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Research Article

Molecularly Imprinted Polymers-Solid Phase Extraction Coupled With HPLC-FLD for the Analysis of Four Fluoroquinolones in Honey and Urine

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ABSTRACT
A new, fast and sensitive method using molecularly imprinted polymers-solid phase extraction (MIPs-SPE) followed by high pressure liquid chromatography with fluorescence detection (HPLC-FLD) has been developed for the separation and determination of four fluoroquinolones (FQs) from honey and urine. Detection has been done by fluorescence measurement with excitation wavelength 300 nm and emission wavelength 450 nm. The best separation was achieved in mobile phase consisting of water, acetonitrile, and 1% formic acid (21:9:70 v/v). Sample preconcentration and sample clean-up was done by molecularly imprinted polymer solid-phase extraction and the developed method has been applied to spiked samples of honey and urine for extraction of four fluoroquinolones namely norfloxacin (NOR), ofloxacin (OFLO), lomefloxacin (LOME) and danofloxacin (DANO). Detection limits are in the low range of 0.02 – 0.287 ng /mL.

KEYWORDS: HPLC –FLD; MIPs-SPE; Fluroquinolones

INTRODUCTION

Fluroquinolones (FQs) are broad spectrum synthetic antibacterial drugs widely used in human and veterinary medicines [1, 2]. They are highly effective against both Gram-positive and Gram-negative bacteria. In Gram-positive bacteria, the mode of their action is the inhibition of DNA gyrase (a topoisomerase II), an enzyme responsible for supercoiling of bacterial DNA during DNA replication, while in Gram-negative bacteria, the primary target is topoisomerase IV, an enzyme responsible for relaxation of super-coiled circular DNA and separation of the inter-linked daughter chromosomes [3, 4]. FQs are only partially metabolized by the patients, so eliminated as parent compounds being major constituent of hospital sewage or municipal wastewater. FQs are not completely removed by sewage treatment and hence irrigation of crops with this water introduces them in surface waters with agricultural runoff [5]. FQs are persistent pollutant due to the great stability of heterocyclic ring and the relative solubility which increases their environmental diffusion [6]. Various products of animal origin like meat, honey, milk or its derivatives have adverse effect on human heath due to presence of FQs in them and they also constitute a resistance selection to the pathogens [7]. The widespread use of these antibiotics has led to resistance of human pathogens against them resulting their appearance in both environmental and various other matrices [8]. Therefore, the study of FQs

nowadays is of special concern because of the ever growing antimicrobial resistant bacteria

The chromatographic techniques are most widely used for the determination of fluoroquinolones in various matrices. Analysis of FQs is mostly carried out by HPLC employing UV [9-11], fluorescence [12-15] and electrochemical detections [16]. FQs have high florescence quantum yield thus allowing for highly sensitive analytical methods. As FQs have different physio-chemical properties, so sample pre-treatments are necessary to extract these drugs [17]. Various sample preparation techniques such as liquid to liquid extraction (LLE) [18,19], solid phase microextraction (SPME) [20,21] , supercritical fluid extraction (SFE) [22] , stir bar sorptive extraction (SBSE) [23], liquid phase microextraction (LPME) [24], dispersive liquid-liquid microextraction (DLLME) [25] have been described for the analysis of FQs in various matrices. The tedious and time consuming procedures, relative low recoveries (>50-70%), poor extraction efficacy and inability to simultaneously extract all FQS has limited those methods practically [7].

Solid-phase extraction is one the most popular routinely used techniques due to its simplicity, rapidness, less organic solvent consumption and convenience to use [26]. Despite their attractive features, the classical SPE sorbents such as C18, ion-exchange and size-exclusion phases are lacking in selectivity leading to co-extraction of matrix interference components with the target analyte [27, 28]. The best solution to this problem is by carrying out selective extraction targeted for the compound of interest. This selective extraction is potentially accomplished by using molecularly imprinted polymers (MIPs) which bind the target molecule in selective manner even when the target analyte is present in complex matrix [26]. MIPs are synthetic materials with artificially generated recognition sites able to specifically rebind a target molecule in preference to other closely related compounds [26-30]. MIPs are synthesized by polymerization and cross linking of monomers around the template molecule. Template molecule with binding sites complementary to target analyte in shape, size and function is extracted after the completion of polymerization. MIPs are stable, robust and resistant to wide range of pH, solvent and temperature. Their synthesis is cheap and easy compared to natural receptors having high mechanical strength and reusability [27, 28, 31, 32]. MIPs-SPE allows not only the pre-concentration of analyte but also the removal of other interfering compounds present in the sample matrix.

This paper describes HPLC method combined with simple, fast and efficient extraction procedure based on SPE combined with MIPs that enables to determine four FQs namely, norfloxacin (NOR), olfoxacin (OFLO), lomefloxacin (LOME) and danofloxacin (DANO) in honey and urine. The variables affecting SPE-MIPs efficiency as eluting solvent, eluting solvent volume and flow rate were systematically optimized and the conditions for HPLC separation were investigated to get good recoveries with minimal matrix effects.

EXPERIMENTAL

Analytical reference standards and reagents

The standards of norfloxacin (NOR), ofloxacin (OFLO), lomefloxacin (LOME), danofloxacin (DANO) were obtained from Sigma Aldrich (Steinheim, Germany) and kept at - 4°C. HPLC-grade acetonitrile and methanol were purchased from Merck. Aqueous and non-aqueous solvents were filtered with 0.45 µm Nylon - 6, 6 membranes (Rankem, New Delhi, India) in a glass filtration assembly (Riveria, Mumbai, India). Prior to injection, all samples were centrifuged and then filtered using 0.22 µm syringe filters (Rankem, New Delhi, India).

Instrumentation

The analytical system consisted of HPLC (Dionex P680, Dionex Softron GmbH, Germany). It is a quaternary solvent delivery pump attached with 20 µL rheodyne sampling valve, a RP-Amide column of 10 cm×4.6 mm, 5 µL. The 'Ultimate 3000 Fluorescence Detector' was used with excitation (λ_{ex} = 300 nm) and emission wavelengths (λ_{em} = 450 nm). Chromeleon software was used for data acquisition. Separations were carried out at room temperature maintained at 23 - 25°C.

Standards preparations

Stock solutions for standards were prepared by dissolving these substances in 0.1 M acetic acid solution at concentration of 1mg/mL. The stock solutions were kept at - 4°C and could be stored over a four week period. Required standard

solutions were prepared daily by diluting the stock solutions.

Sample preparation

Honey sample

Honey sample was purchased from local market. Sample was prepared by dissolving 1 gm honey in 5 mL of methanol and centrifuged for 5 min at 8000 rpm. Prior to use for HPLC-FLD study this supernatant was spiked with known amount of FQs. Then this sample was cleaned with SPE by loading and washing on conditioned MIPs.

Urine sample

Urine sample of a healthy person was taken for sample preparations. Each urine sample was initially diluted 10 times with triple distilled water and then filtered by using Nylon 6, 6 membrane filters in filtration assembly (Riviera, SCHOTT DURAN, Mainz, Germany). It was then degassed with an ultrasonic bath and stored at - 4ºC. It was then spiked with the FQs to obtain the required concentration for MIPs-SPE/HPLC analysis.

Chromatographic Separation

In reversed phase HPLC, the success of separation depends on choosing suitable mobile phase. The mobile phase is usually composed organic solvent (e.g. acetonitrile and water). In this work, the effect of polar fractions (water and acetonitrile) in the mobile phase on separation of target fluoroquinolones was investigated. Different ratios of mixture of acetonitrile, water and buffer (i.e. 1% formic acid) were used to optimize the baseline separation. Finally, a ternary system of water-acetonitrile-buffer was adopted. The isocratic elution of these three solvents in the ratio of (21:9:70 v/v) water, ACN, buffer respectively gives the desired baseline separation with fine peak shapes and good resolution The flow rate was also optimized by the variation of flow from 1mL/min to 1.8 mL/min. To avoid high column pressure, it was optimized at 1.5 mL/min. The separation of four fluoroquinolones was achieved within 10 minutes at λ_{ex} = 300 nm and λ_{em} = 450 nm using fluorescence detector. The injection volume was 20 µL. The column temperature was 25°C.

SPE procedure

SPE was used as sample preparation and sample cleaning technique using molecularly imprinted cartridges (MIPs). Supel MIPs fluoroquinolones SPE cartridges column (25 mg/3 mL) were purchased from Supelco (Sigma Aldrich, India). A Visiprep SPE Vacuum manifold was purchased from Supelco (Sigma Aldrich, India) and used for SPE procedures using molecularly imprinted polymer based cartridge. Spiked sample of honey and urine were taken for SPE using MIPs. Solid phase extraction consists of four steps namely conditioning of cartridges, loading of sample, washing and elution of loaded sample. In first step MIPs were conditioned with 2 mL methanol followed by 2 mL of triply distilled water. In loading step 5 mL of sample spiked with FQs was loaded by following washing step. Washing was done with 2 mL of water. In final step elution of loaded analytes was done in 250 µL of optimized eluting solvent i.e., 0.1 M acetic acid. The eluted samples were injected to HPLC system.

RESULTS

SPE-HPLC-FLD Analysis

The separation of NOR, OFLOX, LOMI and DANO was studied using direct injection of sample and different parameters like selection of suitable wavelength, effect of flow rate and composition of mobile phase were optimized. Flow rate was optimized to 1.5 mL/min. Fluorescence detection was performed at λ_{ex} = 300 nm and λ_{em} = 450 nm. A characteristic chromatogram at 10 ng/mL showing the separation of NOR, OFLO, LOMI and DANO is shown in Fig.1

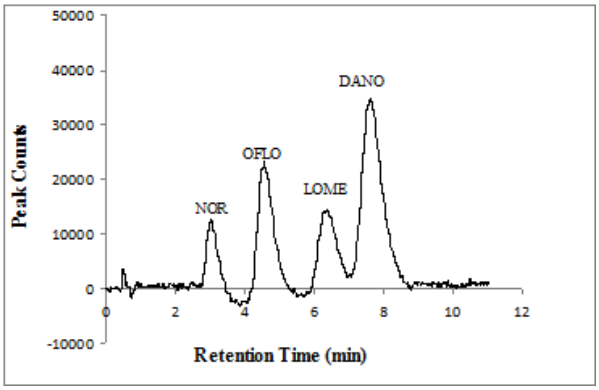

Fig 1. Chromatogram of standard of FQs at10 ng/mL

Optimization of eluting solvent

Different eluting solvents were used to optimize eluting solvent in which maximum elution can be obtained. Solvents like methanol, ACN were used but recoveries were less as FQs are not completely soluble in these solvents. Then mixtures of these with ACOH were used like 0.1 M acetic acid and methanol (50:50), 0.1 M acetic acid and ACN (50:50) and 0.1M acetic acid in water. Among all 0.1 M acetic acid in water was optimized as eluting solvent as maximum amount of FQs was eluted with it as evident from Fig. 2a.

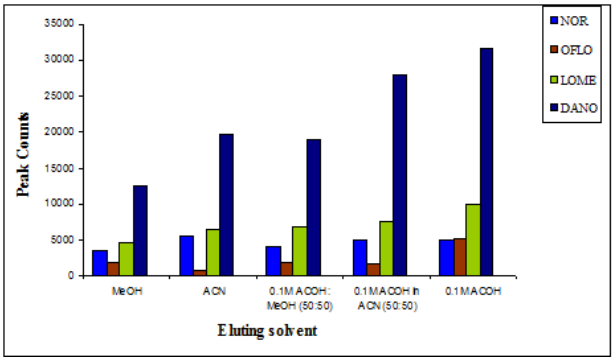

Fig 2 (a) Evaluation of elution efficiency of different solvents using MIP-SPE

Optimization of eluting solvent volume

Different volumes of eluting solvent were applied to elute loaded analytes from cartridge to obtain maximum elution. The different volumes applied to elute analytes were 150 µL, 250 µL and 350 µL. It was found that 250 µL was the volume of eluting solvent which gives maximum elution, as compared to 150 µL, at which elution was not complete and at 350 µL, recovery decreases due to increase in volume, is evident from the Fig.2b.

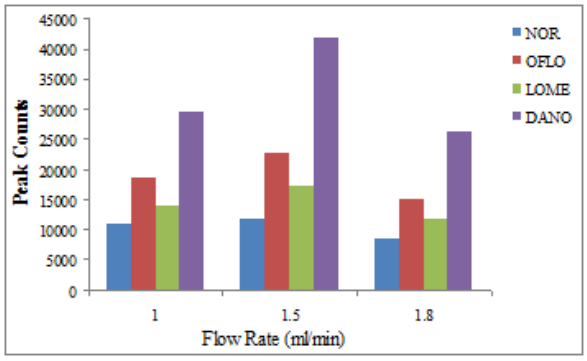

Fig 2 (b) Evaluation of volume of eluting solvent using MIP-SPE

Optimization of flow rate

Different flow rates were applied to get best separation with fine peak shape and good resolution. Flow rates of mobile phase with which it passes through the column were 1.0 mL/min, 1.5 mL/min and 1.8 mL/min. The best separation and resolution was obtained at 1.5 mL/min and it was optimized as evident from Fig. 2c.

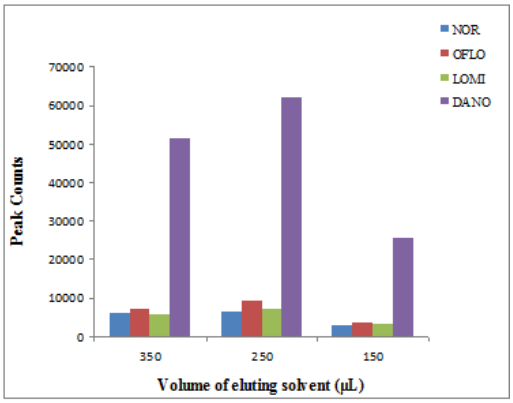

Fig.2 (c) Evaluation of effect of flow rate of mobile phase using MIP-SPE

Method performance

Limit of detection (LOD) and quantification (LOQ)

The optimized conditions established above were then used to prepare calibration curves for these four analytes spiked at 1, 10, 50, 100 and 200 ng/mL. The calibration curves were linear over these ranges. The method detection limits (LOD) were calculated for the analytes as three times the signal to noise ratio (S/N=3). Similarly the method quantification limit (LOQ) can be estimated as 10 times the signal to noise ratio (S/N=10). The characteristics parameters are given in Table 1.

Table 1: Results of the linearity, LOQ and system suitability parameters

Parameter	NOR	OFLO	LOME	DANO
Regression equation (y = mx +c)	4.9157x+3821.4	4.5952x+3266.9	4.765x+2936.7	4.607x+19773
Correlation coefficient (r^2)	0.992	0.991	0.997	0.998
Retention time (min)	3.01	4.50	6.35	7.51
Calibration range (ng/mL)	1-200	1-200	1-200	1-200
LOD, S/N=3 (ng/mL)	0.287	0.108	0.145	0.02
LOQ, S/N=10 (ng/mL)	0.947	0.357	0.478	0.261

Extraction yield and precision

Extraction yield and precision were made at four different concentration levels of analytes, i.e., 1, 10, 50, 100 and 200 ng/mL. The results are reported in Table 2. The results are satisfactory with extraction yield values being in the range 89.12 - 96.58%. The intraday precision was also satisfactory with RSD values being 1.6 - 4.15 % for all analytes. The experiments were conducted six times during the same day to obtain repeatability (intraday precision) and interday precision was obtained by repeating the experiments six times over six different days both values are expresses as RSD % i.e., percentage relative standard deviation values.

Table 2: Recovery of four FQs from spiked honey and urine samples

Analyte	Amount added (ng/mL)	Recovery (%)*	Intraday RSD (%)	Interday RSD (%)
NOR	1	89.12 (90.28)	3.5 (3.3)	4.1 (3.6)
	10	91.89 (90.31)	2.9 (2.6)	3.8 (3.1)
	100	90.02 (91.22)	2.2 (1.9)	3.5 (3.0)
OFLOX	1	90.73 (90.60)	3.2 (2.9)	3.9 (3.5)
	10	90.39 (90.42)	2.9 (2.5)	3.4 (3.0)
	100	89.28 (90.12)	2.7 (2.4)	3.0 (2.5)
LOMI	1	91.23 (91.76)	4.1 (3.8)	4.6 (3.8)
	10	90.56 (91.83)	3.8 (3.5)	4.3 (3.9)
	100	91.06 (91.37)	3.6 (3.4)	4.1 (3.8)
DANO	1	95.26 (96.12)	2.9 (2.3)	3.3 (2.5)
	10	94.80 (96.58)	2.5 (1.9)	2.8 (2.4)
	100	95.82 (96.08)	2.2 (1.6)	2.7 (2.3)

**each value is a mean of 6 independent assays*
( ) Urine sample values
The extraction yield was calculated from the analyte peak area from spiked honey and Urine samples compared with those obtained from the same analyte concentration in standard solution.

Comparison of MIPs-SPE HPLC-FLD with other methods

The proposed method was compared with the SPE reported for the extraction of antibiotics from environmental samples (Table 3).The results indicate that the proposed method has lower LOD as compared to methods previously reported in literature [2,5,7,8,16]. Present method has advantages in the experimental cost, organic solvent consumption, and operation simplicity. The limit of detection values for NOR, OFLO, LOME and DANO are 0.287, 0.108, 0.145 and 0.02 ng/mL respectively.

Table 3: Comparison of purposed method with that of previously reported methods

Analyte	Method	Sample preparation	LOD (ng/mL)	Reference
OFLOX	HPLC-FLD	-	17.9	[2]
NOR			9.8	
NOR	HPLC-FLD	MAE	0.7	[5]
DANO			2.7	
DANO	HPLC-FLD	-	0.7	[7]
NOR			5.0	
LOMI	Capillary	SPE	3.5	[8]
NOR	electrophoresis		6.0	
OFLOX			3.5	
NOR	HPLC-FLD	SPE	1.7	[16]
OFLOX LOMI			8.9	
DANO			5.0	
			0.2	
NOR	HPLC-FLD	MIP-SPE	0.287	Present work
OFLOX LOMI			0.108	
DANO			0.145	
			0.02	

Applications

The performance of the developed method was tested using a spiked sample of honey and urine both containing known spiked amounts of the target analytes. The samples were prepared as described in section 2.4. The absence of peaks in the chromatograms at retention time of the target analytes studied verified the absence of these compounds in the spiked honey sample and spiked urine sample

Fig. 3a and 3b. The chromatograms for honey and urine samples spiked with the target analytes at a concentration of 1 ng/mL are shown in Fig.4a and 4b.

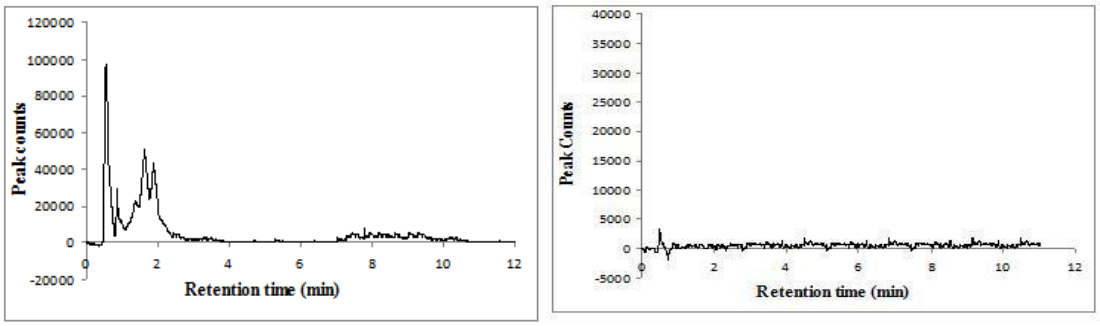

Fig. 3: (a) MIP-SPE-HPLC chromatogram obtained from blank honey; **(b)** MIP-SPE-HPLC chromatogram obtained from blank urine.

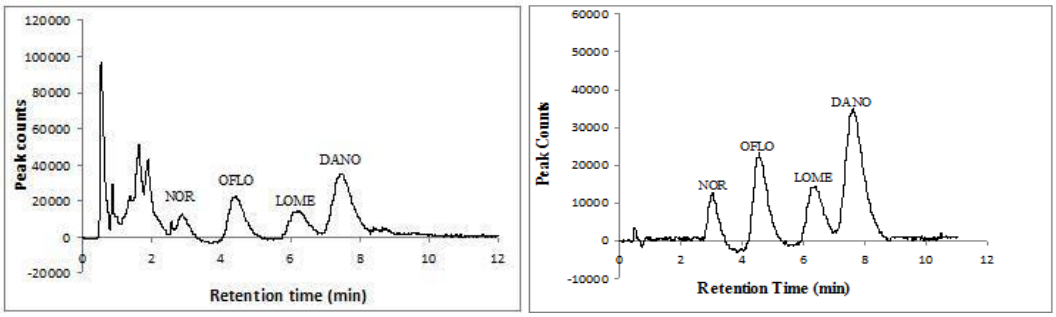

Fig.4: (a) MIP-SPE-HPLC chromatogram obtained from honey spiked with 1 ng/mL of FQs; **(b)** MIP-SPE-HPLC chromatogram obtained from urine spiked with 1 ng/mL of FQs

DISCUSSIONS

The superiority of MIPs as selective SPE sorbents for analysis of various analytes has been well established. The MIP-SPE cartridges specific for fluoroquinolones allow selective retention and elution of these from complex matrices thus eliminating interferences. The results demonstrate that developed method is suitable for the rountine analysis of FQs in biological and food samples. Specificity studies clearly indicate that the analytes can be detected without any interferences. The sensitivity and calibration range obtained here is useful to detect the analyte at the level that can be found in biological and other food samples. This method is very simple and rapid with very good precision and accuracy results. The extraction yield was obtained in the range more than 85% with RSDs less than 5%.

CONCLUSION

The proposed method developed here is simple, rapid, reproducible, precise and economic and can be used for the assay of studied fluoroquinolones and their detection in various environmental samples due to high sensitivity of the method. A fast MIPs-SPE technique has been used as sample preparation technique which is very much simple as compared to other sample preparation techniques. The use of this technique as sample preparation method is that it not pnly reduces the time of determination but economic too. The method validation parameters yielded good results and included the range, linearity, precision and accuracy.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

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