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Novel Liquid Chromatographic Method for the Simultaneous Evaluation of Erythromycin and Azithromycin in Human Urine

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Abstract

A simple new method for the quantitative determination of erythromycin (ERY) and azithromycin (AZI) concentrations in spiked human urine by high-performance liquid chromatography (HPLC) was developed. Separation was performed by using an end-capped ODB RP_{18} column as a stationary phase and mobile phase consisting of acetonitrile –2-methyl-2-propanol–hydrogenphosphate buffer, pH 6.5, with 1.5% triethylamine (33:7: up to 100, v/v/v), delivered at a flow-rate of 1.0 mL min^{-1}. UV detection is performed at 215 nm for erythromycin and 210 nm for azithromycin. The procedure was quantitatively characterized and, results of selectivity, linearity, detection and quantification limits, precision and accuracy were very satisfactory. The suitability of the method for simultaneous evaluation of the both macrolides was demonstrated by the analysis of spiked samples of human urine. Recovery experiments revealed recovery of 96.54–112.07%.

1. Introduction

Macrolide antibiotics constitute, relatively, new class of antibiotics, which have been demonstrated to be highly active not only against gram-positive and gram-negative cocci but also against non-classical pathogens. Macrolides are considered to be medium-spectrum antibiotics, which have therefore been used to treat a wide range of infections in medical and veterinary field. They interfere with RNA–dependent bacterial protein synthesis, resulting in a bateriostatic effects on pathogens [1]. Currently, erythromycin and azithromycin are the most widely prescribed in clinical use.

Erythromycin is a polyhydroxylactone that contains two sugars. The amino sugar, D-desosamine, is attached through a *β*-glycosidic linkage to the C-5 position of the lactone ring, and a tertiary amine of desosamine. Azithromycin is a novel semi-synthetic macrolide similar to erythromycin but composed of 15-membered lactone ring. As at erythromycin, cladinose and desosamine sugar residues are attached at positions 3 and 5 [2]. The chemical structures of erythromycin and azithromycin are depicted in Figure 1.

Since macrolides are used in both animal and human medicine, wide spectrums of samples of different origins are subjected to the analysis of this family of antibiotics. The analysis of animal tissues (such as liver and kidney) mainly involves residue analysis, whereas the analysis of human biological fluids (such as serum and urine) mainly involves pharmacokinetic and clinical studies. The analysis of macrolide antibiotics in urine requires pretreatment, including the clean-up and pre-concentration of urine samples, because the urine medium is very complex, and the concentration levels of macrolide antibiotics are quite low. Liquid–liquid extraction [3,4] and simple organic solvent precipitation [5,6] are the most popular pre-treatment methods.

Determination of antibiotics, including macrolides, is mainly carried out by microbiological assays [7], TLC, paper chromatography, GC, capillary zone electrophoresis [8–10]. Several reports have described the quantitative determination of azithromycin and erythromycin in raw material, drugs and biological matrices included spectrophotometry [11], Spectrofluorimetry [12], NIR spectroscopy [13], voltammetry [14,15], and HPLC with fluorescent [16,17], ultraviolet (UV) [18], electrochemical [19] and mass spectrometric detection [20]. UV detection in concert with HPLC has been conventionally used for the determination of macrolides since introduced by Tsuji and Groetz [21]. However, for the simultaneous determination of the both macrolides in human urine by HPLC, there are no official methods described neither in the official codes nor in the literature. These methods were time-consuming, tedious, and committed to sophisticated and expensive analytical instruments. Therefore, the development of new alternative method for the determination of

macrolides that can overcome the disadvantages of the existing methods is essential. This led us to develop a quantitative HPLC method using conventional materials, reagents and equipment. UV absorption was chosen as principle detection since it is the most universal and it is available in most of laboratories.
The present manuscript describes a simple, rapid, precise, accurate, time saving and validated isocratic reversed-phase HPLC method for the simultaneous determination of azithromycin and erythromycin in the same spiked human urine.

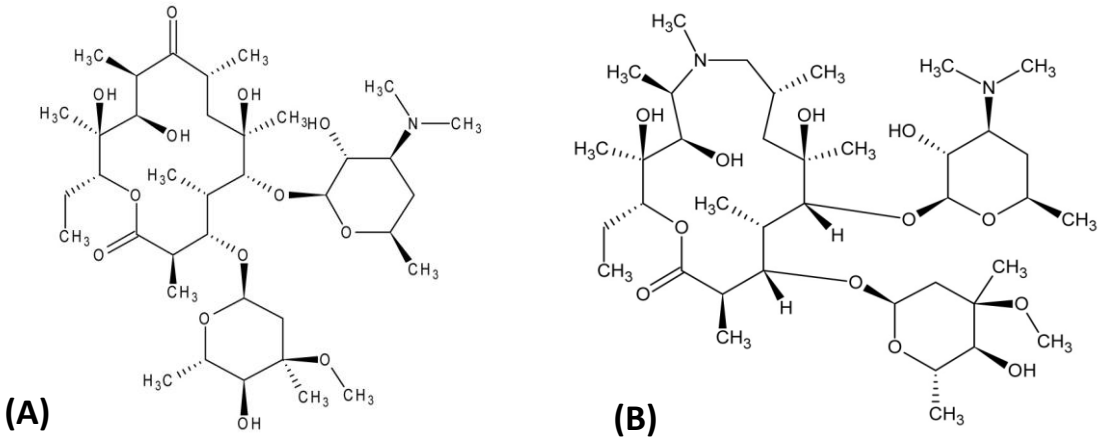

Figure 1: Chemical structures of erythromycin (A) and azithromycin (B).

2. Materials and methods

2.2. HPLC analysis

LC experiments were performed using a LC–10ATVP pump (shimadzu, Japan) equipped with an injector model Rheodyne (C.C, U.S.A) and a 20 µL loop, the SPD –10AVP UV–vis detector (shimadzu, Japan) was at 210 and 215 nm. The column was immersed in a water–bath, heated by a memert D–91126 thermostat (FRG, Germany). ODB column (250×4.6 nm I.D) was packed with silica uptisphere 5µm (interchim, France), the flow–rate was 1.0 mL min^{-1}.

2.1. Materials and reagents

Erythromycin was purchased from Sigma (Steiheim, Germany) as pharmacopeial standard, and azithromycin working standard was kindly provided from SAIDAL–GROUPE (Saidal, Algeria), while pharmaceutical tablets containing AZI were obtained commercially. Azithromycine BEKER® tablets (Laboratoires Beker, Algeria) was labeled to contain azithromycin at concentration of 500 mg/tablet.
Dipotassium hydrogenphosphate, 2-methyl-2-propanol, acetonitrile, triethylamine and absolute ethanol from SIGMA–ALDRICH (Steiheim, Germany) were of analytical–reagent grade. Methanol was of chemical grade from the same source. The following mobile phase was finally used: acetonitrile–2-methyl-2-propanol–0.025 *M* potassium phosphate buffer (pH 6.5) (33: 7: up to 100, v/v/v). The phosphate buffer solution was filtered through a Millipore GS 0.22 µm filter (Milford, MA, USA). Distilled water was used throughout all experiments.

2.3. Preparation of standard solutions

Stock standard solutions of ERY and AZI were prepared by dissolving 4.0 mg of each compound in 100 mL of 0.025 *M* dipotassium hydrogen phosphate (pH 6.5)–acetonitrile (7:3, v/v). The standard solutions were kept at 4 °C in amber glass vessels. From each standard solution a series of dilution was prepared quantitatively in appropriate solvent to obtain standard solutions having concentration ranges of linearity. The solutions were prepared freshly every day and used as working standards (theoretical concentrations of ERY and AZI were 27.0 and 12.5 µg mL^{-1}, respectively).

2.4. Treatment of urine samples

Urine samples were obtained from healthy male volunteers. A 1.0 mL aliquot of urine (blank or containing drug) was transferred to 25.0 mL separating funnel containing 5.0 mL of diethyl ether-dichloromethane (4:1, v/v) (analytical–grade). The mixture was thoroughly shaken well for 15 min. Then, the organic layer was transferred to a glass tube, and the solvent was evaporated in water bath to dryness. The residue was reconstituted in absolute ethanol. An aliquot of 20 µL was injected into the HPLC apparatus. Drug concentrations in urine were determinate using the regression equations.

2.5. Method validation

The concentrations of the macrolides were calculated using peak-height of samples. The evaluation by this procedure was more precise at low concentrations than the peak-area method. In order to determinate linearity, blank urine was spiked with solutions of the macrolides to give concentrations in the range 0.94–32.00 μg mL^{-1} and 0.25–15.00 μg mL^{-1}, respectively for erythromycin and azithromycin, and processed in duplicate. Calibration lines (equation: y= a x) were established by plotting peak-height of each drug against the respective concentration.

Intra-day and inter-day precision were assessed by analysis of control samples of urine (high, medium, low concentration at 80, 100, 120% levels of theoretical concentration for both macrolides) with each run in duplicate. The limit of quantification was estimated by analyzing macrolides at progressively lower concentrations starting at the lower end of the calibration curves. The limit of quantification for each antibiotic was defined as low concentration level where accuracy and precision were still better than 20%. To determine the limit of detection, a dilute (1.0 μg mL^{-1}) solution of the drugs in methanol–water (50:50, v/v) was injected into the HPLC system. The limit of detection was then defined as the amount of antibiotic which caused a signal three times of the noise (S/N = 3).

The recovery from urine was determined by processing spiked samples. The results were compared with those obtained after direct injection of aqueous–methanolic (50:50) reference solution of the analytes.

3. Results and discussion

3.1. Optimization of the chromatographic separation

Preliminary studies showed that end-capped columns offer advantages over than non-end-capped ones with regard to peak symmetry. Therefore, several end-capped C_8 and C_{18} columns were tested using acetonitrile–water mobile phases. On the whole, the ODB RP_{18} column provided the best results, since it showed the highest factors of efficiency and symmetry, and thus, it was selected for further experiments.

The acetonitrile content and pH of the mobile phase strongly affect the chromatographic behavior of the analytes. Although no significant effect on the retention times was observed in the pH range 5–7, peak shape improved as pH increased and pH 6.5 was chosen in order to preserve the column efficiency. At this pH no on-column degradation of compounds was observed, even for ERY which is easily degraded in acid solutions [2].

Acetonitrile content above 30 % (v/v) was required to elute ERY and AZI at acceptable times, whereas the addition of 2-methyl-2-propanol as a second organic modifier reduced the analysis time, and the percentage of 7 % (v/v) was chosen. Thus, elution was applied using the following final composition of the mobile phase: acetonitrile–2-methyl-2-propanol– 0.025 *M* potassium phosphate buffer (pH 6.5) (33:7: up to 100, v/v/v), with 1.5% triethylamine. Nevertheless, drugs show absorption maxima at different wavelengths (215 and 210 nm for ERY and AZI, respectively).

Temperature (25–40 °C) had no effect on retention and although an increase in temperature led to slightly narrower peaks, separation was carried out at room temperature for convenience. Flow-rate (1.0–1.5 mL min^{-1}) had no significant effect on peak heights, and thus further chromatograms were obtained at 1.0 mL min^{-1} so the retention time of the last eluted compound was reduced at below 15 min. The details of the results will be later reported.

3.2. Pretreatment of samples

ERY and AZI are hydrophobic compounds, and a mixture of *n*-hexane–acetone (6:1, v/v) was used as the extracting solvent in the previous method [3–6]. However, the recovery of drugs was not satisfactory with this extracting solvent. Accordingly, several extracting solvents were investigated and satisfactory recovery for ERY and AZI from urine was obtained using diethyl ether–dichloromethane (4:1, v/v). Based on these results, the sample preparation method described in *Section 2.4* was adopted.

3.3. Validation of the proposed method

Method validation was investigated in terms of selectivity, linearity, sensitivity, precision, and recovery. Because the detector responses differed depending on the analyte, calibration curves were constructed using seven triplicate data points.

3.3.1. Selectivity

To ensure the absence of interfering response values, the selectivity of the method was evaluated. The chromatographic method conferred good selectivity for both compounds (ERY and AZI) contained in different sample solutions. Indeed, the chromatogram overlay of the validation standards, the calibration standards and the

sample matrix showed no endogenous peak interference at the retention times of ERY and AZI. Figure 2 shows the chromatograms of drug-free urine by two healthy volunteers. As shown in this figure, all the analytes were well resolved under the optimal conditions.

3.3.2. Linearity

The linearity of the results generated by an analytical procedure is the ability within a given range to obtain test results that are directly proportional to the concentrations (amounts) of an analyte in the sample [22,23]. The calibration curves were obtained by analyzing calibration standards in the concentration ranges of 0.94–32.00 µg mL^{-1} for erythromycin and 0.25–15.00 µg mL^{-1} for azithromycin. Good linearity was observed between the peak-height ratio (Y) and the corresponding urine concentrations (X), with correlation coefficients (r^2) higher than 0.989 (Table 1). The r values were found to be significant with confidence intervals at P=0.05, supporting the linearity of the method. In addition, the values of student's *t*-test and variance ratio *F*-test obtained did not exceed the theoretical tabulated values.

Table 1: Linear curve, LOD, and LOQ for the HPLC analysis of ERY and AZI.

Compound	Liner range (µg mL^{-1})	r^2	LOD (µg mL^{-1})	LOQ (µg mL^{-1})
Erythromycin	0.94–32.00	0.9928	0.37	0.94
Azithromycin	0.25–15.00	0.9971	0.12	0.25

3.3.3. Limits of detection, quantification and range

The limit of detection (LOD) was estimated by injecting a series of extracted solutions until a signal-to-noise ratio of three was obtained. The lower limit of quantification (LOQ) is the lowest amount of the targeted analyte in the sample which can be quantitatively determined under the experimental conditions prescribed with a well defined accuracy [23]. For an injection of 20 µL, the limit of quantification with a signal-to-noise ratio of 10 was: 0.94 µg mL^{-1} for ERY and 0.25 µg mL^{-1} for AZI. The limit of detection with a signal-to-noise ratio of 3 was: 0.37 µg mL^{-1} for ERY and 0.12 µg mL^{-1} for AZI (Table 1). The use of a large sample amount (e.g. 1.0 mL) may offer higher sensitivity, because no interference was observed at the retention time of any analyte (Figure 2). These results indicate the acceptable sensitivity of the proposed method.

3.3.4. Method precision

The precision expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same sample [23]. The precision is evaluated in terms of repeatability (same analytical procedure, same operator, and same day) and intermediate precision (same analytical procedure but different operators and different days) expressed by relative standard deviations (%RSD).

The precision of the method was evaluated at three concentration levels and expressed as the relative standard deviation. The intra-day precision was determined for 1 day (n = 3), while the inter-day precision was determined for three separate days (n=3×3). As shown in Table 2, the intra- and inter-day precisions, which were less than 10.3% and 8.9% for ERY and AZI, respectively, where, nevertheless, they never exceeded 10.5%. This level of precision is suitable for the quantification of drug-urine concentrations in clinical studies.

Table 2: Intra- and inter-day precisions for the HPLC analysis of ERY and AZI.

Compound	Intra-day (n = 3)			Inter-day (n=3×3 days)		
	% RSD			% RSD		
	Low[a]	Medium[b]	High[c]	Low[a]	Medium[b]	High[c]
Erythromycin	7.5	2.3	1.4	7.7	5.8	10.3
Azithromycin	8.7	4.2	5.1	4.2	3.4	8.9

[a,b,c] The concentrations used were at the 80,100, 120% levels.

3.3.5. Accuracy and recovery

The accuracy of an analytical procedure expresses the closeness of agreement between the value found and the value accepted as the conventional true value. The closeness of agreement observed is the resultant (total error) of the sum of the systematic and random errors, also the sum of the trueness and the precision [23].

The absolute recovery from water and urine was 99.88-107.14% and 96.54-112.07% for ERY and AZI, respectively. Table 3 shows the accuracy of the quantified values for calibration standards back- calculated from the calibration curves for urine samples. Results obtained indicate good recoveries (mean percentage recoveries greater than 90%) and confirm the accuracy of the proposed method.

Table 3: Experimental values (%) obtained in the recovery study for ERY and AZI in samples, by the developed HPLC.

Compound	Spiked concentration[a]		
	Low	Medium	High
Erythromycin	99.88	107.14	104.73
Azithromycin	98.02	96.54	112.07

[a] The concentrations used were the same as those for precision study.

3.4. Application of the developed method for the analysis of real human urine samples (Figs. 2-4)

The developed method was tested for the determination of AZI in real urine samples that were provided by two healthy volunteers. The samples were collected 160 min after a single dose of Azithromycine BEKER® and analyzed as described in *Section 2*. Azithromycin was clearly detected in the two samples (Figure 3), and the determined drug concentrations in the urine samples were 0.74 and 0.53 µg mL^{-1}, by adding known amounts of reference substance to the samples at the beginning of the process. For the simultaneous determination, both real urine samples were spiked with pharmacopeial standard of erythromycin. On the chromatograms obtained from isocratic HPLC, no urine endogenous materials interference was observed at the retention times of erythromycin and azithromycin.

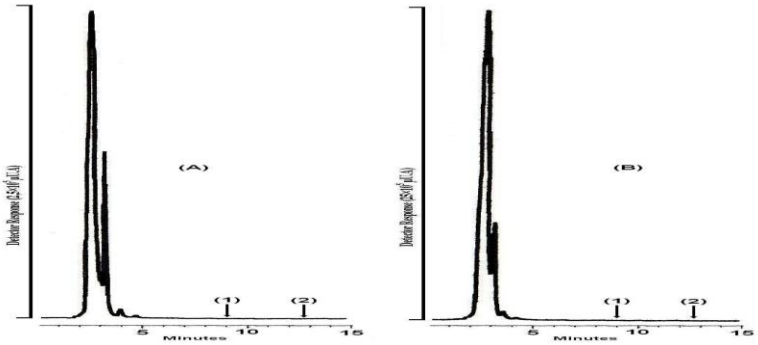

Figure 2: Typical chromatograms of blank urine samples for the first (A) and the second (B) healthy volunteers. Mobile phase: Acetonitrile–2-methyl-2-propanol–0.025 *M* potassium phosphate buffer (pH 6.5) (33: 7: up to 100, v/v/v); stationary phase: ODB RP_{18} 5µm (250×4.6 mm I.D.); flow-rate: 1.0 mL min^{-1}; temperature: 25°C; detection: UV at 210 nm. Retention time of erythromycin (1) was 8.95 min, and azithromycin retention time (2) was 12.83 min.

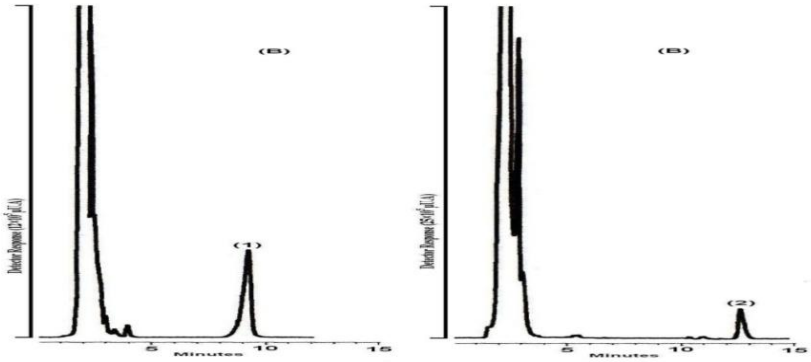

Figure 3: Chromatograms of blank urine obtained from the second healthy volunteer (B) and spiked with solutions of ERY (1) and AZI (2). See fig.2 for experimental conditions.

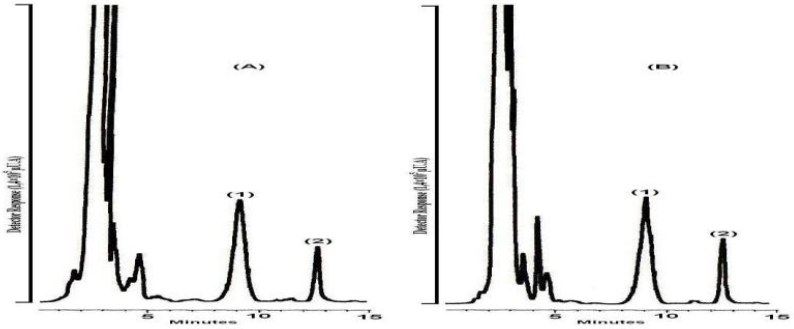

Figure 4: Chromatograms of the simultaneous determination for both real urine samples (A and B) containing AZI (2) and spiked with ERY (1). See fig.2 for experimental conditions.

As shown at Figure 4, each analyte was separated from the endogenous compounds and could be determined with high sensitivity. Therefore, these results indicate that erythromycin can be used as an internal standard and vice versa, for the analysis of the both macrolides in other biological matrices.

Conclusion

1. The quantitative method described in this report of erythromycin and azithromycin in urine is selective and shows a high degree of linearity, sensitivity, precision and accuracy.
2. As the preliminary pharmacokinetic study in real human urine samples indicates, it is adequate for full scale bioavailability and pharmacokintic studies.

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