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DEVELOPMENT OF DISPERSIVE MICRO-SOLID PHASE EXTRACTION FOR THE ANALYSIS OF OFLOXACIN AND SPARFLOXACIN IN HUMAN PLASMA

(Pembangunan Pengekstrakan Fasa Pepejal-Mikro Disesarkan untuk Analisis Oflosaksin dan Sparflosaksin dalam Plasma Manusia)

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Abstract

Dispersive micro-solid phase extraction (D-µ-SPE) using C_{18} adsorbent combined with HPLV-UV was developed for the determination of ofloxacin and sparfloxacin in human plasma. Seven D-µ-SPE parameters namely type and amount of adsorbent mass, sample volume, pH of sample solution, extraction time, desorption solvent and volume were optimized. Under optimum conditions, calibration curves showed good linearity in the range of 0.5–1000 µg L^{-1} with acceptable limit of detection (LOD) of 0.73 and 1.81 µg L^{-1} and limit of quantitation (LOQ) of 2.44 and 6.03 µg L^{-1} for ofloxacin and sparfloxacin, respectively. The D-µ-SPE also demonstrated acceptable precision at the concentration of 500 dan 1000 µg L^{-1} of ofloxacin and sparfloxacin, respectively in human plasma with RSD value of ≤12.5%. A good relative recoveries was obtained between 90.1-109.5%. The developed D-µ-SPE method has proven to be a fast and simple approach which only requires low amount of extraction solvent for drug analysis.

Keywords: dispersive micro-solid phase extraction, high performance liquid chromatography, ofloxacin, sparfloxacin, human plasma

Abstrak

Pengekstrakkan fasa pepejal mikro disesarkan (D-µ-SPE) menggunakan penjerap C_{18} yang digabungkan dengan HPLC-UV telah dibangunkan untuk penentuan oflosaksin dan sparflosaksin dalam plasma manusia. Beberapa parameter D-µ-SPE seperti jenis dan jumlah jisim penjerap, isipadu sampel, pH larutan sampel, masa pengekstrakan, pelarut penyahjerap dan masa penyahjerap telah dioptimumkan. Dalam keadaan yang optimum, lengkung penentu ukuran menunjukkan lineariti yang baik dalam julat 0.5-1000 µg L^{-1} dengan had pengesanan (LOD) 0.73 dan 1.81 µg L^{-1} dan had pengukuran (LOQ) 2.44 dan 6.03 µg L^{-1} yang memuaskan masing-masing bagi ofloksaksin dan sparflosaksin. Kaedah yang dicadangkan juga menunjukkan ketepatan pada kepekatan 500 dan 1000 µg L^{-1} yang baik bagi oflosaksin dan sparflosaksin dari plasma manusia dengan RSD ≤12.5% dan pemulihan relatif yang

baik dalam julat 90.1-109.5%. Kaedah D-μ-SPE terbukti sebagai kaedah yang cepat danmudah untuk analisis ubat-ubatan kerana ia hanya memerlukan pelarut organik dalam jumlah yang kecil sewaktu analisis dijalankan.

Kata kunci: pengekstrakkan fasa pepejal mikro disesarkan, kromatografi cecair berprestasi tinggi, oflosaksin, sparflosaksin, plasma manusia

Introduction

Fluoroquinolones (FQs) are one of the most significant groups of synthetic antibiotics that possess excellent pathogenic behavior [1]. FQs are currently licensed as antibiotics under the Ministry of Health Malaysia's National Pharmaceutical Regulatory Agency (NPRA) and are listed by the WHO as important medicines for human health. Due to the increased resistance to beta-lactams and macrolides, FQs are currently used widely for the treatments of respiratory tract infections [2]. A broad-spectrum of this class of drugs demonstrated good activity against several gram-positive and gram-negative pathogenic bacteria, such as atypical respiratory pathogens [3].

Nevertheless, the concentration of FQs in human plasma are not measured in clinical practice. Considering the toxification and effectiveness of drugs, he concentration of FQs in body fluid should be tracked to change its dosage for regular use in clinical practice [4,5]. Moreover, FQs can become emerging contaminants in the environmental sample as they were partially metabolised inside the human body 20-80% were excreted out from human body in various pharmacologically active states. [6]. Therefore, appropriate analytical methods must be developed for the detection of FQs in the environmental and biological samples [7].

Several analytical techniques based on the spectrofluorimetric [6], high-performance liquid-chromatography (HPLC) with UV [8, 9], or mass spectrometry (MS) detection [10, 11] and capillary electrophoresis (CE) [12] were established for the determination of FQs. The process of sample preparation plays an important role in increasing sensitivity and reducing matrix interference, particularly when dealing with complex human plasma samples. One of the most common techniques for extraction of FQs is a solid phase extraction (SPE) by virtue of its ability to enrich analytes and eliminate matrix interferences [13-16]. However, it take a relatively long time and requires large amount of solvents during the analysis.

A recent trend in analytical chemistry is to miniaturize the separation techniques that enhance the extraction efficiency based on performance and cost consumption of the process [11]. Dispersive micro-solid phase extraction (D-μ-SPE) which is the SPE miniaturization is established to resolve the inherited disadvantages. In this extraction process, the adsorbents are applied directly to the sample solution, wherein the equilibrium can be achieved in a short time, since the surface area between the adsorbent and the aqueous solution is high [17]. D-μ-SPE offers a wide range of advantages compare to SPE due to its convenience, lower solvent use and efficiency of recovery within short time. Enhancement in the extraction efficiency can also be achieved due to the increment of contact area between adsorbent and analytes during dispersion process. It is also an economical and simple technique which can be used with different types of adsorbents [18-20].

The use of dispersed adsorbents was firstly reported by Anastassiades et al. which focused on increasing method selectivity [21]. Recently, there were only two studies of D-μ-SPE reported the determination of FQs involving ofloxacin. Amoli-Diva et al. investigated the modification of MWCNT in combination of surfactant-enhanced magnetic nanoparticles as an adsorbent for extracting FQs from plasma and urine samples [22]. With the advancement of technology, a new approach of an automated magnetic D-μ-SPE which is based on flow system using the strategy of fluidized beds. Zr-Fe-magnetic nanoparticles were used in this case as a powerful adsorbent for the determination of FQs in food samples [23].

Considering the advantages of D-µ-SPE, this study investigated the development of D-µ-SPE utilizing C_{18} adsorbent, coupled with HPLC-UV for the detection of ofloxacin and sparfloxacin. Factors that affect the extraction efficiency were evaluated and optimized. The proposed D-µ-SPE method showed an excellent performance for the detection of ofloxacin and sparfloxacin in human plasma.

Materials and Methods

Chemicals and reagents

Ofloxacin and sparfloxacin from Sigma-Aldrich (Steinheim, Germany). HPLC-grade methanol (>99.99%) and silica particles from Merck (Darmstadt, Germany). Sodium phosphate monobasic monohydrate, phosphoric acid (85%, w/w) and sodium phosphate from Sigma-Aldrich (St. Louis, MO, USA). Center for Drug Research, Universiti Sains Malaysia, Penang, Malaysia have kindly donated human plasma sample for this study. DSC-18 (C_{18} adsorbent, 50 µm particle size) from Supelco (Bellefonte, PA, USA). Ultrapure water (resistivity, 18.2 M Ω cm^{-1}) was used for the preparation of solutions in this study.

Chromatographic conditions

The chromatographic analyses were carried out using Thermo-Fisher Hypersil Gold ODS C_{18} (250 × 4.6 mm × 5 µm) at the wavelength of 254 nm. The mobile phase was prepared in the presence of methanol (MeOH) and phosphate buffer (5 mM, pH 3.0) with a ratio of 50:50, v/v. It was filtered and degassed with a 0.22 µm membrane filter (Sartorius, Germany) before use. The flow rate 1.0 mL/min and the injection volume 20 µL were used.

Preparation of stock and working solution

Stock solutions ofloxacin and sparfloxacin (20 mg L^{-1}) were prepared by dissolving both compounds in MeOH and then kept at 4°C. Working standard solutions (1000 µg L^{-1}) were prepared by diluting stock solution in 10 mL volumetric flask, then top-up to the mark before adjustment to the appropriate pH.

D-µ-SPE procedure

Approximately 1000 µg L^{-1} of the spiked ofloxacin and sparfloxacin solution was put in a 50 mL centrifuge tube that contained 20 mg of C_{18} adsorbent. The mixture was then vortexed at high speed (2400 rpm) for 1 minute to allow adsorbent to disperse in the sample. The solution was modified to pH 4 with 0.1 M HCl. Next, the adsorbent was collected on 20-25 µm filter paper (Whatman, UK) after the extraction. After that, the adsorbent was transferred to a centrifuge tube. A 200 µL of MeOH was introduced to desorb the analytes and sonicated for 5 minutes. Finally, the supernatant was filtered through 0.22 µm nylon membrane filter prior to HPLC-UV analysis.

Optimization of D-µ-SPE parameters

Seven D-µ-SPE parameters including the type and amount of adsorbent mass, sample volume, pH of sample solution, extraction time, desorption solvent and volume were studied. The optimizations were carried out using one variable at a time (OVAT) method, where an independent variable changed, while the dependent variables were kept at a constant level.

Method validation of D-µ-SPE

For D-µ-SPE, the linearity was carried out at different concentrations (0.5, 100, 250, 500, 750, 1000 µg L^{-1}). Intra-day and inter-day were evaluated at the concentration of 500 and 1000 µg L^{-1} in triplicate for the same day (n = 3) analyses. Relative recovery (%RSD) was determined as the percentage of the mean target analyte concentration detected after extraction against the concentration of spiked ofloxacin and sparfloxacin in the sample.

Sample pre-treatment for human plasma

To minimize the matrix effects prior to D-µ-SPE, 2 mL of human plasma sample was spiked prior to pH adjustment to pH 4 using 0.1 M of HCl. Then, the mixture was vortexed for 30 s to ensure complete mixing. MeOH (1 mL) was added to the blank/spiked plasma (2 mL) and the mixture was centrifuged at 4000 rpm for 10 min in order to precipitate the proteins. Then, the supernatant was collected and applied for the D-µ-SPE procedure.

Results and Discussion

Optimization of D-μ-SPE conditions: Selection of adsorbent

Selecting sorbent material is vital to the development of D-μ-SPE methods as it determines the selectivity and absorption ability of target analytes, particularly in complex matrices such as human plasma [24]. In this study, various adsorbent materials, i.e. C_{18}, -CN and -NH were investigated which can be categorized into polar adsorbent (-CN, -NH) and non-polar adsorbent (C_{18}). The retention of the analytes on the adsorbents is due to weak interactions of both compounds through the hydrogen bonding, π-π interactions and Van der Waals. Based on the result obtained, the highest extraction efficiency for ofloxacin and sparfloxacin were achieved by using C_{18} as adsorbent (Figure 1). As ofloxacin and sparfloxacin have the log *P* value of 1.86 and 2.60, respectively which can be considered as mid-polar analytes, C_{18} adsorbent provided greater adsorption as compared to -CN and -NH adsorbent. Thus, it was selected for use in the subsequent analysis.

Mass of adsorbent

To investigate the effect of C_{18} adsorbent mass on ofloxacin and sparfloxacin extraction efficiency, the mass was varied from 10–30 mg. As depicted in Figure 2, an increment of extraction efficiency was observed as the mass of C_{18} adsorbent increased. However, the reduction of extraction efficiencies was observed when more than 20 mg were used. This observation may be attributed to the agglomeration of C_{18} adsorbent, masking the sorbent active sites, thereby reduces its extraction efficiency [25]. Hence, 20 mg of C_{18} was chosen as an adsorbent in the following experiments.

Sample volume

In order to improve the partitioning between ofloxacin and sparfloxacin and C_{18} adsorbent, the effect of the sample volume on the extraction efficiency was investigated in the range of 5-20 mL. The extraction efficiency of ofloxacin and sparfloxacin improved with the increment of the sample volume to 10 mL, as shown in Figure 3. The extraction efficiency of ofloxacin and sparfloxacin sustained when the volume of sample increased more than 10 ml. Therefore, the sample with the volume of 10 mL was selected for use in the subsequent experiments.

Extraction time

The amount of analyte extracted in D-μ-SPE is based on mass transfer from the aqueous phase to the adsorbent [26]. Extraction time therefore is another significant parameter to consider. Extraction times in the range of 30-110 s were studied to evaluate the effects of extraction time on the extraction efficiency. As shown in Figure 4, extraction efficiencies improved as the extraction time increased to 60 s. The availability of abundant active regions on the adsorbent surface enabled ofloxacin and sparfloxacin to react immediately by binding to the adsorbent binding regions at the beginning of the extraction time and resulted in increased extraction efficiency with increasing extraction time [7]. There was no significant improvement on the extraction efficiency when the extraction time was prolonged. Therefore, the extraction time of 60 s was selected for the analysis.

pH of sample solution

pH has a major effect on extraction efficiency by influencing the form of the analyte by transforming the analytes into molecular forms and enhances the surface charge C_{18} adsorbent. The pH of the sample solution has a significant impact on the extraction as it specifies the ionic state of the analytes which influences the extraction process [27]. The pH of the sample solution was set within a range of 2–7 to study its effect on the extraction efficiencies. Since ofloxacin and sparfloxacin are weak base analytes with pK_a values of 5.19 to 6.42, respectively, they existed in molecular forms under acidic environment to allow the extraction to occur. Based on the results shown in Figure 5, ofloxacin and sparfloxacin were converted more in molecular forms in an acidic environment. Nonetheless, under highly acidic condition (pH 2), they could easily be degraded. At pH greater than 4, both antibiotics were in their ionizable form which restricted the extraction process. Further investigations were carried out using sample solution at pH 4.

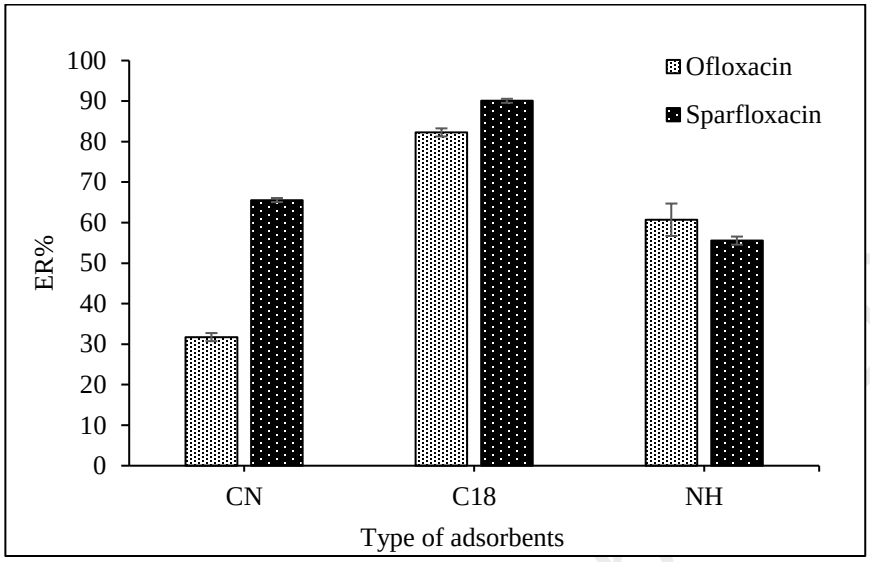

Figure 1. Effect of type of adsorbents on the extraction efficiency of ofloxacin and sparfloxacin. Extraction conditions: 1000 µg L^{-1} of ofloxacin and sparfloxacin; mass of adsorbent: 20 mg; sample volume: 10 mL at pH 4; extraction time: 60 s; desorption solvent: 200 µL of MeOH; desorption time: 5 minutes

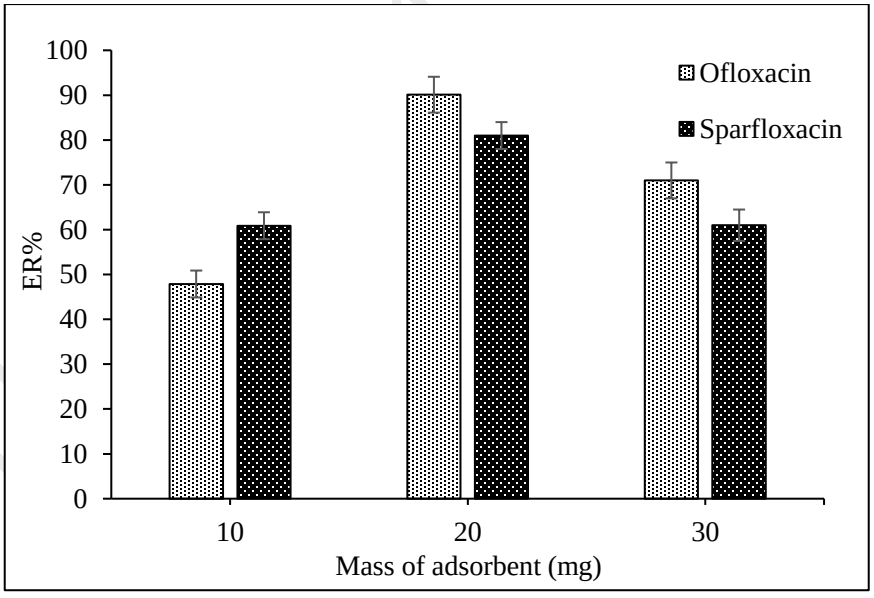

Figure 2. Effect of mass of adsorbent on the extraction efficiency of ofloxacin and sparfloxacin. Extraction conditions: 1000 µg L^{-1} of ofloxacin and sparfloxacin; type of adsorbent: C_{18} ; sample volume: 10 mL at pH 4; extraction time: 60 s; desorption solvent: 200 µL of MeOH; desorption time: 5 minutes

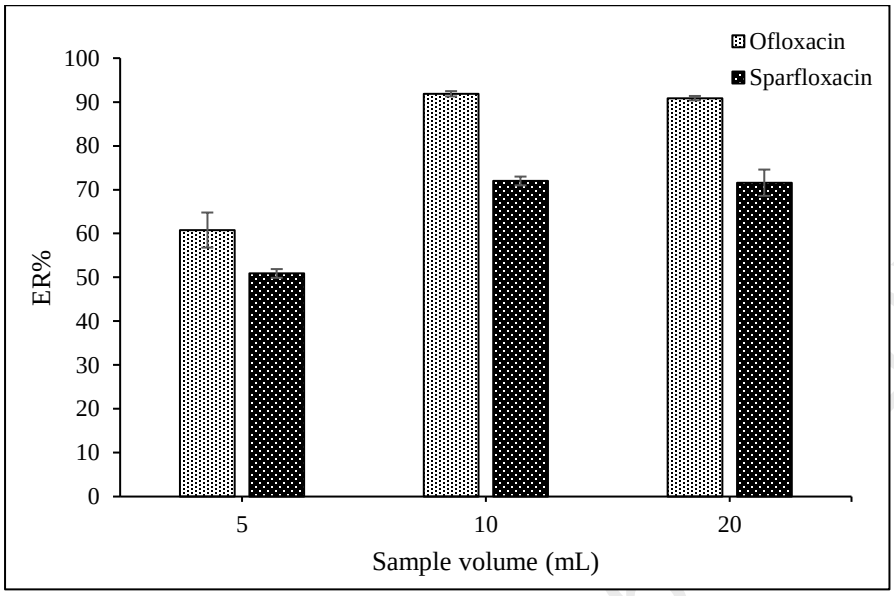

Figure 3. Effect of sample volume on the extraction efficiency of ofloxacin and sparfloxacin. Extraction conditions: 1000 µg L^{-1} of ofloxacin and sparfloxacin; adsorbent: 20 mg of C_{18}; sample solution: pH 4; extraction time: 60 s; desorption solvent: 200 µL of MeOH; desorption time: 5 minutes

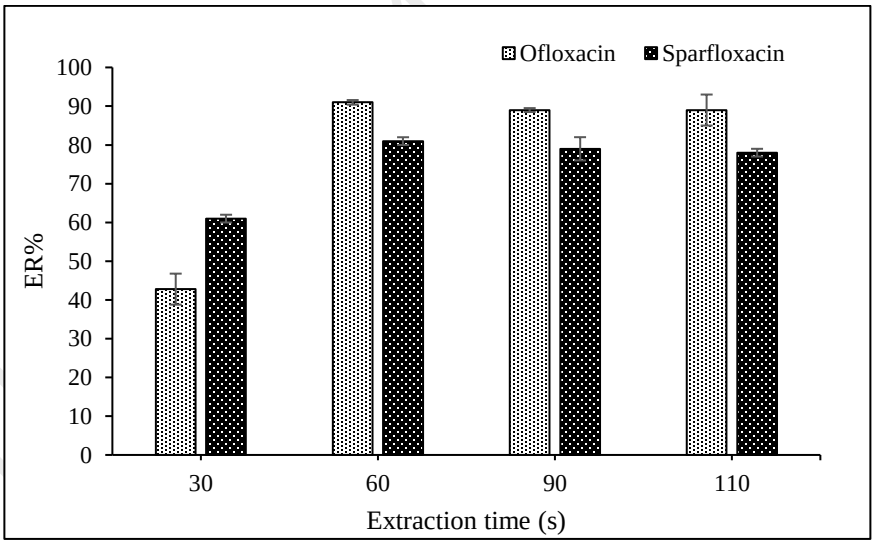

Figure 4. Effect of extraction time on the extraction efficiency of ofloxacin and sparfloxacin. Extraction conditions: 1000 µg L^{-1} of ofloxacin and sparfloxacin; adsorbent: 20 mg of C_{18}; sample solution: 10 mL at pH 4; desorption solvent: 200 µL of MeOH; desorption time: 5 minutes

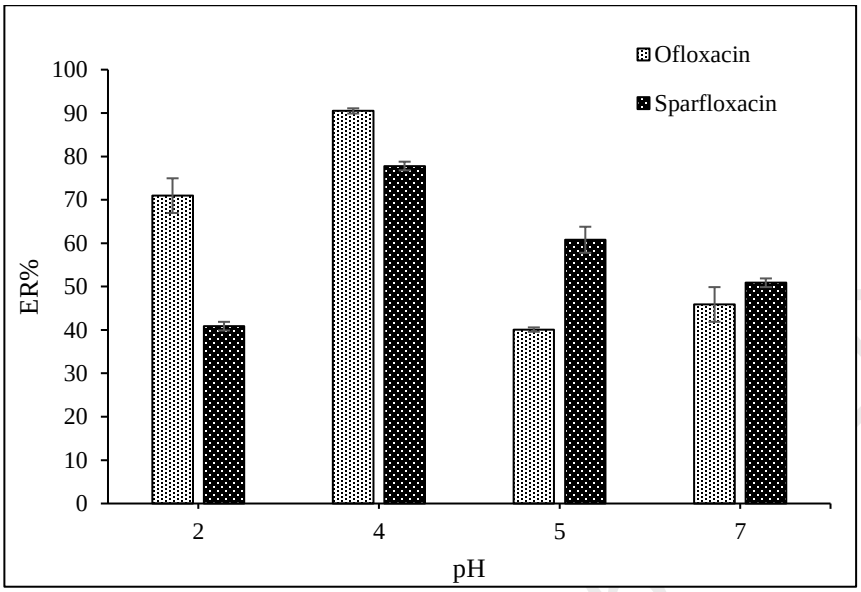

Figure 5. Effect of pH on the extraction efficiency of ofloxacin and sparfloxacin. Extraction conditions: 1000 µg L^{-1} of ofloxacin and sparfloxacin; adsorbent: 20 mg of C_{18}; sample solution: 10 mL; extraction time: 60 s; desorption solvent: 200 µL of MeOH; desorption time: 5 minutes

Desorption solvent

The selection of solvents for desorption depends on the compatibility with the HPLC-UV. This study assessed typical organic solvents, namely dichloromethane (DCM), acetonitrile (ACN) and methanol (MeOH) as desorption solvents for ofloxacin and sparfloxacin. As shown in Figure 6, MeOH showed the highest peak areas followed by ACN and DCM in most of the analytes. This result can be explained by the high solubility of ofloxacin and sparfloxacin in MeOH (both standard stock solutions were prepared in MeOH). Hence, MeOH was selected as the solvent for desorption.

Desorption time

In this analysis, several desorption times in between 3-15 minutes were analysed. At desorption time of 5 minutes, the extraction efficiency was optimum (Figure 7). There was no significant improvement was observed as the desorption time extended to 15 minutes. Therefore, desorption time of 5 minutes was chosen in the subsequent experiments.

Method validations

Analysis of human plasma

The linearity of D-µ-SPE was evaluated using five different concentrations prepared by spiking MeOH with the ofloxacin and sparfloxacin standards. The calibration curve was linear indicated by R^2 values obtained, (0.9973 for ofloxacin and 0.9907) for sparfloxacin over the studied concentration range of 0.5–000 µg L^{-1} with LOD and LOQ were 0.73 - 1.81 µg L^{-1}, respectively as shown in Table 1. Although the endogenous interferences was successfully eliminated in the pre-treatment of human plasma, the low LOD was obtained. This is primarily due to the multiple extractions in smaller quantities during the analysis.

The precisions of the analysis were measured by conducting triplicate analysis of spiked human plasma sample on the same day and on three different days at various concentrations. The proposed approach demonstrated acceptable precision based on the RSD values (Table 2). D-µ-SPE method demonstrated

significant relative recovery of ofloxacin and sparfloxacin in the range of 90.1-109.5% from human plasma samples. The recovery was acceptable with good RSD values suggesting a satisfactory extraction efficiency. Under optimum conditions, the overall extraction efficiency was evaluated by the typical chromatogram as shown in Figure 8. There was no interference in the analytes detected in the blank sample.

Comparison of D-μ-SPE with other published methods

The proposed extraction of method D-μ-SPE-HPLC-UV was compared with other published methods (Table 3). Each approach usually has its own advantages and disadvantages. SPE developed adequate sensitivity, accuracy, and recoveries due to the selective adsorbent of hydrophilic and lipophilic balance against ofloxacin and sparfloxacin [26]. A simpler process for the precipitation of proteins has recently been developed to remove drugs from human plasma. This method was fast and fulfilled the high requirements of bioanalysis for the sample yield. The detection limit was indeed not sufficient [27]. The proposed method D-μ-SPE has many benefits such as time saving, high sensitivity and flexibility, as well as applicable to human plasma. This method only used 20 mg of C_{18} adsorbent, allowing optimum interaction between analytes and adsorbent. This result in short contact time between analytes and adsorbent with greater sensitivity in comparison to other procedures. Furthermore, D-μ-SPE requires simple setup and ultrasonics instrument. Thus, the proposed method could be served as an alternative approach that is beneficial for green microextraction in biological matrices of ofloxacin and sparfloxacin.

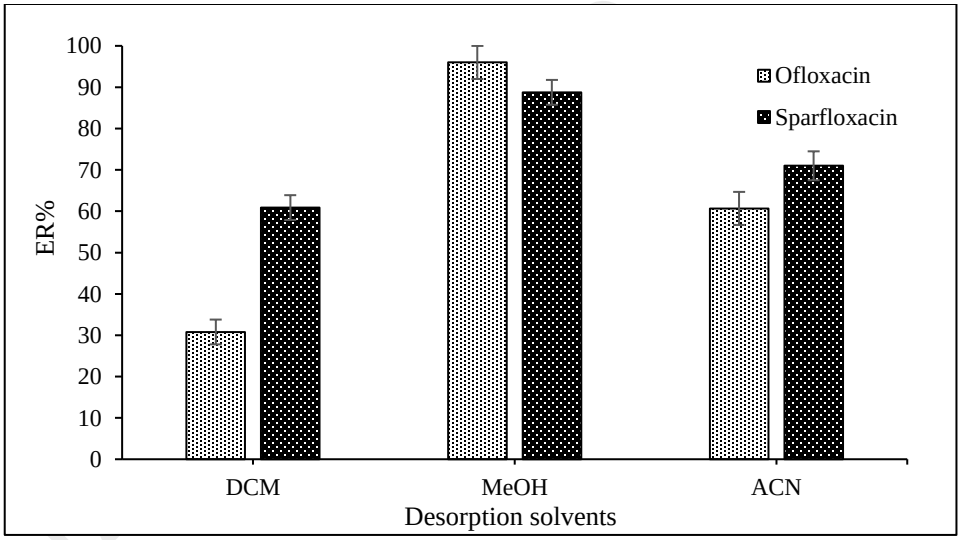

Figure 6. Effect of solvent on the extraction efficiency of ofloxacin and sparfloxacin. Extraction conditions: 1000 μg L^{-1} of ofloxacin and sparfloxacin; adsorbent: 20 mg of C_{18}; sample solution: 10 mL at pH 4; extraction time: 60 s; desorption solvent: 200 μL desorption time: 5 minutes

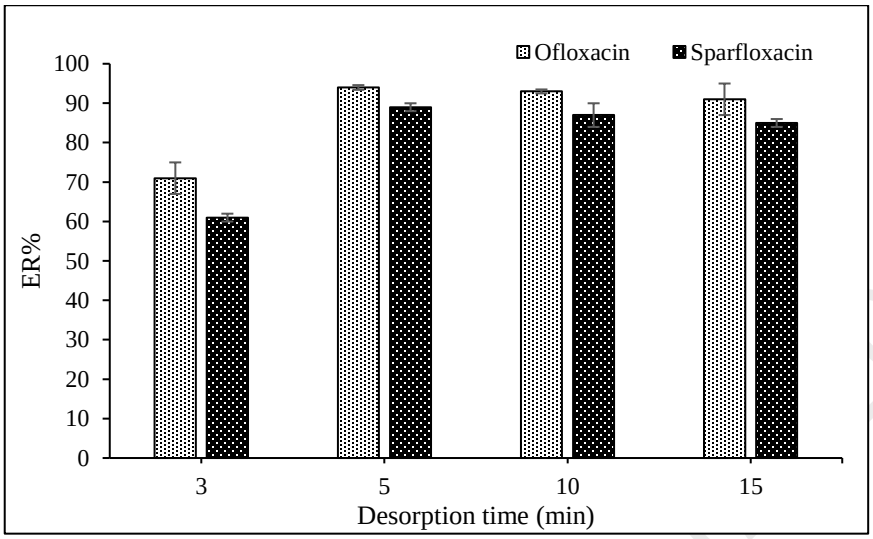

Figure 7. Effect of desorption time on the extraction efficiency of ofloxacin and sparfloxacin. Extraction conditions: 1000 µg L^{-1} of ofloxacin and sparfloxacin; adsorbent: 20 mg of C_{18}; sample solution: 10 mL at pH 4; extraction time: 60 s; desorption solvent: 200 µL of MeOH

Table 1. Validation parameters for the proposed method for ofloxacin and sparfloxacin in plasma

Matrix (Plasma) / Analytes	**Linearity Range (µg L^{-1})**	**Correlation of Determination (r^2)**	**LOD[a] (µg L^{-1})**	**LOQ[b] (µg L^{-1})**	**Precision, RSD (%, n = 3)**
Ofloxacin	0.5-1000	0.9973	0.73	2.44	5.60-9.81
Sparfloxacin	0.5-1000	0.9907	1.81	6.03	3.97-7.35

[a]Calculated from signal - to - noise =3, [b]Calculated from signal - to - noise =10

Table 2. Recoveries (%) and precisions (% RSD) of D-µ-SPE-HPLC-UV of human plasma sample

Concentration Level (µg L^{-1})	**Plasma**	
	Ofloxacin	**Sparfloxacin**
Intra-day recoveries (RSD, %, n=3)		
500	90.1± 1.9	100.1± 9.1
1000	100.6± 8.03	102.1± 5.3
Intra-day recoveries (RSD, %, n=3)		
500	98.6± 4.3	103.6± 7.1
1000	105.6± 6.32	109.5± 12.5

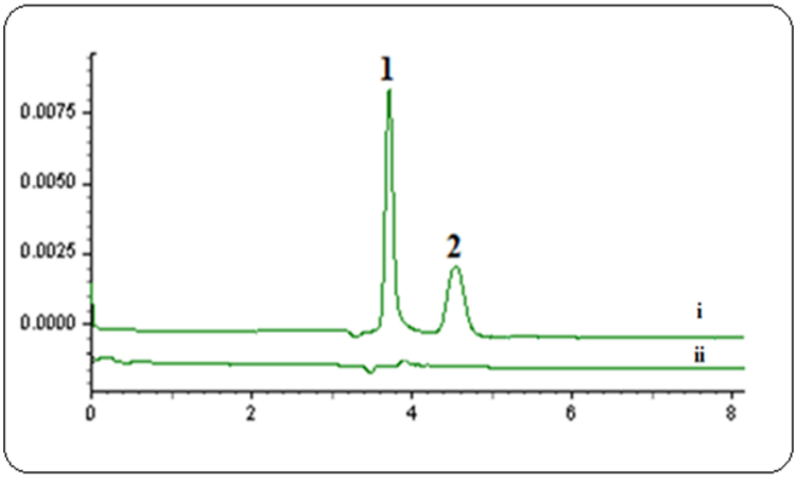

Figure 8. HPLC typical chromatogram of antibiotics drugs subjected to D-µ-SPE - HPLC-UV. i: spiked plasma (1000 µg L^{-1}); ii: unspiked human plasma under the optimized HPLC-UV conditions, Column: Thermo-Fisher Hypersil Gold ODS C_{18} (250 x 4.6 mm x 5µm); mobile phase: MeOH and phosphate buffer (5 mM, pH 3.0), (50:50) λ: 254 nm; flow rate: 1 mL min^{-1}. (1) ofloxacin and, (2) sparfloxacin

Table 3. Comparison of D-µ-SPE with other published methods for the determination of ofloxacin and sparfloxacin

Fluoroquinolone	**Matrix**	**Extraction Technique**	**Analytical Method**	**LOD (µg L^{-1})**	**Recovery (%)**	**Ref.**
Sparfloxacin	Plasma, Urine	PP	HPLC–UV	25	96.7	[28]
Gatifloxacin, Sparfloxacin and Moxifloxacin	Plasma	LLE	HPLC–UV	0.0001	80.8	[29]
Ofloxacin	Edible animal tissue	SPE	HPLC–UV	0.018	60	[30]
Ofloxacin	Plasma	LLE	HPLC–UV	15	92.9	[31]
Ofloxacin and sparfloxacin	Plasma	D-µ-SPE	HPLC–UV	0.73 and 1.81	89.5 - 102.3	Current work

SPE: Solid phase extraction, PP: Protein precipitation, LLE: Liquid -liquid extraction, D-µ-SPE: Dispersive micro-solid phase extraction

Conclusion

In this study, D-µ-SPE method coupled with HPLC-UV for the determination of ofloxacin and sparfloxacin were successfully developed and validated. It is an aqueous-based method that can be used for the direct HPLC analysis . Besides, it was found to be simple and fast approach that only used less amount of solvent for sample extraction The simplicity and sensitivity of the developed D-µ-SPE can be advantageous for the routine drugs analysis of ofloxacin and sparfloxacin in human plasma as drug monitoring system. Therefore, the dosage of drug can be monitored accurately and an appropriate dose of medicines can be given to the patients with sufficient adsorption in the body. Furthermore, the developed D-µ-SPE method might have the potential to be applied for the detection of emerging pollutants such as antibiotics in the environmental water samples.

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