



An ecofriendly green liquid chromatographic method for simultaneous determination of Amoxicillin, Metronidazole and Ciprofloxacin; application to dosage form and human urine

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Abstract

Green liquid chromatographic method using cyano column and ethanol and 0.5% acetic acid in water (pH 2.8) as mobile phase in gradient mode has been developed for analysis of Amoxicillin, Metronidazole and Ciprofloxacin in human urine. Quantification was carried out using a diode-array UV detector. The detection wavelength was 255 nm. The retention times and detection limits for each antibiotic were 4.9 min and 0.5 µg/mL for Amoxicillin, 6.035 min and 0.14 µg/mL for Metronidazole, 8.03 min and 3.07 µg/mL for Ciprofloxacin, respectively. The developed method was applied to examine the urinary excretion pattern of Amoxicillin, Metronidazole and Ciprofloxacin in healthy male volunteer after an oral administration of Metronidazole and Ciprofloxacin in their combination tablets (Ciprodiazole[®]) and combination tablets of Ciprofloxacin and Amoxicillin (Helicocin[®]). The developed HPLC method was successfully used for the analysis of the selected drugs in their dosage forms and human urine without interference from the excipients or urine matrix. The proposed method was rapid, specific, precise, accurate, environmentally friendly and suitable for bioequivalence and pharmacokinetic studies.

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1. Introduction

Ciprofloxacin (CIP) is a second-generation fluoroquinolone, which inhibit bacterial DNA synthesis by inhibiting bacterial DNA gyrase enzyme that makes CIP active against gram-negative

and gram-positive bacteria (Wilson CO et al., 2004).

Metronidazole (MTZ) is an antibiotic used against anaerobic microorganisms and protozoa. Gastrointestinal tract infections and various

anaerobic infections are treated by MET. Moreover, MTZ has been tested for antimicrobial activity (Lamp KC et al., 1999).

Amoxicillin (AMX) is broad spectrum β -lactam antibacterial with activity acting by inhibition bacterial cell wall biosynthesis by binding to the enzymes which produces the cell wall protein (Menelaou AA et al., 1999; Anfossi A et al., 2002). AMX is highly used antibiotic as it is effective, cheap and its high bioavailability (>90%), not affected by food or other taken (Suarez-Kurtz G et al., 2001).

MTZ is used in combination with other antibiotic like CIP for the treatment of mixed aerobic/anaerobic infections (Werk R and Schneider L et al., 1988). Additionally, MTZ combinations with AMX have synergistic effect on the bacterial susceptibility to antibacterial treatment (Pavicic MJ et al., 1991; Baumgartner JC and Xia T et al., 2003). These antibiotics are administered orally and excreted mainly into the urine, therefore its determination in biological fluids gain a great attention.

Many analysis methods have been reviewed for analysis of CIP (Lian Z and Wang J, 2016; Ghoufran Kawas et al., 2018), MTZ (Wang JC et al., 2007; Patel A et al., 2009) and AMX (Carzola RR et al., 2007; Reiriz AG et al., 2007; Bejjani A, 2016) in pharmaceuticals and different matrix, including HPLC that was used for the analysis of the three drugs, such as CIP in human plasma and urine (Kamberia M et al., 1998; Wagenlehner F. M et al., 2006; Zotou A et al., 2002), MTZ in human plasma and urine (Jense, JC et al., 1998; do Nascimento TG et al., 2004; Mustapha KB et al., 2006) and AMX in urine and plasma (Lee T L et al., 1979; Pei Q et al., 2010; Torres RF et al., 2010).

However, these methods use hazardous and polluting organic solvents in the mobile phase, which are harmful to human health and environment. Green analytical chemistry decreasing the negative

impact of these methods on both human health and environment (Armenta S et al., 2008; Yang Y et al., 2011).

Currently Several guidelines exist to develop green HPLC methods (Sandra P et al., 2010; Plotka M et al., 2013). One of them is a solvent-replacement by less harmful and more ecofriendly solvent, such as water (Smith, RM, 2008), ethanol and isopropanol (Rainville PD et al., 2012). Till now there is no reported methods for simultaneous separation and quantification of the three antibiotics in biological fluid.

In this study, a rapid, sensitive, precise and green HPLC method was developed and validated for the simultaneous estimation of CIP, MTZ and AMX in human urine by direct injection of the urine samples using ethanol as green organic solvent in the mobile phase.

2. Experimental

2.1 Instrumentation

A Waters HPLC system consisted of a 2695 binary pump, vacuum degasser, auto sampler tray and a 2996 photo diode array detector covering the range 200–600 nm was used during the study. Empower software was used to monitor and process the output signal.

2.2. Materials and reagents

CIP, MTZ and AMX Pharmaceutical grade were gifted by EIPICO Pharmaceuticals (Cairo, Egypt). Ethanol HPLC grade and acetic acid analytical standard were purchased from Sigma-Aldrich, Germany. Purified and deionized water produced in-house by Millipore's Milli-Q System (USA). Commercial Ciprofloxacin[®] tablets (Batch no JFE1746) was manufactured by Minapharm Egypt, labeled to contain 500 mg CIP and 500 mg MTZ per tablet. Commercial Flagyl[®] tablets (Batch no 9EG064) was manufactured by Sanofi Aventis Egypt, labeled to contain 500 mg MTZ per tablet. Commercial Emox[®] capsules (Batch no 2003452)

was manufactured by EIPICO Egypt, labeled to contain 500 mg AMX per capsule.

2.3. HPLC conditions

The chromatographic analysis was made on Luna CN column (25 cm, 5 μ m, 4.6 mm). Samples were eluted with a mobile phase composed of 0.5% aqueous acetic acid (solvent A) and ethanol (solvent B); the gradient elution used is shown in **Table 1** and pH adjusted to 2.8. Detection and quantification of CIP, MTZ and AMX were by DAD at a wavelength of 255 nm in room temperature with total running time was 10 min.

Table 1: Gradient Used for Elution of AMX, MTZ and CIP

Time (min.)	0.5% aqueous Acetic acid	Ethanol	flow rate (ml/min)
1.0	100	0	1
3.0	100	0	1
5.5	70	30	2
9.0	100	0	1

2.4. Standard solutions and calibration graphs preparation

Stock standard solutions of 1000 μ g mL⁻¹ of CIP, AMX and MTZ were prepared separately in water. Further dilutions for CIP, AMX and MTZ were made to get concentration ranges of 0.1-100 μ g/mL and stored in the absence of light under refrigeration at 4°C. The stock standard solutions with different volumes of each drug were added to 10 mL volumetric flasks. Then added 1 mL of blank human urine to each flask, and complete volume to 10 ml with water to get a concentration range from 0.1 to 100 μ g/mL for each of the studied drugs. The 0.45 μ m disposable membrane filters were used to filter solutions. Triplicate 10 μ l injections were done for each drug concentration of studied drugs and analyzed according to the previous procedures and the calibration curve for each compound was plotted.

2.5. Urine sample preparation (in vivo procedure)

The proposed method was used to examine the urinary excretion pattern of CIP, MTZ and AMX in healthy Egyptian male volunteer (aged 18 years, weighing 86 kg, 183 cm height) after an oral administration of MTZ and CIP in their combination tablets (Ciprodiazole® 500 mg CIP, 500 mg MTZ) and combination tablets of MTZ and AMX (Helicocin® 500 mg AMX and 500 mg MTZ). The volunteer was informed to refrain from all drugs for 2 weeks before and during study. Also, the volunteer was informed to empty his bladder completely just before the administration of one tablet of Ciprodiazole® or Helicocin® with about 300 mL of water. The zero-hour sample of human urine was collected as blank. The urine samples collected at intervals for up to 24 hours. After each collection, the urine sample volume was measured and recorded and stored at -20°C until the time of analysis.

Collected urine samples were diluted with water, depending on its concentration to obtain the three drugs calibration range. The triplicate injections of each diluted urine sample solutions were chromatographed.

This study was done according to the Egyptian Community guidelines for the use of humans in experiments. The Human Ethics Committee of Faculty of Pharmacy, Suez Canal University, approved this study. The approval code is 201901R3.

3. Results and discussion

3.1. Method development and optimization

The objective of this work was to do rapid and selective HPLC for the separation and quantification of AMOX, MET and CIP in human urine. To develop HPLC method it is important to obtain accepted peak symmetry and resolution within a suitable run time for routine analysis. To accomplish this goal, many trials were carried out to optimize mobile phase and stationary phase, pH value.

The reversed phase C8 and CN columns were tested. The satisfactory resolution between the three

antibiotics was obtained by the two columns. However, in C8 column tailed and more retained CIP peaks were observed. The Luna CN column (25 cm, 5 μ m, 4.6 mm) was selected as the working column for this experiment as it gave good separation and accepted peaks symmetry.

To develop a green HPLC method ethanol was used as mobile phase. At the beginning, the mobile phase with an isocratic elution (ethanol 40% or 30%) was used to separate the three drugs. Unfortunately, AMX and MTZ were coeluted. Then a mobile phase contains ethanol 10% and 5% was investigated. AMX and MTZ were not well resolved and CIP take too long time to elute. The gradient elution was examined to improve resolution and analysis time. At first, the ethanol of the mobile phase was set at 0% until 5.5 min with flow rate 1 ml/min to allow a sufficient resolution, and subsequently increased to 30% with flow rate 2 ml/min to achieve appropriate analysis time for routine use. **Table 1** shows the gradient elution used in this method.

The impact of pH of the solutions on the retention times and resolution of three drugs was studied over pH values the range of 2.8–6.0 with 0.5% aqueous acetic acid during varies experiment. It was found that CIP was more retained at pH higher than 5 while the influence of pH on retention time of AMX and MTZ was weak. However, the optimum resolution with acceptable retention time was observed at pH 2.8, so it was chosen in further tests.

Chromatograms of blank urine in **Fig. 1** showed no interfering peaks at the retention times of CIP, AMX, and MTZ. **Fig. 2** shows typical chromatograms for CIP, AMX, and MTZ standard solution spiked with human urine where the three antibiotics were well separated with clear baseline separation. The retention time was 4.9, 6.03 and 8.03 min for AMX, MTZ and CIP, respectively. The system suitability parameters are given in **Table 2**.

To study the effectiveness of the proposed HPLC method on real sample the method was applied to human urine samples taken from male volunteer

takes Ciprodiazole[®] and Helicocin[®] medication.

Table 2: The system suitability parameters for determination of AMX, MTZ, and CIP by HPLC.

Parameters	AMX	MTZ	CIP
Retention time	4.9	6.03	8.03
Capacity factor (K)	3.9	5.07	7.09
Selectivity α	1.3	1.4	-
Resolution R_s	3.5	4.8	-
Tailing factor	1.07	0.99	1.20
%RSD of retention time	1.43	0.43	0.59
Plate count	6970	15100	17100

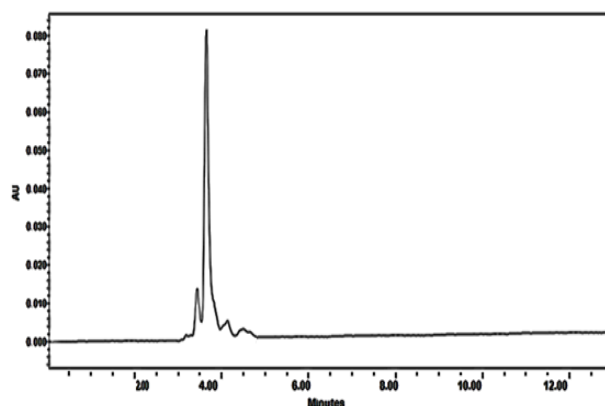


Fig. 1: Typical HPLC chromatograms obtained from analysis of blank human urine

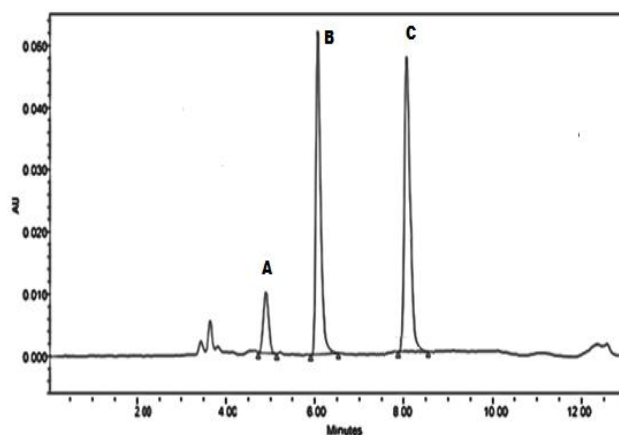


Fig. 2: Typical HPLC chromatograms obtained from analysis of (A) AMX, (B) MTZ and (C) CIP in human urine.

Fig. 3 represents HPLC chromatogram for sample of real human urine after 6 h of administration of Ciprodiazole[®] and Helicocin[®] medication. The concentration of CIP and MTZ in urine sample after 6 h of administration of Ciprodiazole[®] were analyzed and found to be 56.3 $\mu\text{g mL}^{-1}$ and 19.81 $\mu\text{g mL}^{-1}$, respectively. The concentration of AMX and MTZ in urine sample after 6 h of administration of Helicocin[®] were determined and found to be 62.75 $\mu\text{g mL}^{-1}$ and 1.85 $\mu\text{g mL}^{-1}$, respectively.

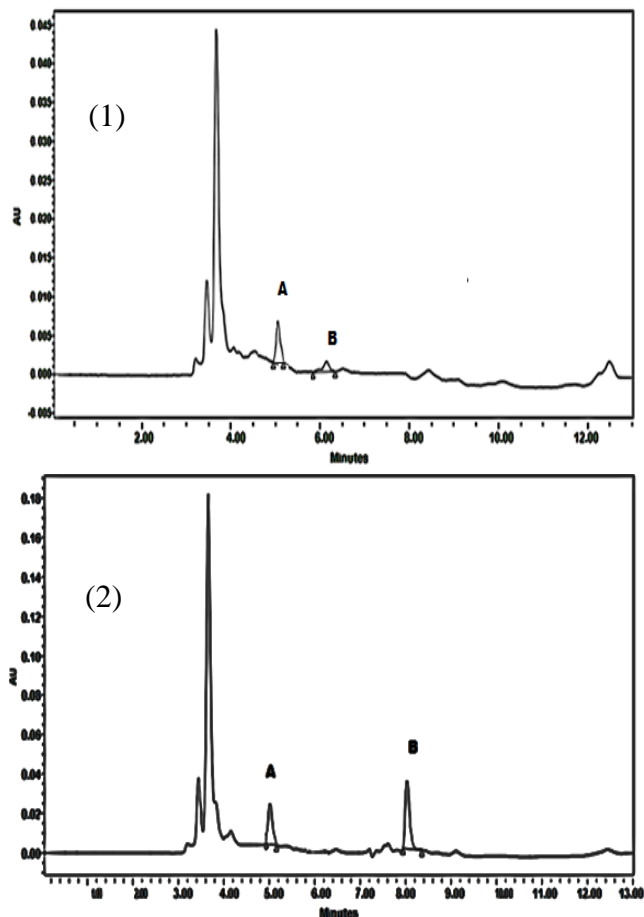


Fig. 3: Typical HPLC chromatograms obtained from analysis of real samples of human urine after oral dosing of (1) Ciprodiazole[®] tablets (A) MTZ, (B) CIP and (2) Helicocin[®] tablets (A) AMX, (B) MTZ.

3.2. Urinary excretion pattern

Following oral administration of the three drugs, urinary excretion is the main excretion pathway. The introduced analytical method was developed for determination of the urinary excretion pattern of CIP and MTZ and after oral administration of

Ciprodiazole[®] tablet (500 mg MTZ, 500 mg CIP) (**Fig. 4**) and for determination the urinary excretion pattern of AMX and MTZ after oral administration of Helicocin[®] tablet (500 mg MTZ and 500 mg AMX) (**Fig.5**). The cumulative urinary excretion data of Ciprodiazole[®] tablets and Helicocin[®] tablets are summarized in **Table 3**.

Apart from MTZ in the Helicocin[®] tablets, the urinary excretion data of the three drugs agreed with the literature (**Lee T L et al., 1979; Jensen J C, and Gugler R, 1983; Wagenlehner F. M et al., 2006**). The maximum excretion rate and maximum excreted amount were observed within 2-4 hours after tablets administration (3 hours as a mid-point). MTZ in the Helicocin[®] tablets, on the other hand, showed maximum excretion rate and maximum excreted amount within 8-10 hours after tablets administration (9 hours as a mid-point). Surprisingly, MTZ has exhibited different urinary excretion patterns in the 2 different drug combinations. MTZ maximum excretion rate was 3 times higher when combined with CIP than when combined with AMX. Additionally, both cumulative excreted amount (mg) and percent of the drug excreted unchanged of MTZ was 2.5 times higher in Ciprodiazole[®] tablets than Helicocin[®] tablets. These results demonstrated possible drug-drug interactions that can affect the therapeutic activity of the MTZ in different drug formulations.

3.3. Method validation

3.3.1. Linearity and range

The HPLC method linearity was estimated by determining CIP, MTZ, AMX in a series of different concentrations. The linearity ranging between 10-50 $\mu\text{g/ml}$ for CIP, 10-90 $\mu\text{g/ml}$ for AMX and 10-90 $\mu\text{g/ml}$ for MET. The calibration curves were set by plotting peak area versus concentrations of AMX, CIP and MTZ. The least-square method was applied to determine the regression data for the proposed method shown in **Table 4**.

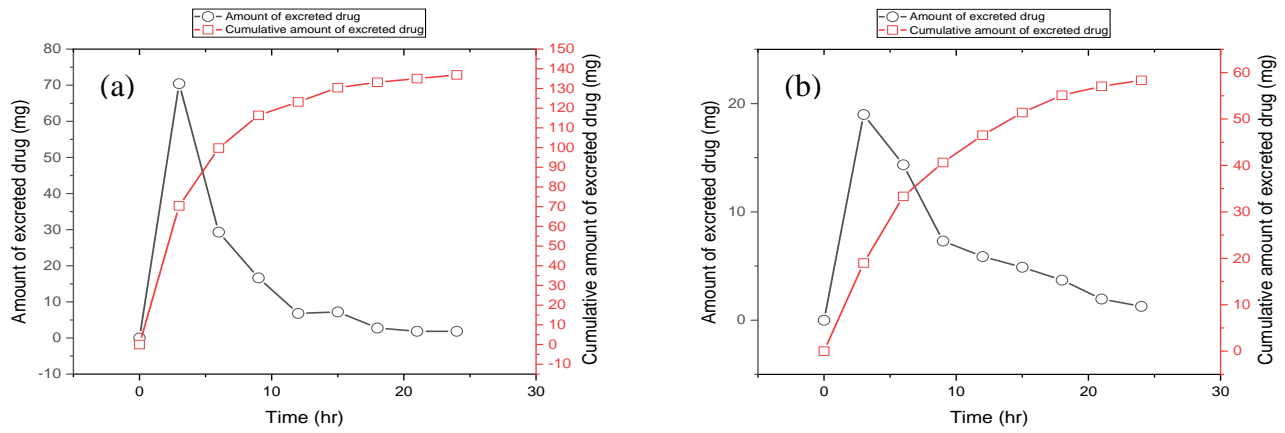


Fig. 4: Cumulative excretion of Ciprodiazole® tablets (a) CIP 500 mg and (b) MTZ 500 mg in the urine sample of a healthy human male volunteer

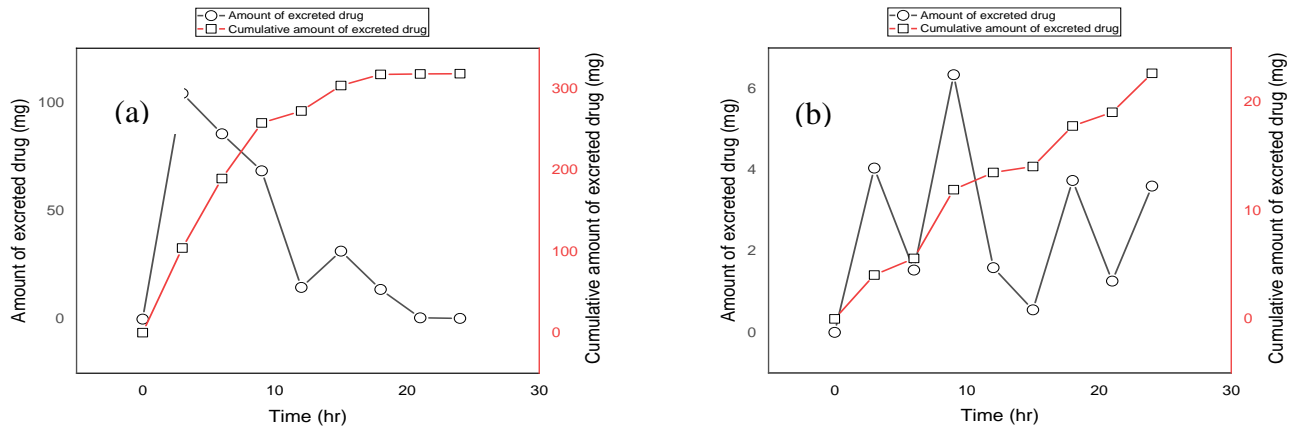


Fig. 5: Cumulative excretion of Helicocin® tablets (a) AMX 500 mg (b) MTZ 500 mg in the urine sample of a healthy human male volunteer

Table 3: The cumulative urinary excretion of Ciprodiazole® tablets and Helicocin® tablets

Active principle	Maximum excretion rate (mg/h)	Cumulative excreted amount (mg)	Percent of the drug excreted unchanged (%)	Reported percent of the drug excreted unchanged (%)	
Helicocin® tablets	Amoxicillin	34.7	318.6	63.7	50-68 ¹
	Metronidazole	2.1	22.7	4.5	Below 10 ²
Ciprodiazole® tablets	Ciprofloxacin	23.5	136.9	27.4	25.9 – 50.3 ³
	Metronidazole	6.3	58.3	11.7	Below 10 ²

¹ Lee T L et al., 1979

² Jensen J C, and Gugler R, 1983

³ Wagenlehner F. M et al., 2006

Table 4: The characteristic regression parameters of the HPLC method for analysis of AMX, CIP and MTZ in samples spiked with human urine.

Parameters	CIP	AMX	MTZ
range ($\mu\text{g mL}^{-1}$)	10-50	10-90	10 - 90
DL ($\mu\text{g mL}^{-1}$)	3.077	0.509	0.148
QL ($\mu\text{g mL}^{-1}$)	9.32	1.54	0.451
SD of the slope (S_b)	317.32	34.81	125.3114
Slope	11284.19	885.07	4235.41
Confidence limit of the slope b	$1.08 \times 10^4 - 1.17 \times 10^4$	$8.3 \times 10^2 - 9.3 \times 10^2$	$4.1 \times 10^3 - 4.3 \times 10^3$
Intercept (a)	-7502.1	2667.34	5713.02
SD of the intercept (S_a)	10524.63	2239.799	6416.9
Confidence limit of the intercept ^b	$-2.24 \times 10^4 - 7.47 \times 10^3$	$-5.2 \times 10^2 - 5.8 \times 10^3$	$1.61 \times 10^2 - 1.12 \times 10^4$
Correlation coefficient (r)	0.9995	0.9995	0.9997

Table 5: Intra-and inter-day results of AMX, MTZ and CIP in human urine

Compound	Concentration ($\mu\text{g ml}^{-1}$)	Intra-day precision		Inter-day precision	
		%Recovery \pm SD ^a	CV (%)	%Recovery \pm SD ^a	CV (%)
AMX	10	106.37 \pm 3.51	0.028	100.95 \pm 2.51	0.021
	60	99.53 \pm 3.05	0.006	99.88 \pm 1.52	0.002
	90	101.98 \pm 3.05	0.004	100.77 \pm 2.64	0.003
MTZ	10	100.32 \pm 2.08	0.004	100.10 \pm 2.08	0.004
	50	101.17 \pm 3.05	0.001	100.04 \pm 2.64	0.001
	90	99.31 \pm 3.50	0.001	100.06 \pm 2.51	0.001
CIP	10	97.19 \pm 2.08	0.002	97.20 \pm 4.04	0.003
	30	99.85 \pm 2.00	0.001	100.75 \pm 2.08	0.005
	50	99.23 \pm 1.52	0.003	99.19 \pm 2.50	0.005

^aMean \pm SD from three determinations

3.3.2. Limits of detection and quantification

According to ICH recommendations, the detection and the quantitation limits were calculated depending the S.D. of the response and the slope and the result is shown in **Table 4**. The intra-day precision for the presented method was examined by replicate analysis of urine spiked samples at different concentrations of each compound within the linearity range at three concentration levels.

3.3.3. Precision

The inter-day precision was tested by the same way in different days up to 5 days and the result is shown in **Table 5**. The inter-day and the intra-day results showed high precision, as the CV% was less than 2%.

3.3.4. Specificity

Six blank human urine samples were randomly selected and collected under controlled conditions. samples injected directly and analyzed to asses that urine matrix does not interfere with the studied compounds.

3.3.5. Robustness

The influence of the the mobile phase pH and the concentration of acetic acid buffer on resolution were examined by modifying pH from 2.8 to 3.2 (± 2) and changing the concentration of 0.5% acetic acid by ± 2 %, the separation between AMX, MTZ, and CIP and other components in the biological matrices was not changed.

4. Conclusion

This is a first reported green HPLC method for the analysis of amoxicillin, metronidazole, and ciprofloxacin in human urine. The method was validated according to ICH criteria and applied with high degree of selectivity and accuracy. Furthermore, the method separates the analyzed compounds in short analysis time.

5. Acknowledgments

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