PERIÓDICO TCHÊ QUÍMICA
ARTIGO ORIGINAL

DESENVOLVIMENTO E VALIDAÇÃO DE MÉTODOS HPLC DE DETERMINAÇÃO QUANTITATIVA DOS ANTIBIÓTICOS DE FLUOROQUINOLONA - HIDROCLORETO DE MOXIFLOXACINA E NORFLOXACINA EM APOIO AO ESTUDO DE ADSORÇÃO EM ZEOLITOS NATURAIS

DEVELOPMENT AND VALIDATION OF QUANTITATIVE DETERMINATION HPLC METHODS OF THE FLUOROQUINOLONE ANTIBIOTICS - MOXIFLOXACIN HYDROCHLORIDE AND NORFLOXACIN IN SUPPORT OF ADSORPTION STUDY ON NATURAL ZEOLITES

ბუნებრივ ცეოლითებზე ადსორბციის შესწავლისათვის ფტორქინოლონური ანტიბიოტიკების - მოქსიფლოქსაცინის ჰიდროქლორიდისა და ნორფლოქსაცინის მესქ მეთოდების შემუშავება და ვალიდაცია

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RESUMO

Os antibióticos da fluoroquinolona têm sido amplamente utilizados na medicina humana e animal. A poluição residual por antibióticos se tornou um dos mais graves problemas ambientais e de saúde humana atualmente. Portanto, tem sido uma grande exigência desenvolver métodos e tecnologias de tratamento eficientes e econômicos para a remoção de antibióticos da água industrial e doméstica contaminada. Existe a técnica mais utilizada - adsorção para o tratamento de águas residuais. Devido à alta capacidade de troca catiônica, bem como às propriedades das peneiras moleculares, as zeólitas naturais podem ser utilizadas como adsorventes para remoção dos antibióticos mencionados acima das águas residuais e no processo de purificação. A presente pesquisa refere-se ao desenvolvimento e validação de novos métodos de HPLC seletivos, sensíveis e rápidos para a determinação quantitativa dos antibióticos fluoroquinolonas mais frequentemente utilizados - cloridrato de moxifloxacina e norfloxacina em soluções aquosas para medir sua adsorção nas zeólitas naturais e para análises de rotina de águas residuais. Os métodos de HPLC propostos foram validados com relação à robustez (estudo de estabilidade da solução padrão, teste de compatibilidade do filtro de membrana, estudo de efeitos de fatores críticos usando o projeto de experimentos - DoE), teste de adequação do sistema, especificidade, faixa de linearidade (acima da faixa de concentração de 0,05 a 2000 µg/mL para ambos os antibióticos), precisão, exatidão, limites de detecção (LOD) e quantificação (LOQ). O LOD e LOQ foram 0,01 e 0,05 µg/mL para cloridrato de moxifloxacina e 0,008 e 0,05 µg/mL para norfloxacina, respectivamente. O experimento de adsorção por zeólitas naturais, em condições estáticas e dinâmicas, foram usadas para a preparação da amostra de ambos os antibióticos de fluoroquinolona.

Palavras-chave: *Cloridrato de moxifloxacina, Norfloxacina, HPLC, Validação e zeólita natural.*

ABSTRACT

The fluoroquinolone antibiotics have been widely used in human and animal medicine. Residual antibiotics pollution has become one of the most serious environmental and human health problems today. Therefore, it has been a great exigency to develop some efficient and cost-effective treatment methods and technologies for antibiotics removal from industrial and household contaminated water. There is the most used technique - adsorption for the treatment of wastewaters. Due to high cation-exchange ability as well as to the molecular sieve properties, natural zeolites can be used as adsorbents for removal of the above-mentioned antibiotics from wastewaters and in the purification process. The present research concerns the development and validation of new, selective, sensitive and rapid HPLC methods for the quantitative determination of the most frequently used fluoroquinolone antibiotics – moxifloxacin hydrochloride and norfloxacin in aqueous

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solutions to measure their adsorption on the natural zeolites and for routine analysis of wastewaters. The proposed HPLC methods were validated with respect to robustness (standard solution stability study, membrane filter compatibility test, critical factors effect study using design of experiments - DoE), system suitability test, specificity, linearity-range (over the concentration range of 0.05 to 2000 µg/mL for both antibiotics), precision, accuracy, limits of detection (LOD) and quantitation (LOQ). The LOD and LOQ were 0.01 and 0.05 µg/mL for moxifloxacin hydrochloride and 0.008 and 0.05 µg/mL for norfloxacin, respectively. Adsorption experiment by natural zeolites in static and dynamic conditions was used for sample preparation of both test fluoroquinolone antibiotics.

Keywords: *Moxifloxacin hydrochloride, Norfloxacin, HPLC, Validation, and Natural zeolite.*

რეზიუმე

ფტორქინოლონური ანტიბიოტიკები ფართო გამოყენებას ჰპოვებს ადამიანისა და ცხოველების სამედიცინო პრაქტიკაში. დღეისათვის, ანტიბიოტიკების ნარჩენებით დაბინძურება ერთ-ერთ ყველაზე სერიოზულ პრობლემას წარმოადგენს გარემოსთვის და ადამიანის ჯანმრთელობისთვის. ამიტომ განსაკუთრებულად მნიშვნელოვანია შემუშავდეს ეფექტური და ნაკლები დანახარჯების მქონე საყოფაცხოვრებო და სამრეწველო ჩამდინარე დაბინძურებული წყლებიდან ანტიბიოტიკების მოცილების მეთოდები და ტექნოლოგიები. ადსორბცია ჩამდინარე წყლების გაწმენდისათვის ხშირად გამოყენებად ტექნიკას წარმოადგენს. ბუნებრივი ცეოლითები, რომლებიც ხასიათდებიან მაღალი იონ-მიმოცვლისა და მოლეკულურ-საცრული თვისებებით, შესაძლებელია გამოყენებული იქნას ჩამდინარე წყლებიდან ზემოთხსენებული ანტიბიოტიკების მოცილებისა და გაწმენდის პროცესში. წარმოდგენილი კვლევა ეხება წყალხსნარებში ბუნებრივ ცეოლითებზე ადსორბციის შესწავლისა და ჩამდინარე წყლების რუტინული ანალიზებისთვის ხშირად გამოყენებადი ფტორქინოლონური ანტიბიოტიკების - მოქსიფლოქსაცინის ჰიდროქლორიდისა და ნორფლოქსაცინის რაოდენობრივი განსაზღვრის ახალი, სელექტური, მგრძნობიარე და სწრაფი მაღალეფექტური სითხური ქრომატოგრაფული მეთოდების შემუშავებასა და ვალიდაციას. შემოთავაზებული მესქ მეთოდები ვალიდირებული იქნა შემდეგი პარამეტრების - მდგრადობის (სტანდარტული ხსნარების სტაბილურობის შესწავლა, მემბრანული ფილტრის შესაბამისობის შემოწმება, კრიტიკული ფაქტორების გავლენის შესწავლა ექსპერიმენტის დიზაინის გამოყენებით), სისტემის ვარგისობის შემოწმების, სპეციფიკურობის, სწორხაზოვნება-დიაპაზონის (0.05-დან 2000 მკგ/მლ კონცენტრაციის დიაპაზონში ორივე ანტიბიოტიკისთვის), სიზუსტის, სისწორის, აღმოჩენის ქვედა და რაოდენობრივი განსაზღვრის ზღვრების გამოყენებით. აღმოჩენისა და რაოდენობრივი განსაზღვრის ზღვრებია შესაბამისად 0.01 და 0.05 მკგ/მლ მოქსიფლოქსაცინის ჰიდროქლორიდისათვის, ნორფლოქსაცინისათვის - 0.008 და 0.05 მკგ/მლ. ორივე ფტორქინილონური ანტიბიოტიკისათვის ნიმუშების მომზადებისთვის გამოყენებული იქნა ბუნებრივ ცეოლითებზე ადსორბციის ექსპერიმენტი სტატიკურ და დინამიკურ პირობებში.

საკვანძო სიტყვები: მოქსიფლოქსაცინის ჰიდროქლორიდი, ნორფლოქსაცინი, მესქ, ვალიდაცია და ბუნებრივი ცეოლითები.

1. INTRODUCTION

Today, one of the most serious environmental and human health problems is residual antibiotics pollution. Therefore, it has been a great exigency to develop some efficient and cost-effective treatment technologies for removal of antibiotics residues from industrial and household contaminated waters. One of the most used, cost-effective, and modern technique for wastewaters treatment is adsorption method. Natural zeolite with high cation-exchange abilities and molecular sieve properties has been widely used as an adsorbent in purification processes, for removal of various toxic inorganic and organic compounds from aquatic environments. To conduct adsorption study in order to give information about various solid adsorptive materials for removal antibiotics residues from contaminated water, there are two main separate activities: first of these activities is to develop a method for adsorption study including the investigation of the main factors affected on adsorption process and calculation of the uptake adsorbate and the adsorption capacity. Second is to develop a selective, precise, and a sensitive analytical method for quantitative determination of each adsorbate (contaminant) in sample solution.

Quinolone antibiotics are synthetic

antibacterial drugs with the 4-quinolone basic structure. The most commonly used quinolones are fluoroquinolone (FQ) antibiotics. The presence of fluorine atoms is a critical factor for high pharmacological activity. FQs can inhibit the proliferation of many Gram-negative and Gram-positive bacteria [1], so, they have been widely used in human and animal medicine. However, as a result of antibiotic overuse, public concern about FQs has been greatly increasing in the past decades. The environmental concern of FQ residues in the aquatic environments is not only on their potential to increase antibiotic resistance but also on their unfavorable ecotoxicity profile [2]. Moxifloxacin (MOX) and norfloxacin (NOR) belong to the fluoroquinolone antibiotics family. These active pharmaceutical ingredients (API) are frequently used in medical and veterinary practice. The presence of the above-mentioned FQ residues in effluents from households, hospitals, and pharmaceutical industries is a major cause of acute and chronic toxicity, as well as the emergence of resistant bacteria. Consequently, the removal of the FQ residues from aquatic environments, wastewaters is a crucial issue [3]. The structure, main physical-chemical properties, and the content in surface waters [4-6] are shown in Table 1.

Various HPLC methods for the quantitative determination of moxifloxacin [7-13] and norfloxacin [14-17] have been reported in several papers and pharmacopeias [4,5]. A literature review revealed that no adequate and suitable HPLC methods with method validation data for the quantitative determination of MOX and NOR residues at a very low concentration level in aqueous solutions for adsorption study on natural zeolites are to be found. introduction must clearly state the problem, the reason for doing the work, the hypotheses or theoretical predictions under consideration and the essential background. It should not contain equations or mathematical notation. A brief survey of the relevant literature so that a non-specialist reader could understand the significance of the presented results.

The present research concerns the development and validation of new, selective, sensitive and rapid high performance liquid chromatographic (HPLC) methods for the quantitative determination of the most frequently used fluoroquinolone antibiotics – moxifloxacin HCl and norfloxacin in aqueous solutions to measure their adsorption on the natural zeolites namely clinoptilolite, mordenite, and their modified forms.

2. MATERIALS AND METHODS:

2.1. Reagents and chemicals

The certified analytical standards of moxifloxacin hydrochloride and norfloxacin, HPLC grade acetonitrile (ACN), trifluoroacetic acid, orthophosphoric acid, trimethylamine, sodium hydroxide, and hydrochloric acid were purchased from Merk (Germany).

2.2. Instrumentation and materials

HPLC grade purified water was obtained using Milli Q Adventage A10 purification system (France). The chromatographic analysis was performed using LC-20AD Prominence Shimadzu HPLC System (Japan). Analytical balance ALX-210 (USA) and Hanna Instruments HI 2211 pH-meter (USA), also Durapore polyvinylidene difluoride (PVDF) membrane syringe filters were used for standard and sample preparation. All the measuring equipment was appropriately calibrated. The experiment was carried out in climate parameters controlled laboratory area (temperature, t=22±3^{0}C, relative humidity, RH=45±15%). For sample preparation, local natural zeolites – clinoptilolite and mordenite (particle sizes of 0.5-1.0 mm; canal sizes of 0.39 x 0.54 nm) obtained from the regions of Georgia were used as adsorbents which were prepared and treated previously for the method development and validation using the laboratory standard procedure [18].

2.3. Chromatographic system conditions

HPLC analytical methods for the quantitative determination of MOX HCL and NOR in sample aqueous solutions were developed using HPLC column - Agilent SB-C18 4.6 x 250 mm, 5 µm (USA). For MOX HCl determination a mixture of buffer pH 2.5 and ACN 60:40 v/v was used as a mobile phase (MP) with isocratic elution, and for NOR determination - a mixture of buffer - orthophosphoric acid solution (1:1000) and ACN 85:15 v/v; The flow rate of MP was 1.0 mL/min; Detector wavelengths were 293 nm and 275 nm, and Injected volumes were 10 µL and 20 µL for methods of MOX HCl and NOR determination, respectively; The column temperature was maintained at 40°C. The MP was used as a diluent in both analytical procedures. The run time was 5 min for MOX HCl and 11 min for NOR.

2.4. Standard and sample preparation

20 mg of the analytical standard of each FQ antibiotic - MOX HCl/NOR was weighed and transferred to 20 mL volumetric flask, dissolved in 15 mL of diluent and then diluted to volume with the same diluent, mixed well (standard stock solution – 1 mg/mL). The obtained solution was filtered through 0.45 µm PVDF membrane syringe filter and transferred 1 mL of this solution to 10 mL volumetric flask, diluted to volume with diluent, mixed well (0.1 mg/mL).

The sample stock solutions (adsorbate influent solution) were prepared the following procedure: MOX HCl and NOR analytical standards were diluted in HPLC grade purified water in order to obtain the concentration of 1.0 mg/mL.

For adsorption process study by static method, 0.2 g of zeolitic adsorbent was transferred to 50 mL Erlenmeyer flask and added 20 mL of each FQ antibiotic sample stock solution. Initially, zeolite sample with adsorbate FQ antibiotic solution was left on an orbital shaker at 150 rpm for 15 min, then statically for the determined time interval. Then the adsorbent sample was centrifuged at 3000 rpm for 5 min. 1 mL of the obtained supernatant was used, transferred 10 mL of volumetric flask and diluted to volume with diluent, mix well.

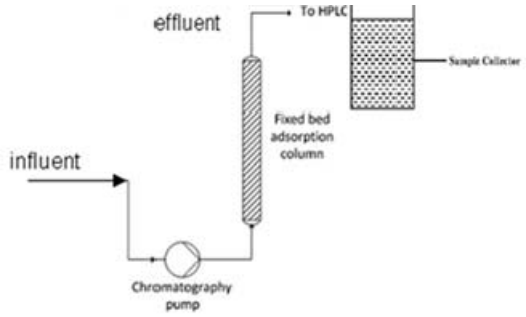

Figure 1. *The laboratory dynamic type equipment for adsorption study.*

For adsorption process study by dynamic method using the specially constructed laboratory dynamic type equipment with fixed bed adsorption glass column (internal diameter - 1.0 cm and length - 8 cm) packed with 9 g of the selected natural zeolite adsorbent and chromatography pump (Figure 1). MOX HCl and NOW influent solutions were added to a glass beaker and pumped with two different flow rates of the influent stream - 1.5 and 5.0 mL/min into the adsorption column. The effluent samples were collected initially, at different time intervals and the end of the adsorption experiment after the saturation state occurred. The obtained solutions were filtered through 0.45 µm PVDF membrane syringe filter, and 1 mL of these solutions were diluted to 10 mL with diluent, mix well. The initial pH value of adsorbate effluent sample solution was adjusted by adding 0.1 M NaOH and HCl solution.

2.5. Analytical method validation

The developed HPLC methods were validated with respect to robustness (standard solution stability, PVDF membrane filter compatibility test, critical factors effect study using design of experiments - DoE) system suitability test (SST), specificity, linearity-range, precision, limits of detection (LOD) and quantitation (LOQ) according to methodologies [19-21] and ICH Q2 guideline requirements [22]. Microsoft Excel was used for statistical assessment and graphical analysis.

2.6. Design of experiments

For the robustness parameter of method validation, the qualitative critical factors were considered and selected, which are given with their levels summarized in table 2. The SST parameters - column efficiency (theoretical plates – N), tailing factor (USP symmetry - As), relative standard deviation (RSD) of peak areas (RSD_A) and RSD of retention times (RSD_{RT}) (n=6) and peak purity (PP) obtained from standard solution chromatograms were used as the response variable for analytical HPLC method. The experiment was conducted in 2^{5-2}=8 runs for five two-level factors.

2.7. Calculation of the content moxifloxacin HCl and norfloxacin in the sample solution

The concentration of each FQ antibiotic – C_u in an effluent sample solution, mg/mL was calculated by the following formula:

$$C_u=A_u\times W\times D_1\times P/A_s\times D_2\times 100 \qquad \text{(Eq. 1)}$$

Where, A_u – The peak area of each FQ obtained with influent/effluent sample solution; A_s – The peak area of each FQ obtained with standard solution; W – The weight of FQ standard, mg; D_1 – The dilution factor of sample solution; D_2 – The dilution factor of standard solution; P – The purity of standard, %.

3. RESULTS AND DISCUSSION:

3.1. Robustness study

The final chromatographic conditions were determined by optimizing the system operational parameters: the wavelength for detection, the composition of the mobile phase, the flow rate, the nature of stationary phase and checking the system suitability test parameters: theoretical plates, tailing factor, peak purity, etc. The calibration curve showed very good linearity for a wide concentration range at 293 and 275 nm for MOX and NOR, respectively. Five critical factors were selected, and small variations (low and high levels) were induced in the nominal values of both methods. 8-run design experiment was performed to assess the effect of each factor on the SST results. Table 3 shows the DoE results of the robustness test for the developed HPLC methods. The variability of the checked STT parameters is within the acceptance criteria, and the results show that these analytical procedures are robust.

The stability of the standard solution was studied initially, after 24 hours, 3, 5, 7, 9 days stored at room temperature against a freshly prepared standard solution. The stability was checked using two standard solutions by calculating the percentage difference between peak areas of standard solutions stored and freshly prepared which should not be more than 5.0 % and the bias in terms of peak area between two standard solutions should be within 0.98-1.02 (acceptance criteria). The percentage differences between peak areas obtained with standard solution stored at room temperature for 9 days and freshly prepared one are 4.4 % and 3.9 % for MOX HCl and NOR, respectively. This gives the confidence that FQ antibiotics residues are stable within 9 days, and the concentration does not change in sample solutions during the experiment of adsorption study.

The PVDF membrane syringe filter compatibility was checked using standard solution and by calculating the percentage difference between peak areas of standard solutions filtered and non-filtered, which should not be more than 0.5 % (acceptance criteria). The percentage difference between peak areas of standard solutions filtered and non-filtered is 0.14 % and 0.32 % for MOX HCl and NOR, respectively, which gives the confidence that adsorption of each analyte does not occur on the used membrane syringe filter.

3.2. Specificity

The specificity parameter was checked by injecting standard solution and the background control (without FQ antibiotic) sample solution - blank. The results show that there is no interference from blank and diluent at the retention time of analyte (adsorbate) peak. The MOX and NOR peaks were pure. Purity factor (975 for MOX and 980 for NOR) was more than purity threshold (950). Figure 2, 3 shows the 3D spectra and the chromatogram obtained from the standard solution of MOX HCl and NOR, respectively.

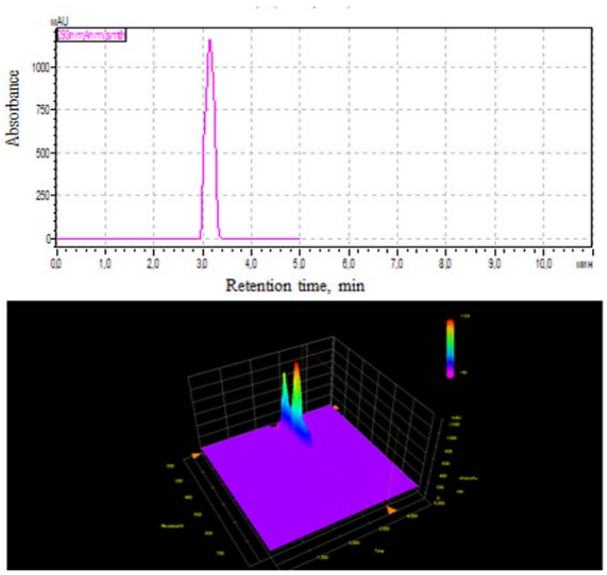

Figure 2. *The chromatogram and the 3D spectra of MOX HCl peak obtained from standard solution with 0.1 mg/mL concentration*

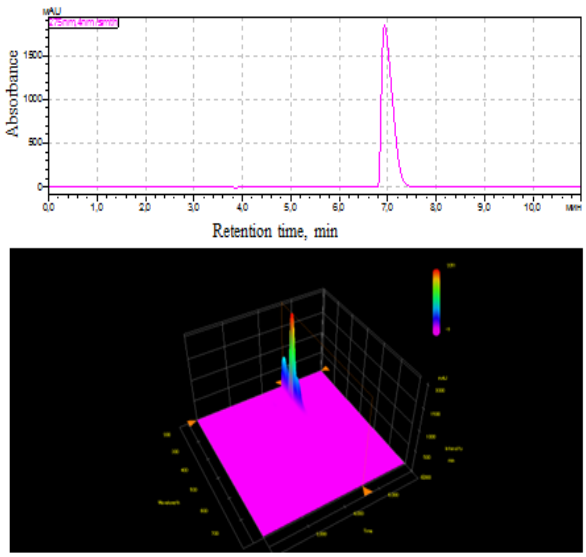

Figure 3. *The chromatogram and the 3D spectra of NOR peak obtained from standard solution with 0.1 mg/mL concentration*

Figure 4, 5 shows the chromatogram obtained from the blank solution of MOX HCl and NOR, respectively.

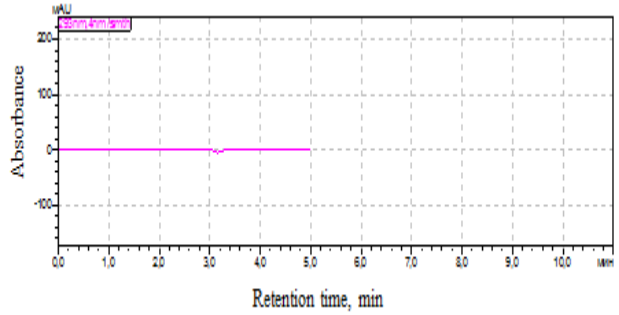

Figure 4. *The chromatogram of blank solution for MOX HCl*

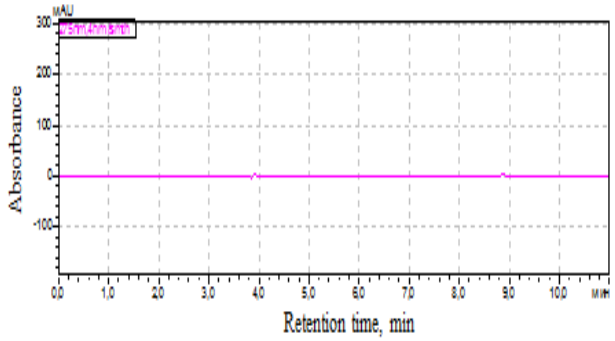

Figure 5. *The chromatogram of the blank solution for NOR*

3.3. Linearity and range; Limits of quantitation (LOQ) and detection (LOD)

The standard working solutions were prepared at six different concentration levels ranging from 0.00005 mg/mL to 2.0 mg/mL from the standard stock solution of both FQ antibiotics - MOX HCl and NOR. Six replicate injections (n=6) were carried out for each concentration level. The linearity over the given range was checked by the square of correlation coefficient - R^2 (acceptance criteria: >0.998), RSD of peak areas - RSD_A (acceptance criteria: <2.0 % for >1.0 mg/mL concentration levels and RSD_A<5.0 % for >0.00005 mg/mL concentration levels and for the last concentration <10 %), RSD of retention times - RSD_{RT} (acceptance criteria: <1.0 %).

The calibration curves (linearity graph) were constructed by plotting the peak area versus the corresponding concentration of the injected working standard solution. The value of the square of the correlation coefficient indicates very good linearity (R2>0.9996 for MOX HCl and R2>0.9999 for NOR). Figure 6, 7 shows the linearity graph for MOX HCl and NOR, respectively.

The limits of quantitation (LOQ) and detection (LOD) were established by injecting a series of stepwise diluted working solutions. The LOQ was estimated by calculating RSD of peak areas - RSD_A for six replicate injections which should be <10 % and the signal-to-noise ratio - S/N - >10 (acceptance criteria). The LOD was estimated to be three times and more of s/N ratio (acceptance criteria). The determined LOQ and LOD for MOX HCl and NOR are presented in Table 4.

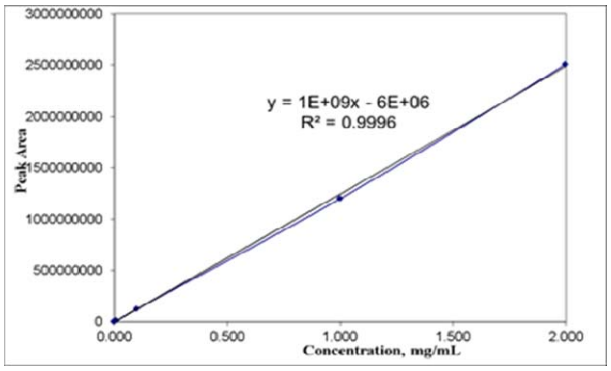

Figure 6. *The calibration curve for MOX HCl over the concentration range 0.00005-2.0 mg/mL*

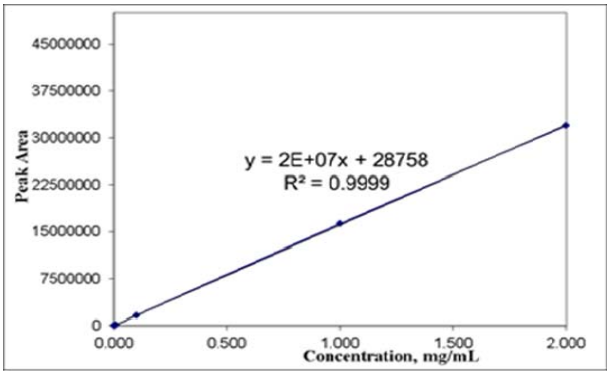

Figure 7. *The calibration curve for NOR over the concentration range 0.00005-2.0 mg/mL*

3.4. System suitability test parameters

The SST parameters were measured in order to check the chromatographic system performance. This test was carried out by six replicate injections (n=6) of the standard solution of each FQ antibiotic. The main SST parameters including RSD of peak areas - RSD_A, RSD of the retention times - RSD_{RT}, peak tailing factor - A_s

(USPcoefficient of the peak symmetry $S=W_{0.05}/2f$) and column efficiency - the number of theoretical plates was measured. The results are summarized in Table 5.

3.5. Accuracy

The accuracy was expressed as the percentage of each FQ antibiotic recovered from a spiked sample solution (effluent sample solution + added standard solution) with the corresponding RSD. The average recovery should be within 95.0–105.0 % and RSD of the percentage recoveries should be <3.0 % for each concentration level of spiked solution (acceptance criteria). The recovery - Rec, % was calculated by the following formula:

$$Rec,\%=(C_1-C_2)\times 100/C_s \qquad \text{(Eq. 2)}$$

Where, C_1 – the concentration of each FQ antibiotic obtained with the spiked sample solution, mg/mL, C_2 - the concentration of each FQ antibiotic obtained with the effluent sample solution, mg/mL and C_s – the concentration of each FQ antibiotic obtained with a standard solution, mg/mL [19-21]. The results of the recovery are given in Table 6 which is well within the usually accepted limits indicating the accuracy of both methods.

3.6. Precision

The precision of the developed analytical methods was estimated by measuring repeatability (intra-day) on six replicate injections of standard solution and on six individual determinations of MOX HCl, and NOR in sample solution obtained using adsorption static method. For sample preparation, 0.2-0.2 g of zeolite adsorbent samples were transferred separately to six 50 mL Erlenmeyer flasks and added 20 mL of each FQ antibiotic standard stock solutions at 1.0 mg/mL concentration. Initially, zeolite samples with adsorbate FQ antibiotic solutions were left on an orbital shaker at 150 rpm for 15 min, then statically for 3 hours. After that zeolite samples were centrifuged at 3000 rpm for 5 min and 1-1 mL of the obtained supernatants were used and diluted to 10 mL with diluent, mix well. The precision was checked by calculating RSD of the concentration of each FQ antibiotic, mg mL-1 and retention times of MOX and NOR for six individual determinations which should not be more than 5.0 % and 1.0 %, respectively. The results are shown in Table 7. Figure 8, 9 shows chromatograms obtained from sample solutions. The results were within than acceptance criteria which indicate that this method has good precision.

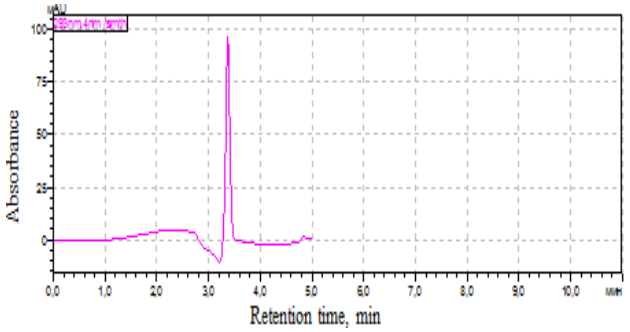

Figure 8. *The chromatogram of effluent sample solution of MOX HCl*

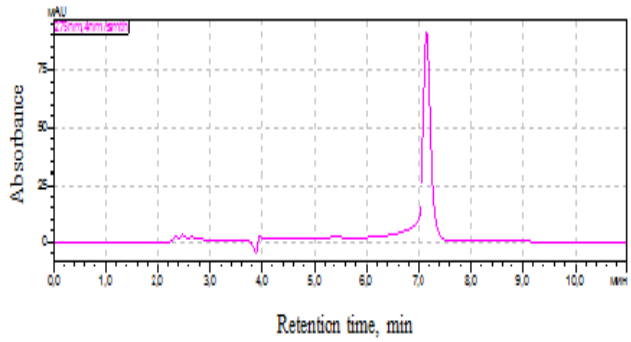

Figure 9. *The chromatogram of effluent sample solution of NOR*

4. CONCLUSIONS:

Analytical procedures were developed and validated for the quantitative determination of moxifloxacin HCl, and norfloxacin in aqueous solutions was found to be robust, precise, linear and accurate. No interferences from blank solution were observed. Hence, the proposed and validated HPLC methods can be successfully used to measure adsorption of moxifloxacin HCl and norfloxacin on the natural zeolites and their modified forms; also, they can be applied by other laboratories for routine analysis of wastewaters and pharmaceutical formulations containing the above-mentioned APIs.

5. ACKNOWLEDGMENTS:

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Table 1. *The structure, main physical-chemical properties and the content in surface water of MOX and NOR*

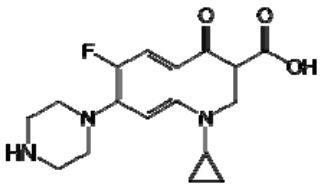
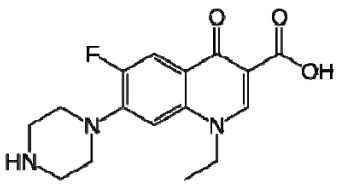
Parameter	**FQ antibiotics**	
	MOX	**NOR**
Molecular formula	$C_{21}H_{24}FN_3O_4$	$C_{16}H_{18}FN_3O_3$
CAS number	354812-41-2	70458-96-7
Molecular structure		
Molecular weight, g/mol	401.438	319.331
The acid dissociation constants, pKa	pKa1=6.43; pKa2=10.63	pKa1=0.16; pKa2=8.68
Chemical name	(4aS-cis)-1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(octahydro-6H-pyrrolol[3,4-b]pyridin-6-yl)-4-oxo-3-quinolinecarboxylic acid	1,4-Dihydro-1-ethyl-6-fluoro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid
Water solubility, mg/mL at 25^0C and pH 5-7.5	1.15-5	0.45-161
Concentration in surface water, μg/L	0.006-0.017	0.0023-0.12
Toxicity, oral LD50 (rat), g/kg	1.32	4

Table 2. *Robustness factors and design of experiment*

#	**Factor (Xi)**	**Unit**	**Low Level (-)**	**Nominal Level (0)**	**High Level (+)**
1	Flow rate of MP (X1)	mL/min	0.9	1.0	1.1
2	MOX/NOR standard stock solution (X2)	pH	4.0	7.0	-
3	ACN percentage in MP for MOX/NOR (X3)	%	35 10	40 15	45 20
4	Column temperature (X4)	°C	35	40	45
5	DAD wavelength for MOX/NOR (X5)	nm	291 273	293 275	295 277

Table 3. *The results of the robustness parameter (critical factors effect study)*

#	Factors					Parameters							
						N		A_s		RSD_A, %		RSD_{RT}, %	
	X1	X2	X3	X4	X5	MOX	NOR	MOX	NOR	MOX	NOR	MOX	NOR
1	+	+	+	+	+	2865	3895	1.22	0.96	1.362	0.736	0.698	0.365
2	+	+	-	+	+	2563	3645	1.35	0.93	1.568	0.987	0.745	0.498
3	+	-	+	-	+	3256	3712	1.27	0.91	1.032	0.898	0.125	0.245
4	+	-	-	-	-	2463	3100	1.18	0.89	1.500	1.456	0.632	0364
5	-	+	+	-	-	3125	3564	1.23	0.93	1.952	1.365	0.455	0.522
6	-	+	-	-	+	3145	3666	1.09	0.86	1.300	1.112	0.245	0.601
7	-	-	+	+	-	2699	3056	1.11	0.87	0.856	1.354	0.112	0.471
8	-	-	-	+	+	2491	3444	1.13	0.89	1.505	1.866	0.733	0.326
Acceptance criteria						>2000		<2.0		<2.0		<1.0	

Table 4. *The LOQ and LOD of MOX HCl and NOR methods*

Parameter	**Value**	
	MOX HCl	**NOR**
LOQ, mg/mL	0.00005	0.00005
LOD, mg/mL	0.00001	0.000008
RSD of peak areas for LOQ (n=6)	3.561	2.956
RSD of retention times for LOQ (n=6)	0.671	0.583
s/N for LOQ	25	21
s/N for LOD	4	7

Table 5. *The system suitability parameters results*

Parameter	**MOX HCl**	**NOR**	**Acceptance criteria**
N	3166	4532	>2000
RSD_A (n=6), %	1.796	1.629	<2.0
RSD_{RT} (n=6), %	0.762	0.866	<1.0
A_s	1.12	0.93	<2.0

Table 6. *The results of the accuracy study*

Added standard solution, mg/mL	Average concentration of spiked sample solution, mg/mL (n=3)	Concentration of effluent sample solution, mg/mL	Acceptance criteria
MOX HCl			
0.0788	0.1319	0.05650	97.5
0.0985	0.1527		98.5
0.1182	0.1750		100.2
Average recovery - Rec, %			98.7
RSD, % of the percentage recoveries			1.383
NOR			
0.0797	0.1654	0.0862	99.7
0.0996	0.0961		96.5
0.1195	0.1222		102.3
Average recovery - Rec, %			99.5
RSD, % of the percentage recoveries			2.920

Table 7. *The results of the precision study*

The number of sample solution	MOX HCL		NOR	
	Concentration, mg/mL	Retention time, min	Concentration, mg/mL	Retention time, min
1	0.0351	3.326	0.0401	7.674
2	0.0385	3.388	0.0421	7.537
3	0.0355	3.390	0.0435	7.578
4	0.0341	3.347	0.0445	7.495
5	0.0377	3.345	0.0412	7.529
6	0.0366	3.352	0.043	7.480
Average	0.0363	3.358	0.0424	7.549
SD	0.002	0.026	0.002	0.070
RSD	4.582	0.762	3.774	0.931