2.5 POLÍMERS AMB EMPREMTA MOLECULAR PER A L'EXTRACCIÓ SELECTIVA DE FLUOROQUINOLONES EN MOSTRES BIOLÒGIQUES

Degut a que són minoritàries les aplicacions de la MISPE a l'extracció de compostos en teixits animals i als resultats obtinguts en l'estudi anterior, en aquest apartat es presenta la síntesi de tres nous MIPs, dos d'ells empremtats amb enrofloxacina (ENRO) i aplicats a l'extracció d'aquest compost en orina i en teixit d'animal, i un tercer MIP preparat amb ciprofloxacina (CIPRO) com a *template* i aplicat a l'extracció d'aquest mateix compost en mostres d'orina.

L'ENRO i la CIPRO formen part d'una família d'antibiòtics coneguda com fluoroquinolones i són àmpliament utilitzades per al tractament d'infeccions, principalment urinàries, intestinals i biliars, tant en medicina humana com en veterinària, i en el cas dels animals també són emprades per potenciar el seu creixement. Així doncs, igual que les tetraciclines, la presència de fluroquinolones en teixit animal està regulada per la legislació europea. Degut a la complexitat que presenten aquests tipus de mostres l'ús de MIPs com a sorbents pot resultar interessant per a eliminar amb més facilitat la matriu de la mostra i així poder quantificar els compostos als nivells exigits per la normativa, que en el cas de la ENRO, el Consell Regulador 2377/90 [1] estableix els MRLs per aquest compost com la suma de la ENRO i el seu metabòlit CIPRO (Taula 2.5.1).

La síntesi dels MIPs empremtat amb l'ENRO es va portar a terme en dos solvents diferents: diclorometà i cloroform, degut a que l'ENRO era soluble en tots dos i així estudiar l'efecte del porogen en la selectivitat del MIP. D'aquesta manera es va poder triar finalment el MIP que resultava més adient per a l'aplicació a portar a terme.

Inicialment, tots dos MIPs es van avaluar cromatogràficament. El perfil d'elució observat per a l'ENRO en cadascun dels MIPs era l'esperat per a qualsevol polímer empremtat: pics amb llargues cues i temps de retenció elevats. Dels factors d'empremta molecular que es mostren a la Taula 2.5.2 es pot deduir com, efectivament, els dos polímers estaven empremtats i dels valors de RI es dedueix com el MIP preparat en DCM té una reactivitat creuada per la CIPRO

molt més marcada. Per tant, sembla evident que el porogen juga un paper important en les característiques finals de cada MIP.

Taula 2.5.1 MRLs per a la enrofloxacina en diversos teixits d'animals destinats al consum humà

Substància farmacològicament activa	Residu	Espècie Animal	MRL (µg kg^{-1})	Teixit
Enrofloxacina	Enrofloxacina + Ciprofloxacina	Pollastre	100	Múscul
		Boví	100	Múscul
			100	Greix
			300	Fetge
			200	Ronyó
			100	llet
		Porcí	100	Múscul
			100	Pell+greix
			200	Fetge
			300	Ronyó
		Pollastre	400	Múscul
			250	Pell+greix
			800	Fetge
			1000	Ronyó

Taula 2.5.2 Valors dels Factors de capacitat (K'), dels Factors d'empremta molecular (IF) i de l'Índex de Retenció normalitzat (RI) per a cadascun dels MIPs sintetitzats amb l'ENRO com a *template* i amb diclorometà i cloroform com a poròsgens.

	DCM				cloroform			
Compost	k'_{MIP}	k'_{NIP}	IF	RI	k'_{MIP}	k'_{NIP}	IF	RI
Enrofloxacina	3.9	0.2	17.4	1.0	2.2	1.1	2.2	1.0
Ciprofloxacina	6.0	0.1	75.3	4.3	13.4	4.7	2.8	1.3

Tots dos MIPs es van provar com a sorbents en SPE. El procés d'optimització de la MISPE es va dur a terme tant amb una mostra d'aigua Milli-Q com amb metanol que contenien una barreja de tres fluoroquinolones (norfloxacina, CIPRO i ENRO) i es va comprovar com l'efecte d'empremta molecular era

màxim quan la mostra s'aplicava en en metanol. Per aquest motiu es va desenvolupar un sistema d'extracció en dues etapes; primer la mostra es preconcentrava a través d'un cartutx comercial (OASIS HLB) i l'extracte recollit en metanol de l'etapa d'elució es transferia cap al MIP. D'aquesta manera els analits passaven a través del MIP en el solvent on el MIP presentava un major efecte d'empremta molecular. Per una altra banda, cal remarcar que aquest sistema d'extracció permetrà eliminar millor les restes de matèria orgànica que puguin quedar a la mostra d'orina després de filtrar-la i a la mostra de teixit després de triturar-la, homogenitzar-la i centrifugar-la i, per tant, allargar la vida útil del cartutx. En altres estudis en què s'analitzen teixits [2,3], s'ha comprovat com l'etapa d'extracció també es realitza mitjançant l'ús de dos cartutxos.

En aplicar 25 ml d'un blanc d'orina a través d'aquest doble sistema d'extracció, es va poder comprovar com el MIP preparat en cloroform presentava més selectivitat, ja que tal i com s'observa a la Figura 2.5.1 el cromatograma corresponent a aquest MIP era molt més net.

Tot i així es va continuar treballant amb els dos polímers i en preconcentrar una mostra d'orina fortificada amb una mescla de CIPRO i ENRO es va comprovar com el MIP preparat en DCM mostrava una reactivitat creuada per la CIPRO molt més gran que el MIP preparat en cloroform. Aquesta diferència era d'esperar degut als resultats obtinguts en l'avaluació cromatogràfica. Per aquest motiu es va decidir treballar amb el MIP preparat en DCM, ja que l'objectiu de l'estudi era extreure l'ENRO de mostres de teixit animal i degut que la normativa europea estableix els MRLs per a l'ENRO com la suma d'ENRO i CIPRO, aquest MIP resultava idoni.

El MIP preparat en DCM es va aplicar finalment per a l'extracció d'ENRO i CIPRO en fetge de porc. En aquest cas, no va ser necessari introduir una etapa de neteja abans de l'elució dels analits del MIP, no només perquè l'extracte obtingut ja era prou net, sinó perquè a més a més, d'aquesta manera s'evitaven possibles pèrdues de la CIPRO durant l'etapa de neteja. D'aquesta manera es

poden quantificar les dues fluoroquinolones tal i com estableix la normativa europea [1].

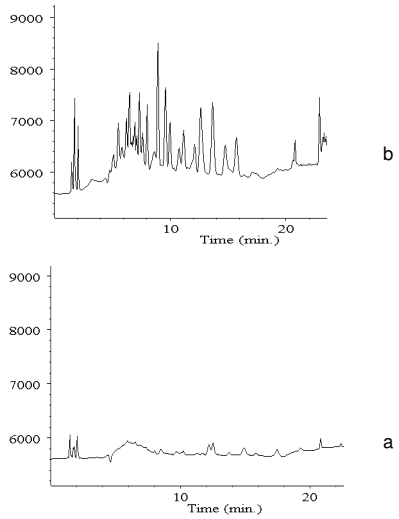

Figura 2.5.1 Cromatogrames obtinguts després de preconcentrar 25 ml d'un blanc d'orina a través del cartutx d'OASIS i del MIP preparat en cloroform (a) i del MIP preparat en DCM (b).

El tercer MIP sintetitzat amb una fluoroquinolona es va preparar amb la CIPRO com a *template* i emprant DCM com a porogen degut a que en cloroform aquest compost era menys soluble. La síntesi es va dur a terme en les mateixes condicions que el MIP empremtat amb la ENRO en DCM i es va aplicar a l'SPE d'un grup de fluoroquinolones.

L'avaluació cromatogràfica d'aquest MIP va permetre comprovar com a diferència del seu homòleg preparat amb la ENRO com a *template*, la reactivitat creuada que presentava aquest nou MIP era molt inferior.

Aquest MIP també es va aplicar com a sorbent en processos d'extracció en fase sòlida i, igual que en els dos MIPs empremtats amb ENRO, es va observar que

quan la mostra s'aplicava en MeOH, l'efecte d'empremta molecular es veia afavorit. Per tant, quan es va dur a terme l'extracció de la CIPRO de mostres d'orina va ser necessari aplicar el procés d'extracció en dues etapes descrit prèviament. D'aquesta manera no només era possible fer un canvi del solvent de la mostra sinó que el procés de MISPE era tan eficient que després d'una etapa de neteja amb un solvent orgànic polar i apròtic, dimetilformamida, s'obtenien cromatogrames molt nets i els extractes de les mostres d'orina es van poder injectar directament a un espectròmetre de masses (MS). La selectivitat del MIP junt amb la selectivitat i sensibilitat del MS, va permetre la quantificació i confirmació de la CIPRO a baixos nivells i reduir el temps d'anàlisi en eliminar la columna cromatogràfica.

Tant el primer dels estudis descrits, on el MIP s'ha preparat amb ENRO com a *template,* com l'estudi dut a terme amb la CIPRO com a *template* han estat enviats a la revista *Journal of Chormatography A* per a la seva publicació.

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2.5.1 *Novel enrofloxacin imprinted polymer applied to the solid-phase extraction of fluorinated compounds from urine and tissue samples*

NOVEL ENROFLOXACIN IMPRINTED POLYMER APPLIED TO THE SOLID-PHASE EXTRACTION OF FLUORINATED QUINOLONES FROM URINE AND TISSUE SAMPLES

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Abstract

A new molecularly imprinted polymer, prepared following a non-covalent approach, was synthesised using enrofloxacin as a template molecule. The imprinting effect of the polymer was verified by chromatographic evaluation and, interestingly, this evaluation also revealed that the imprinted polymer showed a high degree of cross-reactivity for ciprofloxacin, the major metabolite of enrofloxacin. The molecularly imprinted polymer was then applied as a selective sorbent in a two-step, solid-phase extraction method focussing upon complex biological matrices, specifically urine and pig liver. This two-step solid-phase extraction protocol, in which a commercial OASIS HLB cartridge and a molecularly imprinted solid-phase extraction (MISPE) cartridge were combined, allowed enrofloxacin and ciprofloxacin to be determined by liquid chromatography coupled to a UV detector at levels below the maximum residue limits established by the European Union.

Keywords: Molecularly imprinted polymer; Solid-phase extraction; Enrofloxacin; Human urine; Pig liver

INTRODUCTION

Quinolones are antibiotic widely to prevent and treat a large variety of infectious diseases in human and veterinary medicine [1]. In veterinary medicine quinolones also promote animal growth. It is well known that residues of such antibiotics may persist in edible animal tissues, which makes quinolones potentially hazardous to human health.

To ensure that consumers are not exposed to quinolone residues at potentially harmful concentrations, the European Union (EU) has established maximum residue limits (MRLs) for such compounds present in edible tissues [2]. Thus, it is important to develop analytical methods for determining these drugs present in biomatrices at low levels. Most of the analytical methods established thus far for quinolones are based on liquid chromatography (LC) [3-6] and, given the chemically complex nature of sample matrices such as biological fluids and animal tissues, this makes a sample preparation step necessary prior their analysis.

Over the last few years, solid-phase extraction (SPE) has become the most commonly used sample preparation technique, not only for cleaning of the sample matrix but also to enrich the analytes present prior to analysis. Nevertheless, due to the lack of selectivity of the commercial SPE sorbents, other materials with high selectivity, such as immunosorbents (IS) and molecularly imprinted polymers (MIPs) [7], have been developed and applied to extraction procedures. The expensive and time-consuming proce-dure for the production and isolation of antibodies as well as their lack of availability, have led to MIPs being exploited widely in molecularly im-printed solid phase extraction (MISPE) [8].

MISPE has been applied successfully to the extraction of drugs from several different biological fluids, including urine, serum and plasma [9-15], but there are relatively few studies focussing upon tissue samples [16-17]. In these two papers, two-step SPE processes were developed to extract clenbuterol from calf and bovine liver, respectively. Brambilla *et al* [16] used an Extrelut 20 column to extract clenbuterol in the first instance; the eluate collected was then applied to a MIP. In contrast, Crescenzi *et al* [17] used a matrix solid-phase dispersion pre-treatment to prepare the samples, a technique that greatly simplifies the pre-treatment of solid samples, but an additional clean-up step was, never-theless, still required. For this reason, Crescenzi et al. combined MSPD with MISPE.

In the present paper, we describe the first example of a fluorinated quinolone, enrofloxacin, used as template to prepare a MIP which is applied as a selective sorbent, to extract ENRO via a two-step MISPE procedure from human urine but also to extract ENRO and ciprofloxacin (another fluorinated quinolone) from pig liver. This SPE protocol allowed matrix compounds to be nearly virtually removed from the sample and ENRO to be quantified at MRL levels by a liquid chromatographic system (HPLC) coupled to a UV detector.

EXPERIMENTAL

Reagents and standards

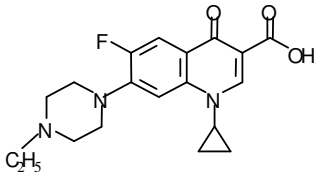
The reagents for the polymer synthesis were as follows: enrofloxacin (ENRO), which was kindly donated by Cenavisa (Reus, Spain), methacryclic acid (MAA) and ethylene glycol dimethacrylate (EGDMA), from Aldrich (Steinheim, Germany), 2,2'-azobisisobutyronitrile (AIBN) from Acros Organics (Geel,

Belgium), and dichloromethane (DCM) from Rathburn Chemicals (Walkerburn, U.K.). The monomers were purified prior to use *via* standard procedures in order to remove stabilisers, and the solvent dried over 4 Å molecular sieves. The AIBN was recrystallised from acetone.

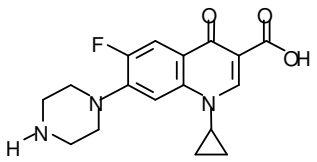
HPLC-grade solvents used to prepare the LC mobile phases were acetonitrile (ACN), provided by SDS (Peypin, France), and water, which was collected from a Millipore water purification system (Milli-Q water). Phosphoric acid and sodium dihydrogen phosphate, which were from Probus (Badalona, Spain) and Panreac (Barcelona, Spain), respectively, were used also to prepare the mobile phases. Other pH modifiers used were acetic acid and hydrochloric acids from Probus, formic acid from Merck (Darmstad, Germany) sodium hydroxide (NaOH) from Prolabo (Fontenay, France) and triethylamine (TEA) from Aldrich.

Some fluorinated quinolones such as norfloxacin (NOR) and ciprofloxacin (CIPRO) and the quinolone flumequin (FLU), all them related structurally to ENRO (Figure 1), were used to investigate the cross-reactivity of the MIP. NOR and FLU were supplied by Sigma (Steinheim, Germany) and CIPRO was kindly donated by Cenavisa (Tarragona, Spain). Standard solutions for each compound were prepared monthly at a concentration of 100 mg l^{-1} in 0.1 M NaOH.

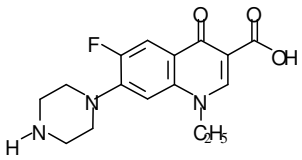
Several tetracycline compounds, including tetracycline (TC), oxy-tetracycline (OTC), and doxycycline (DC), were also used in certain experiments to probe the selectivity of the MIP for other group of antibiotics. The tetracyclines were all purchased from Sigma.



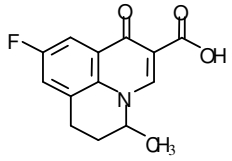
ENROFLOXACIN (ENRO)



CIPROFLOXACIN (CIPRO)



NORFLOXACIN (NOR)



FLUMEQUIN (FLU)

Figure 1. Chemical structures of the fluorinated quinolones studied.

Preparation of the Imprinted Polymer.

For the preparation of the enrofloxacin imprinted polymer, the template (ENRO, 0.21g, 0.58 mmol) was dissolved in the porogen (DCM, 6.66 ml) in a 25 ml thick-walled glass tube. The functional monomer (MAA, 0.40g, 4.64 mmol), the

cross-linking monomer (EGDMA, 4.60g, 23.20 mmol) and the initiator (AIBN, 0.08g, 0.51 mmol) were then added. The resultant solution was cooled on an ice bath and degassed with oxygen-free nitrogen for five minutes before being sealed under nitrogen. The polymerisation was allowed to proceed at 58ºC for 24h in a water bath. After this period, the glass tube was broken and the monolith obtained was ground mechanically and wet sieved using acetone to obtain regularly sized particles with diameters between 25 and 38 μm suitable for the MISPE evaluations.

A non-imprinted polymer (NIP) was prepared and treated in an identical manner to the MIP, the only difference being that there was no ENRO present during polymerisation

Instrumentation

High performance liquid chromato-graphy (HPLC) was the primary tool used for the evaluation of the imprinting effect. In this regard, stainless steel HPLC columns (15 x 0.46 cm i.d.) were slurry packed with the ground polymer (MIP or NIP) particles (25-38 μm) using an air-driven fluid pump (Haskel) with acetone as the slurrying and packing solvent at 2500 psi. An SP 8800 ternary HPLC pump with an automatic injector and an SP 8450 UV detector (Spectra-Physics, Mountain View, CA, USA) were used in the pre-screening work.

For the two-step MISPE of biological samples, a commercial SPE sorbent, such as OASIS HLB (3 ml, 60 mg) (Milford, MA, USA), was used in the first step. The eluate collected from the commercial sorbent was then applied to a 6 ml polyethylene SPE cartridge which contained 200 mg of the MIP. All the cartridges were connected to a manifold, supplied by Teknokroma (Barcelona, Spain), coupled to a vacuum pump.

For the analytical method, the liquid chromatographic system consisted of two LC-10AD pumps, a DGU-14A degasser, a CTO-10A oven and an SPD-10A UV spectrophotometric detector from Shimadzu (Tokyo, Japan). The injection volume was 20 μl and the analytical column was a 15 x 0.46 cm i.d. Eclipse XDB-C8, 5 μm, supplied by Hewlett Packard (Palo Alto, USA).

Chromatographic Conditions

Prior to the chromatographic evaluation of the MIP and the NIP, the columns were washed on-line with a mixture of chloroform/acetic acid (95:5 *(v/v)*) to remove the template and monomers arising from the synthesis. Thereafter, the columns were washed with a chloroform/acetic acid (99:1) mobile phase until a stable baseline was observed. 20 μl of 10 mM enrofloxacin in chloroform/acetic acid (99:1) and 1 μl of the void marker (acetone) were then injected. The NIP was evaluated under identical chromatographic condi-tions. The analysis was performed in isocratic mode at 1 m min^{-1} and the UV detector wavelength was set at 280 nm. The analysis was performed at ambient temperature.

The chromatographic conditions for the MISPE experiments were as follows. The mobile phase was a mixture of two solvents, ACN (solvent A) and an

aqueous phase (solvent B) which contained 0.02 M of sodium dihydrogen phosphate and 2 ml of TEA. The pH of the aqueous phase was adjusted to 3.0 with phosphoric acid and filtered through a 0.22 µm filter. The flow-rate of the mobile phase was 1 ml min^{-1} and the gradient profile was from 5 to 10% B in 5 min, isocratic elution for 6 min and the B content was increased to 20% in 5 min, to 50% B in 2 min and then isocratic elution for 2 min, i.e., the overall run time was 25 min. The column temperature was 35 ºC and all compounds were detected at 280 nm, except for FLU which was detected at 324 nm.

MISPE Conditions

For the two-step MISPE experiments, urine and tissue extracts (adjusted to pH 3) were passed through the OASIS cartridge conditioned previously with 5 ml of MeOH and 2 ml of Milli-Q water. After sample application, a washing step with 2 ml of Milli-Q water was applied and the analytes then desorbed using 10 ml of MeOH. This methanolic solution containing all the compounds from the commercial cartridge was then passed through the MIP previously conditioned with 6 ml of MeOH. The MIP was washed with 6 ml of ACN/acetic acid (95:5) only when urine samples were analysed. For the elution step 3 ml of ACN/(4%) formic acid (1:4) were used and 20 µl of each sample was then injected onto the analytical column.

Analysis of biological samples

The urine samples were collected from a healthy volunteer. Urine was store in a freezer at -20ºC until use and filtered through a 0.22 µm syringe filter before being applied to the OASIS and MIP cartridges.

For the extraction of quinolones from pig liver, a standard protocol was followed to prepare the matrix prior to the SPE [18]. To 5 g of tissue 5 ml of 0.1 M HCl was added. The mixture was homogenised for 1 min using an Ultra-Turrax T-25 (Jankle & Kunkel, IKA-Labortechnik, Staufen, Germany) and the mixture then centrifuged at 9,000 r.p.m. for 20 minutes. After this period, the pellet was blended and centrifuged again with 5 ml of 0.1 M HCl. The supernatants obtained were combined and centrifuged for 15 min and, after filtration of the supernatant through a 0.45 µm syringe filter, the filtrate was passed through the OASIS and MIP cartridges.

RESULTS AND DISCUSSION

Chromatographic Evaluation of the Polymers

Chromatographic evaluation was used as the first tool to investigate the imprinting effect of the MIP. Chloroform was chosen as the mobile phase, but due to the problems associated with the application of MIPs as stationary phases in HPLC (very broad, tailing peaks and time-consuming analyses) a modifier, such as acetic acid, was added to the mobile phase. In this way, the MIP and the modifier compete for rebinding to the analyte, which represents an im-provement of the peak symmetry and reduces simultaneously the analysis time. Chloroform/acetic acid (99:1) was then used as an optimal mobile phase in the

present. From the retention times of ENRO on the MIP and the NIP columns, the capacity factors and the imprinting factor were calculated (Table 1). CIPRO was also injected onto the MIP and the NIP columns to probe the selectivity of the MIP for other structurally related analytes. The capacity factors and the imprinting factor for CIPRO were also calculated in a similar fashion to those for ENRO and are also presented in Table 1.

Table 1. Capacity factors and imprinting factors arising from the chromatographic evaluation of the enrofloxacin imprinted polymer.

Compound	k'^{a}_{MIP}	k'^{a}_{NIP}	IF[b]	RI[c]
Enrofloxacin	3.90	0.22	17.38	1.00
Ciprofloxacin	6.02	0.08	75.30	4.33

[a]k' (Capacity Factor) [b]IF (Imprinting Factor)
[c]RI (Normalised Retention Index)

The Normalised Retention Index (RI) was calculated to enable the k' values of ENRO and CIPRO to be compared [19]. As can be seen in Table 1, the MIP shows high affinity for the template, ENRO, as would be expected for an imprinted material, but also higher affinity for CIPRO than ENRO. The latter observation can be rationalised by considering the chemical structures of ENRO and CIPRO, as shown in Figure 1. The only difference between the two structures arises in the piperizinyl ring. In terms of their relative ability to form an electrostatic interaction with carboxylic acid, in the absence of steric effects one would expect the interaction with ENRO (tertiary amine) to be stronger than that with CIPRO (secondary amine). Given that the expected order of elution was reversed, we can only assume that steric effects are playing a role in the binding.

The selectivity and retention of analytes on the MIP compared to the control polymer has thus been verified. Moreover, it has also been demons-trated that the MIP shows a high degree of cross-reactivity for CIPRO. The chromatographic evaluation has provided useful information about the MIP for the subsequent development of the MISPE method.

MISPE

Several parameters must be optimised in MISPE experiments in order to maximise the selective recognition of the analyte. In order to demonstrate the selectivity of the MIP for the ENRO, other structurally related compounds such as NOR, CIPRO and FLU were also added to the sample. The conditioning and the loading steps were optimised first of all. Thus, 10 ml of a mixture containing 0.3 mg l^{-1} of NOR, CIPRO, ENRO and FLU was prepared in two solvents, methanol (MeOH) and Milli-Q water at different pH values (3, 7, 11) and passed through the MIP. The cartridge had been conditioned previously with 6 ml of the same solvent in each case.

When the sample was applied in MeOH, all the analytes were retained on the MIP mainly by selective interactions, except for FLU which was not retained at all during the loading step. This may well be because FLU lacks a piperizinyl ring, believed to be one of the key structural features necessary for binding to the MIP.

For the elution step several solvents were investigated. The best conditions found to quantitatively elute and recover all the retained analytes was to use 3 ml of ACN/formic acid (4%) (1:4).

When an identical mixture of quinolones was prepared in Milli-Q water at pH 3, all the analytes were completely retained, even FLU, although it was only recovered in 60%. In this situation (aqueous environment) hydrophobic (non-specific) interactions dominate the retention process. When other pH values were investigated (pH 7 and pH 11), the compounds were completely retained at neutral pH but not at basic pH (recoveries from 47-69% for all compounds). Retained analytes were eluted using the same conditions as described previously.

In order to enhance the selectivity of the MIP and decrease the cross-reactivity, a clean-up step (selective washing step) was included in the MISPE procedure. This step was first optimised when the sample was applied in MeOH. Thus, DCM, toluene, chloroform and ethyl acetate were investigated as potential washing solvents for the clean-up step, but the cross-reactivity of the MIP was not decreased. The same behaviour was observed when the effect of more polar solvents, such as ACN and dimethylformamide (DMF), was also investigated. It should be pointed out that these experiments were also performed in the NIP and a clean-up with 4 ml of DMF was enough to completely remove the fluorinated quinolones. The strong cross-reactivity shown by the MIP was expected because of the previous results obtained from the chromatographic evaluation. Therefore, acetic acid was added to the previously described solvents in order to decrease the cross-reactivity since this modifier competes with the binding sites for rebinding the analytes. The best results were obtained using 6 ml of acetic acid in ACN (5:95). Under these conditions there was an improvement since NOR and CIPRO were recovered at a level of 40% whereas ENRO remained completely bound to the MIP. ACN/acetic acid was used as the wash solvent in all subsequent experiments.

The clean-up step was also studied when the sample was applied to the cartridge in acidified Milli-Q water (pH 3). After drying of the cartridge for 15

min, the effect of using the same set of wash solvents was investigated. In this situation, when ACN or DMF were used all the compounds were close to being completely removed from both the MIP and NIP, even although only 2 ml of solvent was used. For this reason, in all subsequent experiments the analytes were applied from MeOH.

A mixture of tetracyclines (TC, OTC and DC) in MeOH was percolated through the MIP together with the quinolones to explore the selectivity of the MIP for another group of antibiotics. Tetracyclines are also used extensively to treat animal and human diseases. When 10 ml (0.3 mg l^{-1}) of a mixture of these tetracyclines and fluorinated quinolones in MeOH was applied to the MIP cartridge, it was found that the tetracyclines were only retained at a level of 40%, presumably because they were not able to establish specific interactions with the MIP. A subsequent clean-up step involving ACN/acetic acid (95:5) eliminated the tetracyclines from

the MIP, leaving only the quinolones remained still bound to the MIP (Figure 2).

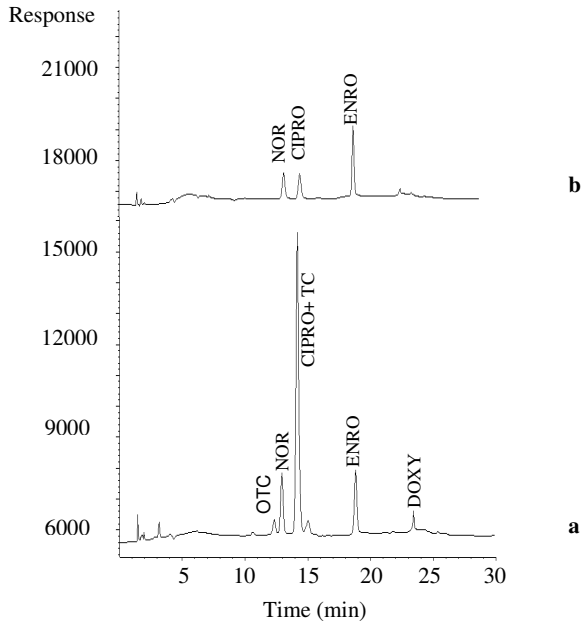


Figure 2. Chromatograms obtained from the percolation through the MIP of 10 ml of sample prepared in MeOH containing 0.3 mg l^{-1} of a mixture of several quinolones and tetracyclines. A) When a clean-up step was not performed; b) with a clean-up step using 6 ml of ACN/acetic acid (95:5).

It has been demonstrated that the MIP was imprinted. However, to make best use of the selective interactions between the analytes and the MIP the sample must be applied in MeOH. For this reason, a two-step SPE method was developed which allowed us to not only apply the biological samples to the MIP from a methanolic solution, but also to obtain cleaner extracts.

Analysis of Biological Samples

To demonstrate the value of the MIP as a sorbent for sample pre-treatment, two different real samples, such as human urine and pig liver, were investigated. These complex matrices make the determination of quinolones difficult at low concentrations, especially when the analytes have to be extracted from tissue samples and quantified at MRL levels. In order to completely suppress matrix interferences, a two-step SPE procedure suitable for monitoring quinolones in urine and pig liver samples was developed. In

the two-step MISPE method used to extract quinolones from biological samples, the urine or the liver extract were first passed through an OASIS cartridge. It had been demonstrated [20] that OASIS offers higher recoveries than other commercial cartridges when drugs have to be extracted from fatty tissues extracts, thus OASIS HLB (60 mg) was used for this particular purpose and the MISPE procedure described in section 2.5 was then applied.

Urine was selected as the first biological fluid for study and a series of experiments carried out aiming at the extraction of ENRO from 25 ml of this sample matrix. Figure 3a shows the chromatogram arising from a blank urine sample that has passed through the OASIS and MIP cartridges without a clean-up step being applied. When 6 ml of ACN/acetic acid (95:5) was used in a washing step, the matrix interferences were considerably reduced (Figure 3b) enabling the identification and quantification of ENRO.

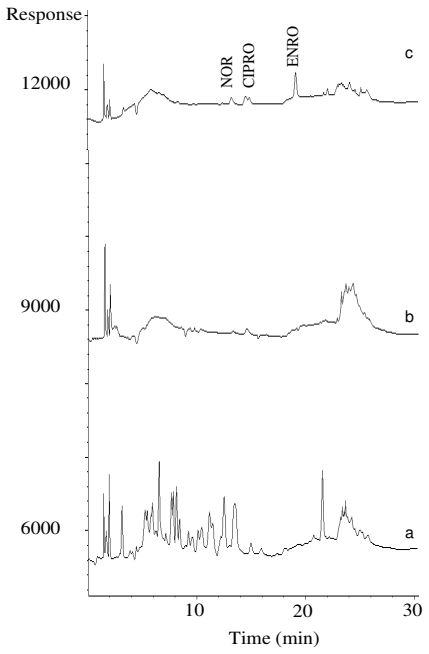


Figure 3. Chromatograms obtained from the percolation of 25 ml of urine through the OASIS cartridge and the MIP cartridge. a) blank of urine; b) blank urine when a clean-up with 6 ml ACN/acetic acid (95:5) was performed; c) urine spiked at 15 μg l^{-1} with norfloxacin, ciprofloxacin and enrofloxacin and with a clean-up step involving 6 ml of ACN/acetic acid (95:5).

In a subsequent experiment, 25 ml of urine spiked with the mixture of quinolones at 15 μg l^{-1} was pre-concentrated (Figure 3c) and ENRO was recovered at 80%. Although an impurity co-eluted with CIPRO, it was possible to quantify the quinolone and it was found that the recoveries for this analyte and NOR were lower than 35%. The ACN/acetic acid wash allowed us to not only reduce the cross-reactivity of the MIP, but also to eliminate all the matrix interferences which interfere with the quantification.

The linearity of the method was evaluated for ENRO. To establish the linear range, 25 ml of urine, which did not contain any quinolone, was spiked with ENRO at concentrations between 120 and 5 μg l^{-1}. The MISPE method was then applied and good linearity was obtained with a determination coefficient (r^2) greater than 0.9999. The repeatability for 25 ml of spiked urine (15 μg l^{-1} of ENRO), expressed as RSD (n=5), was lower than 4%.

The ability of the MIP to extract quinolones from tissue samples was also investigated. In veterinary use, ENRO

must be quantified at the MRL levels, which are described by the EU Council Regulation 2377/90 [2]. In pig liver for the sum of ENRO and CIPRO the MRL level is 200 µg/kg. The two-step SPE method described previously for use with urine samples was used once more, however, in this case the percolation of the sample through the MIP was sufficient in itself to obtain clean chromatograms, making a subsequent clean-up step unnecessary (Figure 4a and b). Moreover, loses of CIPRO due to the washing solvent were also avoided.

To demonstrate the feasibility of using the two-step MISPE method to extract ENRO and CIPRO from tissue samples at lower MRL levels (200 µg/kg), 5 g of pig liver was treated using the protocol described in the Experimental section. In a first instance, the method was evaluated only to check the performance of the MIP when an extract from a tissue sample was percolated, for this reason the tissue extract and not the initial amount of tissue, was spiked with different volumes of standard solutions of NOR, CIPRO and ENRO at 100 mg l^{-1}, to obtain spiked extracts at 500, 200, 100 and 50 µg/kg. Following the two-step SPE, CIPRO and ENRO were recovered at 80% and 96%, respectively (values for spiked extract at 100 µg/kg). Moreover, it was also possible to determine NOR, the quinolone forbidden in veterinary use (recoveries about 70%).

In view of the good results obtained, the two-step MISPE method was then applied to the whole extraction procedure. Therefore, the 5 g of tissue was spiked with different volumes of standard solutions of NOR, CIPRO and ENRO. The liver was homogenised and the resultant extract passed through the OASIS and MIP cartridges (Figure 4c).

In this case, a slight decrease in the recoveries of the analytes was observed due to the tissue treatment. Thus, NOR was almost completely lost and ENRO and CIPRO were quantified at MRL/2 with a linear response for both quinolones and a determination coefficient (r^2) greater than 0.999 which demonstrates clearly that the application of MIP columns to the selective extraction of analytes from heterogeneous biological matrices is both effective and practicable.

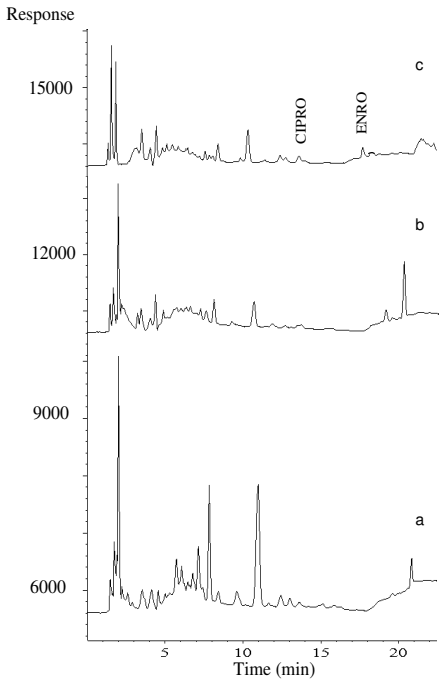


Figure 4. Chromatograms obtained from the extraction of ciprofloxacin and enrofloxacin from tissue samples. a) Blank tissue. 10 ml of tissue extract was percolated through the OASIS cartridge; b) blank tissue. 10 ml of

tissue extract was percolated through the OASIS cartridge and MIP cartridge; c) chromatogram obtained when 5 g of tissue was spiked with the mixture of quinolones at 100 µg/Kg.

CONCLUSIONS

This paper describes the first synthesis and evaluation of an enrofloxacin imprinted polymer. The chromato-graphic evaluation of the polymer established the imprinted properties of the MIP and revealed a high degree of cross-reactivity for the structurally related analyte ciprofloxacin. These attractive features were exploited in an application where the MIP was used in combination with an OASIS cartridge as SPE sorbents to clean and extract quinolones from complex biological samples. The ability of the MIP to selectively recognise quinolones from a mixture of widely used antibiotics, such as TCs, has also been demonstrated. Moreover, a two-step SPE method has been developed for the extraction of ENRO from urine and ENRO and CIPRO from tissue samples. This efficient method allowed cleaner extracts to be obtained and interfering peaks arising from the complex biological matrices to be suppressed. Of particular note is the fact that this method appears to be very well-suited indeed for the control of ENRO and CIPRO in pig liver at values lower than MRL levels.

Acknowledgements

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2.5.2 *Direct determination of ciprofloxacin by mass spectrometry after two-step solid-phase extraction using a molecularly imprinted polymer*

DIRECT DETERMINATION OF CIPROFLOXACIN BY MASS SPECTROMETRY AFTER TWO-STEP SOLID-PHASE EXTRACTION USING A MOLECULARLY IMPRINTED POLYMER

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Abstract

A molecularly imprinted polymer (MIP) has been prepared for the first time with ciprofloxacin (CIPRO) as the template molecule by a non-covalent synthetic procedure. Prior to it use a sorbent in solid phase extraction (SPE), the MIP was chromatographically evaluated which revealed a good imprinting effect. This MIP was used to selectively extract CIPRO from urine samples by a two-step SPE in which a commercial OASIS cartridge and a molecularly imprinted solid-phase extraction (MISPE) cartridge were combined. This SPE procedure allowed the matrix compounds to be effectively removed. The clean urine extracts obtained after this two-step SPE made possible to directly inject the extract to a mass spectrometer to determine the presence of CIPRO in urine samples at low levels.

Keywords: molecularly imprinted polymer; solid-phase extraction; fluorinated quinolones; human urine; mass spectrometry

INTRODUCTION

Over the last few years, molecular imprinting technology has been widely exploited to prepare imprinted polymers, which have been mainly used as sorbents in solid-phase extraction procedures (SPE). The use of molecularly imprinted polymers (MIPs) in SPE is very useful when analysing complex samples such as biological (urine, plasma, serum, tissue) since the selectivity of these imprinted materials allows a better cleaning of the matrix sample to be achieved.

It has been demonstrated in several

studies that MIPs can be successfully applied to the selective extraction of the target analyte from complex biological samples [1-7]. In nearly all these studies [1-6], the molecularly imprinted solid-phase extraction (MISPE) has been used before a liquid chromatographic system (LC). There are only few studies in which MISPE has been used before a detection system [7-14], which is very useful because the time of analysis can be reduced. When a selective elution of the target analyte can be achieved [7-11] or the clean-up performed by the MIP is so effective that allows the interferences (which can hinder the quantification of the target analyte) to be completely removed [12-14], then the separation system can be avoided. Mullet *et al*. developed for the first time a pulsed elution (PE) [8] and differential pulsed elution (DPE) [7,9-11] systems which allowed a selective elution of the target analyte. Nevertheless the number of studies in which the chromatographic system can be avoided because a clean extract is obtained is still limited [12-14]. The first study was performed by Sellergren in 1994 [12] in which a MIP imprinted with pentamidine was directly coupled to a UV detector. In the study developed by Xie *et al*. [13] and Zhu *et al*. [14] flavonoids are isolated from herb extracts. The eluate collected after the MISPE procedure was so clean that it was possible to directly inject it in a mass spectrometer detector.

In the present paper a novel MIP material prepared with ciprofloxacin (CIPRO) as the template molecule has been used as selective sorbent in the SPE. CIPRO is a fluorinated quinolone generally used to treat urinary infections in human medicine [15]. A two-step SPE method combining an OASIS cartridge with the MIP cartridge has been established in order to selectively extract CIPRO from human urine samples. Due to the clean extracts obtained after this two-step SPE, the LC system could be avoided and the extracts collected from the extraction procedure directly injected into the MS.

EXPERIMENTAL

Reagents and standards

The chemicals used for the polymer synthesis were ciprofloxacin (CIPRO), which was kindly donated by Cenavisa (Reus, Spain), methacryclic acid (MAA) and ethylene glycol dimethacrylate (EGDMA), from Aldrich (Steinheim, Germany), 2,2'-azobisisobutyronitrile (AIBN) from Acros Organics (Geel, Belgium), and dichloromethane (DCM) from Rathburn Chemicals (Walkerburn, U.K.). The monomers were purified prior to use *via* standard procedures in order to remove stabilisers, and the solvent dried over 4 Å molecular sieves. The AIBN was recrystallised from acetone.

To prepare the mobile phases or the solutions used in the SPE process we used acetonitrile (ACN) (SDS, Peypin, France), chloroform (Rathburn Chemicals), water from a Millipore water purification system (Milli-Q water), phosphoric acid (Probus, Barcelona, Spain), sodium dihydrogen phosphate (Panreac, Barcelona, Spain), acetic, formic and hydrochloric acids from Probus, sodium hydroxide (NaOH) (Prolabo, Fontenay, France) and triethylamine (TEA) (Aldrich).

Other fluorinated quinolones were used to investigate the selectivity of the MIP for structurally related compounds to CIPRO. Thus, norfloxacin (NOR) from Sigma (Steinheim, Germany), enrofloxacin (ENRO) kindly donated by Cenavisa, and the quinolone flumequin (FLU) from Sigma, were utilised. Standard solutions of each compound were prepared monthly at concentration of 100 mg l^{-1} in NaOH 0.1 M.

Another group of antibiotics such as tetracyclines (tetracycline (TC), oxytetracycline (OTC), 4-epichlorotetracycline (EPI) and doxycycline (DC)) all them from Sigma were also used in some experiments.

Preparation of the Imprinted Polymer.

A ciprofloxacin-imprinted polymer was prepared with CIPRO as the template (0.21g, 0.58 mmol) dissolved in DCM as the porogen (6.66 ml) in a 25 ml thick-walled glass tube. Then, MAA as the functional monomer (0.40g, 4.64 mmol), EGDMA as the cross-linking monomer (4.60g, 23.20 mmol) and AIBN as the initiator (0.08g, 0.51 mmol) were added and the polymerisation mixture was cooled on an ice bath and sparged with oxygen-free nitrogen for five minutes. The tubes were sealed and heated in a water bath at 58ºC for 24h. After this period, the glass tube was smashed and the monolithic polymer obtained was ground mechanically and sieved using acetone to obtain regularly sized particles with diameters between 25 and 38 μm suitable for the MISPE evaluations.

A control non-imprinted polymer (NIP) was prepared following the same procedure but with omission of CIPRO.

Instrumentation

Prior to any use in SPE, the retention capacity and selectivity of the MIP was assessed by high performance liquid chromatography (HPLC). Consequently, stainless-steel HPLC columns (15 x 0.46 cm i.d.) were slurry packed with the ground polymer (MIP or NIP) particles (25-38 μm) using an air-driven fluid pump (Haskel) with acetone as the slurrying and packing solvent at 2500 psi. An SP 8800 ternary HPLC pump with an automatic injector and an SP 8450 UV detector (Spectra-Physics, Mountain View, CA, USA) were used in this pre-screening work.

For the two-step MISPE of urine samples, a commercial SPE sorbent, such as Oasis HLB (3 ml, 60 mg) (Milford, MA, USA), was first used and the eluate collected from this commercial cartridge was then applied through the MIP contained in a polyethylene SPE cartridge (6 ml, 200 mg). All the cartridges were coupled to a manifold supplied by Teknokroma (Barcelona, Spain) connected to a vacuum pump.

The liquid chromatographic system consisted of two LC-10AD pumps, a DGU-14A degasser, a CTO-10A oven and an SPD-10A UV spectrophotometric detector from Shimadzu (Tokyo, Japan). The injection volume was 20 μl and the analytical column was a 15 x 0.46 cm i.d. Eclipse XDB-C8, 5 μm, supplied by Agilent Technologies (Barcelona, Spain).

The MS analyses were performed in a flow injection analysis (FIA) mode. The instrument utilised was a chromatographic system HP1100 series LC-mass selective detector (Agilent Technologies) with an electrospray (ESI) interface and equipped with an automatic injector, a degasser and a quaternary pump.

Chromatographic Conditions

HPLC was used as a primary tool for the evaluation of the imprinting effect, for this reason, before any injection, columns were washed with a mixture of chloroform/acetic acid (95:5 *(v/v)*) to eliminate unreacted monomers arising from the synthesis until no signal was observed.

Then, columns packed with the MIP and NIP were washed with the mobile phase chloroform/acetic acid (90:10). When a stable base line was obtained, 20 µl of 10 mM CIPRO in chloroform/acetic acid (90:10) and 1 µl of the void marker (acetone) were injected. The analysis was performed in isocratic mode at 1 ml min^{-1}. The UV detector wavelength was set at 278 nm and the analysis performed at room temperature.

For the chromatographic conditions in MISPE experiments the mobile phase was a mixture of ACN (solvent A) and an aqueous phase (solvent B), which contained 0.02 M of sodium dihydrogen phosphate and 2% of TEA. The pH of the aqueous phase was adjusted to pH 3.0 with phosphoric acid and filtered through a 0.22 µm membrane filter. The flow-rate of the mobile phase was 1 ml min^{-1} and the gradient profile was from 5 to 10% B in 5 min, to 10.5% B in 11 min, to 20% in 5 min, to 50% in 2 min and then isocratic elution for a further 2 min. The column temperature was 35 ºC and all compounds were detected at 280 nm, except FLU which was detected at 324 nm.

For the MS analysis the mobile phase was ACN/acetic acid pH 3 (1:4) based on a previous work [16]. The mass spectrometer operated in positive mode simultaneously acquired in full scan (from 200m/z to 400 m/z) and under selected-ion monitoring (SIM) acquisition mode. The parameters of ESI-MS were optimised for ciprofloxacin and were the following: the drying gas was operated at a flow-rate of 13 l min^{-1} and 350^{o}C, the nebulizer pressure was set at 40 p.s.i. and the capillary voltage was at 2000 V. The fragmentor voltage was operated at 100 V, and at 125 V for confirmation purpose. The ions acquired for ciprofloxacin were 332, 314 and 231.

MISPE Conditions

The two-step MISPE conditions for the analysis of urine samples were based on the good results obtained from a previous study developed by our research group to extract ENRO from this sample matrix [17]. Thus, 25 ml of urine (adjusted to pH 3 with HCl) was percolated through the OASIS cartridge previously conditioned with 5 ml of MeOH and 2 ml of Milli-Q water. After sample application, a washing step with 2 ml of Milli-Q water was applied and the analytes then desorbed using 10 ml of MeOH. This methanolic solution containing all the retained compounds from the commercial cartridge was then passed through the

MIP, previously conditionated with 6 ml of MeOH and then a clean-up step with an organic solvent (specified later) was performed. The retained analytes were desorbed with 3 ml of ACN/4% formic acid (1:4) and 20 µl of sample was injected onto the analytical column. For the MS analysis 20 µl of sample was injected to the ESI-MS by FIA.

Analysis of urine samples

Urine was collected from a healthy volunteer and kept in the freezer at -20ºC until their use. The urine was filtered through a 0.22 µm syringe filter before SPE process.

RESULTS AND DISCUSSION

Chromatographic Evaluation of the Polymers

The imprinting effect of the MIP was first checked by a chromatographic evaluation. To this end, the procedure described in section 2.4 was followed. In these conditions, it was observed that the use of chloroform/acetic acid (90:10) allowed the chromatographic peak shape and retention time to be improved. When less amount of acetic acid was added, very broad chromatographic peak was observed and longer chromatographic analysis time, which confirms that hydrogen bonds were the dominant interactions in the re-binding step. From the retention times of CIPRO in the MIP and NIP the capacity factors ($K'_{CIPRO}MIP$= 3.95 $K'_{CIPRO}NIP$= 0.045) and the imprinted factor (I_f= 87.78) were calculated

To check the selectivity of the MIP for other structurally related analytes ENRO was also injected onto the MIP and NIP polymers. The capacity factors ($K'_{ENRO}MIP$= 1.278 $K'_{ENRO}NIP$= 0.031) and the imprinted factor (I_f= 40.831) were calculated in a similar manner to those for CIPRO. The Normalised Retention Index (RI) [18]. was calculated from these results. RI enables the K' values of CIPRO and ENRO to be compared RI of the template is 1 by definition and in this case the RI value for the ENRO was 0.47, which demonstrates that the MIP shows higher affinity for the template molecule.

The chromatographic evaluation verifies the selectivity of the MIP for the molecule used during the imprinting process. Moreover, the low RI value for the analogue of the template indicates that this MIP could show low cross-reactivity levels. The MIP chromatographically evaluated was then applied as sorbent in SPE in order to exploit its selectivity to the selective extraction of CIPRO from complex matrices.

MISPE

To determine the selectivity of the MIP in SPE and achieve good recoveries for the analyte of interest, the conditioning, loading and elution steps must be first optimised. In a previous study developed by group to extract ENRO from urine and tissue samples [17], it was observed that the MIP showed the best recognition properties when the sample was percolated in MeOH. The same behaviour was observed for the MIP prepared in the present study, thus, 10 ml of sample containing 0.3 mg l^{-1} of three FQs (NOR, CIPRO and ENRO) and a quinolone

(FLU) was prepared in MeOH and percolated through the MIP previously conditioned with MeOH. All the compounds were completely retained on the polymer, except FLU which was not retained during this loading step because lacks a piperizinyl ring [17]. The retained analytes were quantitatively recovered when 3 ml of ACN/4% formic acid (1:4) was used in the elution step.

From the chromatographic results it was expected that the MIP showed lower cross-reactivity levels. For this reason, in order to enhance the selectivity of the MIP a clean-up step with an organic solvent was included. Several solvents, such as DCM, ACN, toluene and ethyl acetate were investigated as a possible washing solvents resulting in maximum selectivity of the MIP. Although different volumes of these solvents were used the analytes were still retained on the MIP. Nevertheless, when 4 ml of a polar non-protic, solvent such as dimethylformamide (DMF) was applied, ENRO was only retained in a 25%, while CIPRO and NOR were still bound in a 70% and 85%, respectively. It should be noted that in these conditions, the analytes were also completely removed from the control polymer. In order to decrease the cross-reactivity of the MIP for NOR, acetic acid was added to the organic solvent; however, the selectivity of the MIP was not improved. For this reason, DMF was used for further experiments.

The selectivity of the MIP was also explored for another group of antibiotics extensively used to also treat human diseases such as TCs. 10 ml of sample spiked at 0.3 mg l^{-1} with a mixture of TC, OTC, EPI, DC and the fluorinated quinolones NOR, CIPRO and ENRO was prepared in MeOH and percolated through the MIP. TCs were only retained in about 40% because they were not able to establish the specific interactions with the MIP. Therefore, the clean-up step involving 4 ml of DMF removed all the TCs from the MIP and only the fluorinated quinolones remained still bound to the MIP as it is shown in Figure 1.

The focus of the present work was to use the MIP as SPE sorbent to clean and enrich biological samples. For this reason, the MISPE protocol was tested for the extraction of CIPRO from human urine samples.

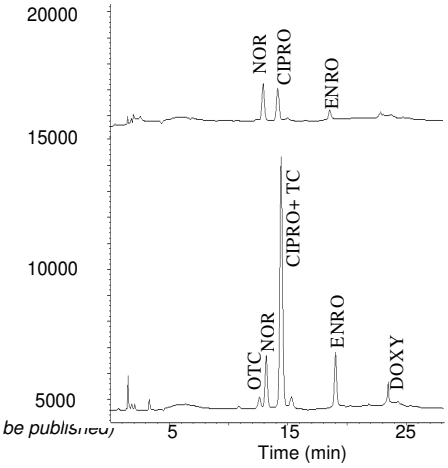

Figure 1. Chromatograms obtained from the percolation through the MIP of 10 ml of sample prepared in MeOH containing 0.3 mg l^{-1} of a mixture of several fluorinated quinolones and tetracyclines. a) When a clean-up step was not performed; b) with a clean-up step using 4 ml of DMF.

Analysis of Biological Samples

In the previous study developed by our group [17], it was demonstrated the feasibility of the two-step SPE procedure, which allowed the biological sample to be passed through the MIP in an organic solvent, since it has been demonstrated that in this way the imprinting properties of these MIPs are enhanced. Moreover, this two-step MISPE procedure also allows matrix interferences arising from biological matrices to be effectively removed. In this study, this feature was exploited to quantify CIPRO in human urine.

For the two-step MISPE method, the procedure described in section 2.5 was then applied. Figures 2a and 2b show the chromatograms obtained after percolating a blank of urine, without and with the clean-up step with 4 ml of DMF, respectively. In a subsequent experiment, 25 ml of urine spiked with 15 µg l^{-1} of each compound was pre-concentrated (Figure 2c) and after the clean-up with DMF, CIPRO was recovered in 80%, whereas NOR and ENRO in 50% and 20%, respectively. As can be seen in Figure 2c the clean-up with DMF allowed CIPRO to be quantified because all the matrix interferences were almost completely removed. This demonstrated that the MIP was still able to exhibit the strongest affinity to its template in a complex biological system. This two-step MISPE also allowed the sample to be percolated in MeOH, so that the selective interactions between the analyte and the MIP are enhanced. Moreover, due to the extracts passed through the MIP cartridge are so clean, the MIP can be reutilised more than 100 times.

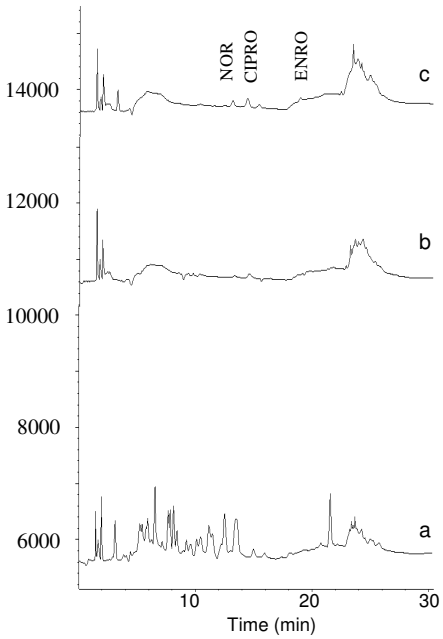

Figure 2. Chromatograms obtained from the percolation of 25 ml of urine through the OASIS cartridge and the MIP cartridge. a) blank of urine; b) blank urine when a clean-up with 4 ml DMF was performed; c) urine spiked at 15 µg/l with norfloxacin,

ciprofloxacin and enrofloxacin and with a clean-up step involving 4 ml of DMF.

The linearity of the method with UV detection was evaluated for CIPRO. To check the linear range, 25 ml of urine, which did not contain any FQ, was spiked with CIPRO at concentrations between 120 and 7 μg l^{-1}. Good linearity was obtained with a determination coefficient (r^2) greater than 0.9932. The repeatability for 25 ml of spiked (15 μg l^{-1} of ENRO) urine, expressed as RSD (n=3), was lower than 11%.

MISPE and ESI-MS

Since the extract of the samples after the OASIS-MIP extraction was quite clean, the next step was to eliminate the chromatographic separation and, therefore, the extract was directly injected by FIA to the ESI-MS operating in positive mode. The full scan spectra of ciprofloxacin, enrofloxacin and norfloxacin were different and therefore the ciprofloxacin could be quantified without interference. The ions selected for the SIM acquisition at 100 V of fragmentor voltage for ciprofloxacin were 332 (100%), 314 (15%) and 231 (10%) corresponding to the $[MH]^+$, $[MH\text{-}H_2O]^+$ and $[MH\text{-}H_2O\text{-}C_3H_4\text{-}NC_2H_5]^+$, respectively. In order to confirm the presence of the ciprofloxacin, a higher fragmentor voltage was applied, 125 V, since the fragmentation was different (332 (100%), 314 (65%), 288 (20%) and 231 (15%)). The 288 ion corresponds to $[MH\text{-}CO_2]^+$ fragment. The spectrum (from 200 to 400 m/z) for CIPRO at 100 V and 125 V are shown in Figure 3. In order to quantify from the FIA-MS analysis, different standard solutions of ciprofloxacin were analysed and the base peak 332 was quantified. Good linearity (r^2>0.990) was obtained between 1 and 500 μg l^{-1} of CIPRO.

When a blank urine sample was spiked at different concentrations of ciprofloxacin (15 μg l^{-1} and 60 μg l^{-1}) the recoveries were 80% and 87%, respectively; therefore, no significant matrix suppression was observed which is typical in MS due to the matrix compounds [19]. This may be explained because of selective extraction after the OASIS-MISPE. The relative standard deviation (%RSD n=4)) for urine samples spiked at 15 μg l^{-1} was 12.3%.

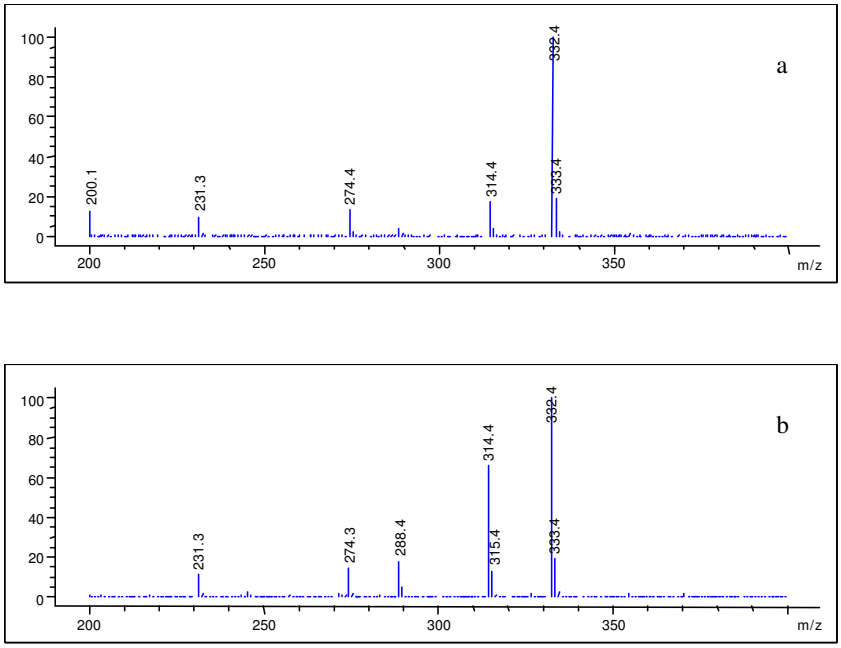

Figure3. Full scan Spectra for ciprofloxacin at different fragmentor voltage a) 100 V and b) 125 V.

CONCLUSIONS

In this study ciprofloxacin imprinted polymer has been synthesised and applied for the first time a as selective sorbent in SPE. Prior to it use, a chromatographic evaluation of the MIP allowed the imprinting effect and the cross-reactivity levels for another structurally related compound (ENRO) to be evaluated. From these results it was expected a high selectivity of the MIP for the template in SPE applications. Nevertheless it was necessary to include a clean-up step to enhance the selectivity of the MIP. When this MIP was applied in combination with an OASIS cartridge to the extraction of CIPRO from urine samples, a clean chromatogram was obtained after the optimised clean-up, which allowed the matrix compounds to be almost completely removed. Because of the efficient two-step MISPE method it was possible to directly inject into a MS the urine extracts to determine CIPRO at μg l^{-1} levels, avoiding the chromatographic column and reducing the time of analysis.

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