was determined by scanning the extracted chromogen in the visible region (350 – 800 nm). To study if Beers Law was obeyed, a calibration curve was constructed by plotting the measured absorbance versus concentration.

Determination of azithromycin in tablet formulation

Twenty tablets of the commercial product were weighed and the mean tablet weight was calculated before they were ground to fine powder. A known quantity of the powder was accurately weighed and transferred into a 50 mL volumetric flask. This was dissolved with HCl and the volume made up to mark with distilled water, shaken well and filtered through Whatman filter paper no. 40. Suitable aliquots of this solution were taken for the assay of Azithromycin. Four different brands of azithromycin were analyzed using the proposed method as described in the general procedure above.

METHOD VALIDATION**Accuracy**

The accuracy of a proposed method was evaluated through recovery studies whereby a known quantity of the pure drug was used to spike pre-analyzed samples and subjected to the analytical procedure previously described.

Precision

Precision of the proposed method was determined by evaluating the inter-day and intra-day variation which were obtained by replicate analysis ($n = 5$) of calibration standards at three different concentration levels, five times per day on five consecutive days.

LOD and LOQ

The limit of detection (LOD) and limit of quantitation (LOQ) of the method was established using the formula: $LOD = 3 s/k$ and $LOQ = 10 s/k$, where s is the standard deviation of replicate determination values under the same conditions as for the sample analysis in the absence of the analyte, and k is the slope of the calibration graph.

OPTIMIZATION OF EXPERIMENTAL CONDITIONS.

The optimal reaction conditions for complex formation were also investigated in order to achieve complete reaction and also obtain maximum absorbance of the complex formed. Preliminary experiments were therefore conducted to study the effects of pH of the buffer, reaction time, shaking time, reagent concentration, extraction solvent and reagent volume.

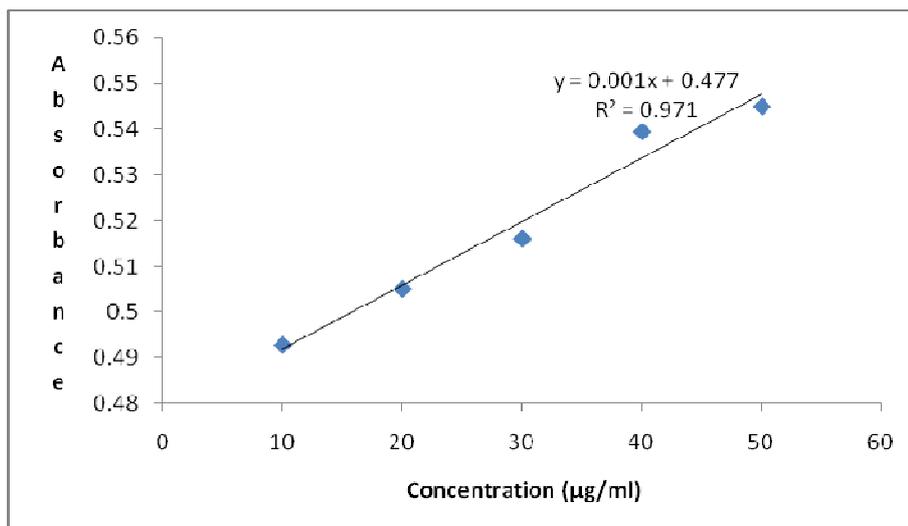
RESULTS

Figure 1: Calibration curve for Azithromycin-methyl orange ion pair complex

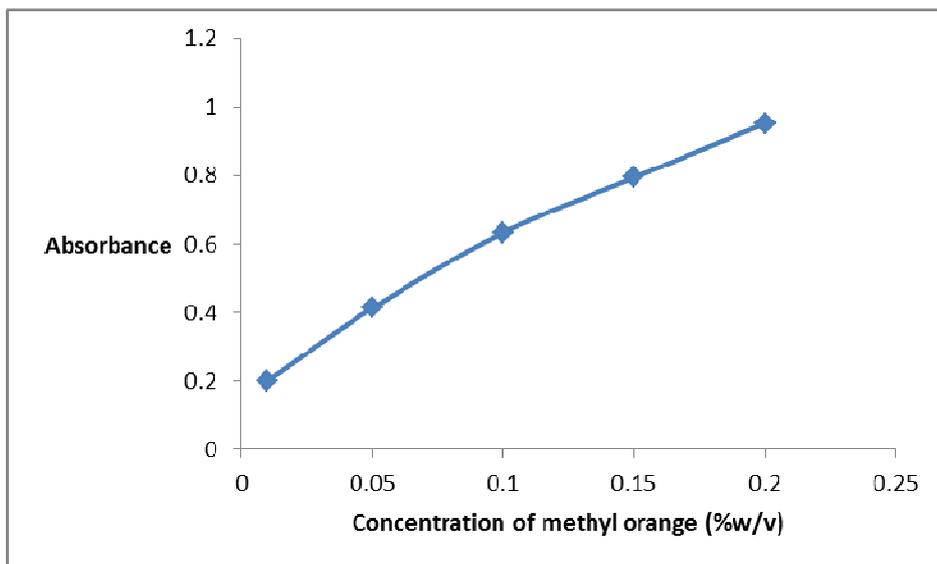


Figure 2: Effect of methyl orange concentration on Azithromycin-methyl orange ion pair complex

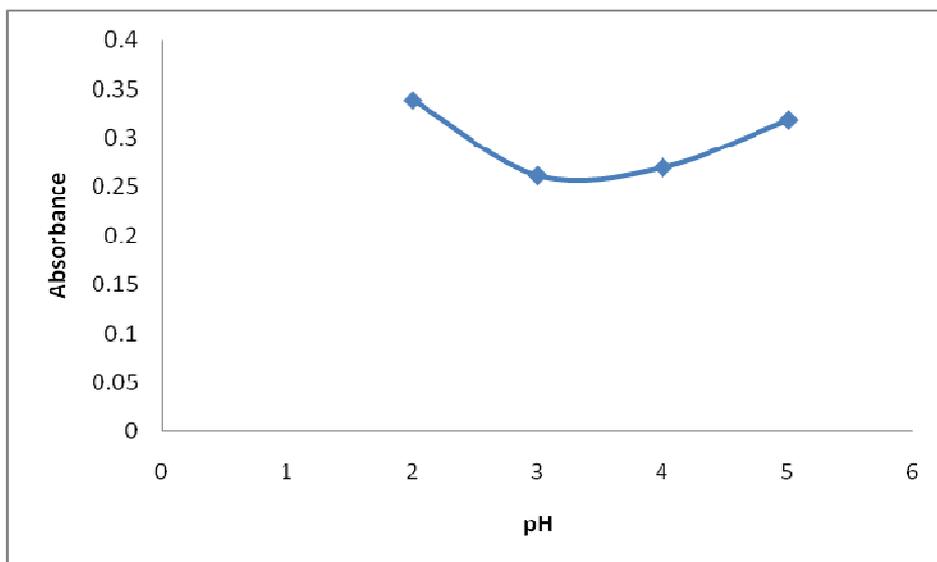


Figure 3: Effect of pH on Azithromycin-methyl orange ion pair complex

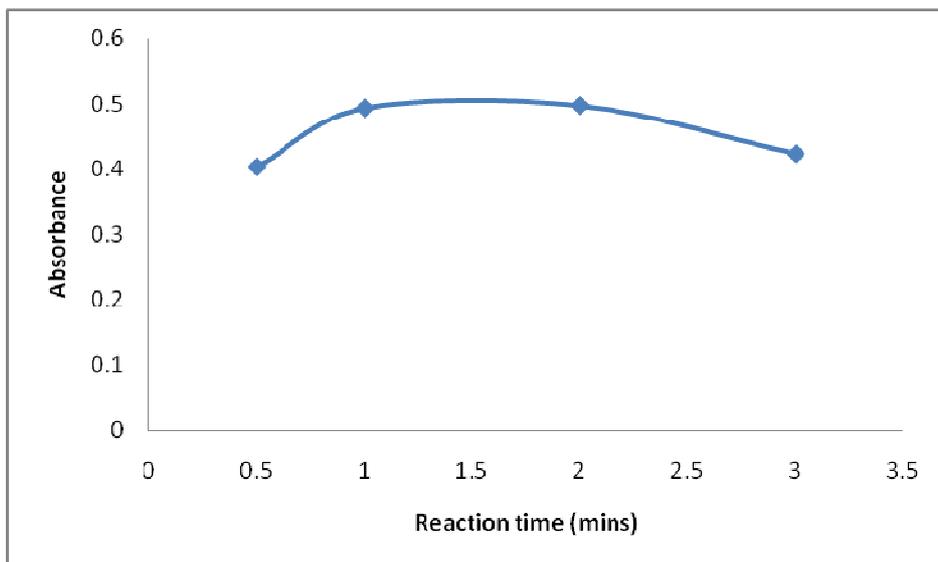


Figure 4: Effect of reaction time on Azithromycin-methyl orange ion pair complex

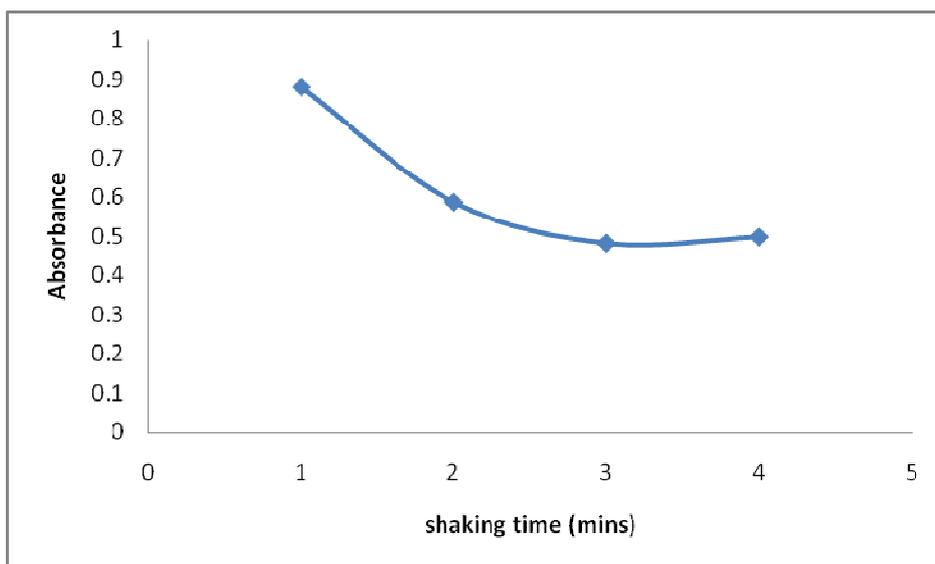


Figure 5: Effect of shaking time on Azithromycin-methyl orange ion pair complex

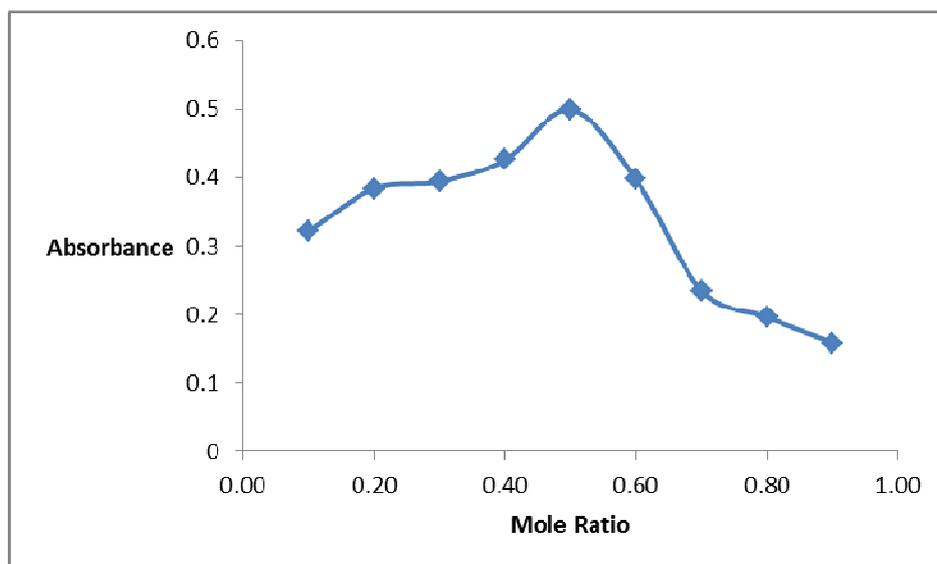


Figure 6: Continuous variation plot for Azithromycin-methyl orange ion pair complex by Jobs method

Table 1: Percentage content of brands by proposed method

Brand	Label claim	% Content by proposed method \pm SD
A	500	95.6 \pm 0.0096
B	250	102.7 \pm 0.330
C	500	114.1 \pm 0.005
D	500	101.3 \pm 0.038

From the preliminary experiments, it was found that potassium hydrogen phthalate buffer was most suitable and butanone was also discovered to be the most effective solvent for extracting the ion pair complex among the various solvents tested which include Chloroform, dichloromethane, butanone, dimethylformamide, acetonitrile. A scan of the extracted Azithromycin-methyl orange complex showed maximum absorbance at 422.5 nm. Beers Law was also obeyed in the range of 10 – 50 μ g/ml. The regression equation for the curve was: $y = 0.001x + 0.477$ ($r^2 = 0.971$). The limit of detection (LOD) and limit of quantitation (LOQ) were determined to be 1.1 μ g /mL and 5.7 μ g /mL respectively. Molar absorptivity and Sandells' sensitivity were similarly calculated to be 61,003.66 $L mol^{-1} cm^{-1}$ and 0.802 μ g/cm²/0.001 absorbance units respectively. The inter-day variation ranged between 0.675 – 0.883 % and the intra-day variation ranged from 0.712 – 1.072 % for the method.

DISCUSSION

EFFECT OF REAGENT CONCENTRATION

The influence of methyl orange concentration on the colour formation was studied by testing different concentrations (0.01-0.2) % w/v of methyl orange. Results showed that 0.01 % w/v of methyl orange solution was required to achieve maximum color intensity and maximum absorbance (Figure 2).

EFFECT OF PH

The ion-pair complex between azithromycin and methyl orange depends largely on the pH of the medium. It was found that maximum color intensities was obtained at pH 2, showing that optimum reaction occurred at very strongly acidic pH (Figure 3).

EFFECT OF REACTION TIME

The optimum time for reaction as illustrated by maximum colour development when the reagents are mixed was also determined. It was found that the reaction product attained maximum absorbance about 2 minutes after Azithromycin was mixed with methyl orange (Figure 4).

EFFECT OF SHAKING TIME

The optimum shaking time which is the time required for quantitative extraction of the ion pair complex with butanone was studied. It was determined to be one minutes (Figure 5).

EFFECT OF REAGENT VOLUME

The effect of reagent volume (methyl orange) on the absorbance of the complex was studied. 1 ml of methyl orange dye was found to be optimal for formation of azithromycin-methyl orange complex.

EFFECT OF EXTRACTION SOLVENT

Different extraction solvents were tested for the extraction of azithromycin - methyl orange complex in order to determine the one that gave the most quantitative extraction. Butanone was found to produce the best extraction as it was able to quantitatively extract the yellow colored complex.

In the acidic medium provided by the phthalate buffer, the anionic dye methyl orange formed an ion pair complex with the positively charged quaternary amine of Azithromycin with the complex being held together by the electrostatic force of attraction between the opposite charged ions. The yellow ion pair complex formed showed maximum absorbance at 422.5 nm and Beers Law was obeyed (Figure 1). The formation and extraction of the Azithromycin-methyl orange complex depends on the concentration and volume of methyl orange, pH of the medium, reaction time, shaking time and nature of organic solvents used. All these parameters were optimized by a number of experiments as discussed above. The absorbance of the ion-pair complex was assessed at intervals from the time of extraction and it was found to remain stable for over six (6) hours. The stoichiometry of the reaction between the drug and the dye was also determined using Jobs continuous variation method and it was found to be a 1:1 ratio (Figure 6). The developed method was applied on commercially available brands of azithromycin and results showed the drug content of these products was in accordance with the label claims (Table 1).

CONCLUSION

The developed colorimetric method is sensitive, simple and reproducible for the estimation of azithromycin in tablet and capsule formulation. The sensitivity of the method makes it suitable for the quantitative determination of pure azithromycin and azithromycin in dosage formulations.

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