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A new molecularly imprinted polymer for the selective extraction of naproxen from urine samples by solid-phase extraction

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Abstract

A non-covalent molecularly imprinted polymer (MIP) was synthesised using naproxen (a non-steroidal, anti-inflammatory drug (NSAID)) as a template molecule. The MIP was chromatographically evaluated to confirm the imprinting effect, and was then applied as a selective sorbent in solid-phase extraction (SPE) to selectively extract naproxen. After this study, the MIP was used to extract naproxen from urine samples; it was demonstrated that by applying a selective washing step with acetonitrile (ACN) the compounds in the sample that were structurally related to naproxen could be eliminated.

Keywords: Molecularly imprinted polymer; Solid-phase extraction; Naproxen; Human urine samples

1. Introduction

Non-steroidal, anti-inflammatory drugs (NSAIDs) are the analgesics used most commonly across the world today. They are used mainly to treat pain, inflammation and fever in animal and human species, although they can lead to severe toxic side-effects in cases of over dose or chronic abuse.

Several analytical techniques can be used to determine these analgesic compounds in biological samples [1]. In nearly all such techniques a suitable sample preparation step, such as liquid–liquid extraction (LLE) or solid-phase extraction (SPE), is an important pre-requisite to the analysis in order to clean and pre-concentrate the sample. In the last few years the clear advantages of SPE over the widely used LLE have made SPE the most important technique for sample preparation. However, typical SPE sorbents lack selectivity and this constitutes a problem when a selective extraction from a complex matrix has to be performed. To enhance the molecular selectivity in SPE, molecularly imprinted polymers (MIPs) [2] have been developed. MIPs allow the analyte of interest to be not only selectively extracted, but also to be pre-concentrated and interferents arising from the sample matrix to be removed simultaneously [3].

The majority of previous studies reported thus far in respect of naproxen imprints have focused on enantiomeric separations where the MIP was used as a chiral stationary phase in HPLC [4–7]. Moreover, in nearly all studies, the MIPs were prepared by following a long and tedious synthetic procedure called the multi-step swelling and polymerisation method [4,5,8]. Only in a study described by Haginaka and Sanbe [8], involving a restricted-access material in conjunction with a molecularly imprinted polymer (RAM-MIP), are the imprints used in an on-line SPE system coupled to an HPLC, for direct injection serum assay. In this particular study two MIPs were prepared, one using naproxen as the template and the other using ibuprofen. However, the naproxen RAM-MIP could not be used for assays of naproxen, and neither could the ibuprofen RAM-MIP be used for assays of ibuprofen, because in both cases leakage of the template from the imprinted poly-

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mers prevented accurate and precise assays of the drugs. The naproxen RAM-MIP was used finally to extract ibuprofen from rat plasma.

The aim of the present work was to demonstrate the feasibility of using MISPE for the selective clean-up and quantification of trace amounts of naproxen from human urine. To the best of our knowledge this is the first time that a MIP synthesised following a conventional non-covalent imprinting protocol using naproxen as the template molecule has been used as a sorbent in SPE of biological samples to extract naproxen selectively.

2. Experimental

2.1. Reagents and standards

For the polymer syntheses, the chemicals used were (*S*)-naproxen, 4-vinylpyridine (4-VP) and ethylene glycol dimethacrylate (EGDMA), from Aldrich (Steinheim, Germany), 2,2′-azobisisobutyronitrile (AIBN) from Acros Organics (Geel, Belgium), and HPLC-grade toluene 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 acetonitrile (ACN) was provided either by Rathburn Chemicals or SDS (Peypin, France) and the water collected from a Millipore water purification system (Milli-Q water). The acetic, hydrochloric and phosphoric acids were from Probus (Badalona, Spain) and dichloromethane (DCM) from SDS (Peypin, France).

The structurally related NSAIDs (Fig. 1) used to investigate the selectivity of the imprinted polymer were ibuprofen from Fluka (Buchs, Switerland), diclofenac sodium from Sigma (Steinheim, Germany) and fenoprofen and benzoic acid from Aldrich. Standard solutions at 1000 mg l^{-1} for each compound were prepared in methanol.

Other compounds such as naphthalene, phenol (Ph), 4-nitrophenol (4-NP), 2,4-dinitrophenol (2,4-DNP), 2-chlorophenol (2-CP), 4-chlorophenol (4-CP), 2-nitrophenol (2-NP), 2,4-dimethylphenol (2,4-DMP), 2-naphthylamine-1-sulfonic acid (1-NS-2-NH_2), 1-naphthol-4-sulfonic acid sodium salt (1-NS-4-OH), naphthalene-2-sulfonic acid sodium salt (2-NS), naphthalene-1,5-disulfonic acid disodium salt (1,5-NDS), 2-naphthylamine-1,5-disulfonic acid disodium salt (1,5-NDS-2-NH_2), naphthalene-2,7-disulfonic acid disodium salt (2,7-NDS), 1-naphthol-3,6-disulfonic acid disodium salt (3,6-NDS-1-OH), supplied by Aldrich and Fluka, were used to check the selectivity of the MIP for other aromatic compounds.

2.2. Preparation of the imprinted polymer

A non-covalent molecular imprinting approach was followed to prepare the MIP. The pre-polymerisation mixture comprised 0.26 g (1.14 mmol) of the template (S-naproxen), 0.48 g (4.56 mmol) of the functional monomer (4-VP), 4.52 g (22.8 mmol) of the cross-linking monomer (EGDMA) and 0.08 g (0.50 mmol) of the initiator (AIBN) dissolved in 6.66 ml of the porogen (toluene) in a 25 ml thick-walled glass tube. This solution was cooled on an ice bath, sparged with oxygen-free nitrogen for 5 min, sealed under nitrogen and then left in a cool bath at −5 °C for 24 h for a UV polymerisation (50 Hz Black-Ray Non UV Semi-conductor Inspection Lamp, Model B 100 AP). The polymer obtained was then placed in a water bath at 60 °C for 24 h for a thermal cure.

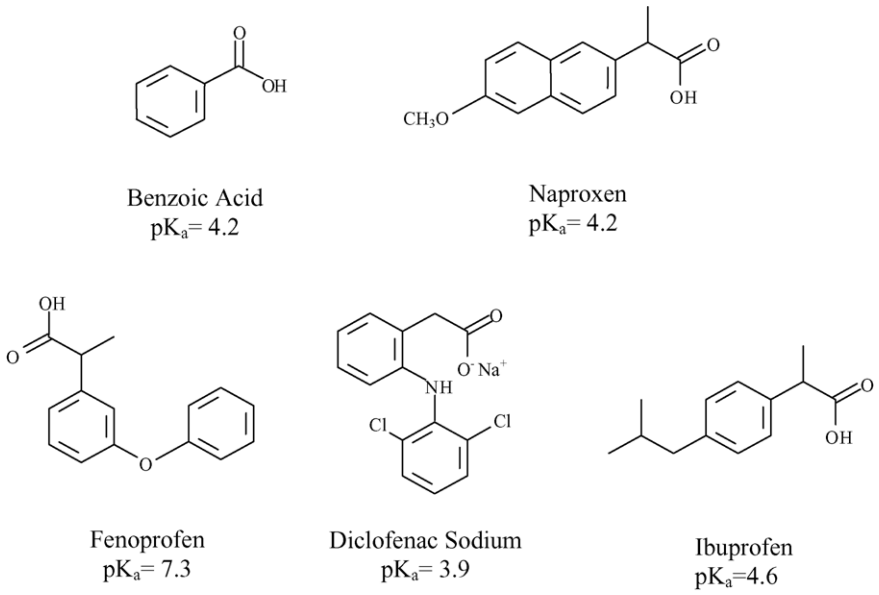

Fig. 1. Chemical structures of the non-steroidal anti-inflammatory drugs (NSAIDs) used to probe the selectivity of the MIP.

The polymer monolith obtained was crushed, ground and wet-sieved using acetone to obtain polymer particles with diameters between 25 and 38 μm suitable for the chromatographic and MISPE evaluations.

A non-imprinted control polymer (NIP) was synthesised, in the absence of template, following the same procedure described above.

2.3. Instrumentation

The polymers were evaluated initially in analytical columns to confirm the imprinting effect. 15 cm × 0.46 cm i.d. stainless steel HPLC columns were slurry packed with the ground polymer 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 prescreening work.

The MISPE study was developed in an off-line mode using a solid-phase extraction manifold supplied by Teknokroma (Barcelona, Spain) connected to a vacuum pump. Two hundred milligrams of each polymer (MIP and NIP) suspended in MeOH was packed into a 6 ml polyethylene cartridge. 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 25 cm × 0.4 cm i.d. Tracer Extrasil ODS2, 5 μm, supplied by Teknokroma.

2.4. Chromatographic conditions

Before the chromatographic evaluation of the polymers, the chromatographic columns were washed with a mixture of acetonitrile/water/acetic acid (92.5:2.5:5 (v/v/v)) for about 20 h to eliminate interfering compounds arising from the synthesis (template and unreacted monomers).

For the chromatographic evaluation, 15 μl of 10 mM naproxen in ACN/acetic acid (99:1) and 2 μl of the void marker (acetone) were injected. The mobile phase was acetonitrile/acetic acid (99:1) and the flow rate was set at 1 ml min^{-1} in isocratic mode. The NIP was evaluated under identical chromatographic conditions. The UV detector wavelength was set at 232 nm and the analysis performed at room temperature.

The HPLC parameters for the MISPE experiments were as follows. The mobile phase was a mixture of two solvents: Milli-Q quality water adjusted to pH 3 with phosphoric acid (solvent A), and acetonitrile (solvent B). The flow rate of the mobile phase was 1 ml min^{-1} and the gradient profile was from 40 to 66.5% B in 21 min, to 100% B in 6 min and then isocratic elution for a further 2 min. The column temperature was 30 °C.

2.5. MISPE conditions

Sample solutions (aqueous or urine adjusted to pH 3) were percolated through the cartridges which had been conditioned sequentially with 6 ml of ACN/H_2O/acetic acid (60:30:10), 6 ml of ACN and 6 ml of Milli-Q water (pH 3). The polymers were then washed with an organic solvent (specified later) and the retained analytes desorbed with 3 ml of ACN containing 1% of acetic acid. Twenty microliters of each sample was injected onto the analytical column.

2.6. Analysis of urine samples

Urine samples were kept in the freezer at −20 °C until their use. The urine was filtered through a 0.22 μm syringe filter before being applied to the MISPE cartridge.

3. Results and discussion

3.1. Chromatographic evaluation of the polymers

To confirm the imprinting effect the MIP was evaluated chromatographically. ACN was chosen as the mobile phase, with 1% of acetic acid being added to this solvent to enable naproxen to be completely eluted from the MIP and to avoid long retention times and peaks with extensive tailing. From these results, the retention factors of naproxen in the MIP (k naproxen$_{MIP}$ = 2.29) and NIP (k naproxen$_{NIP}$ = 0.88) columns and the imprinting factor (IF = $k^{TC}{}_{P1}/k^{TC}{}_{NIP}$) could be calculated (IF = 3). These values, taken together with the elution profiles, demonstrated that the MIP showed higher affinity for naproxen than the NIP and that the MIP was indeed imprinted.

The selectivity of the naproxen MIP for other structurally related analytes was also evaluated. For this purpose, ibuprofen, a NSAID with a structure similar to naproxen (Fig. 1), was injected onto the MIP and NIP columns as a test analyte. Thus, 15 μl of a 10 mM solution of ibuprofen was injected onto each column and the retention factors calculated (k ibuprofen$_{MIP}$ = 1.00 and k ibuprofen$_{NIP}$ = 0.51).

Naproxen and ibuprofen gave different retention times on the NIP, and for this reason the normalised retention index (RI) [9,10] was calculated to enable the k values of naproxen and ibuprofen to be compared. The RI value for the template (naproxen) is 1 by definition; for ibuprofen it was 0.75. From these results it can be concluded that the recognition of the template (naproxen) by the MIP is better than for ibuprofen, in spite of the similarity in their structures.

Exactly as one would expect for a naproxen imprint the chromatographic evaluation demonstrated clearly that the polymer was indeed imprinted. Thus, the MIP was taken forward and applied as an SPE sorbent to selectively extract naproxen from urine samples.

3.2. MISPE

The conditioning and the loading steps were first optimised, then 10 ml of a standard solution, spiked with 1.5 mg l^{-1} of naproxen and 3 mg l^{-1} of the other structurally related compounds (benzoic acid, fenoprofen, diclofenac sodium and ibuprofen), passed through the cartridge. The cartridge was conditioned with Milli-Q water at pH 3, and the sample was prepared in the same solvent. Under these conditions the compounds are in their non-dissociated (protonated) form (the pK_a values are shown in Fig. 1) and non-covalent interactions can be established between the hydroxyl groups of the NSAIDs and the 4-VP residues in the polymer. The analytes were strongly retained on the MIP with recoveries greater than 95% in all cases. When the sample was applied in organic solvents, or the pH of an aqueous sample was modified to neutral or basic, very little of each analyte was retained ($R\% < 30$).

The next step was to optimise the elution solvent. Five aliquots of ACN, each of 1 ml in volume, were used initially to elute the compounds from the MIP. The concentration of naproxen and the other compounds were measured in each individual eluate fraction. Three milliliters of ACN was sufficient to elute all the compounds except for naproxen and diclofenac sodium which were still retained to some degree. For this reason, 1% of acetic acid was added to the ACN. As was expected given the chromatographic evaluation results, the addition of acetic acid as a polar modifier to ACN (total volume of ACN/acetic acid = 3 ml) enabled the efficient elution of naproxen and diclofenac sodium from the MIP (Table 1). Furthermore, no bleeding of the template from the MIP was observed.

It is well known that under aqueous loading conditions all the analytes present in the sample are retained on the MIP because non-specific hydrophobic interactions dominate. Thus, a washing (clean-up) step with an organic solvent was included to remove the non-specifically bound compounds from the MIP, such that only naproxen remained selectively bound through specific interactions with the imprinted binding sites. The behaviour of the NIP under these SPE conditions was also evaluated and compared with the MIP. Prior to the clean-up step, the cartridge was dried by applying a vacuum for 15 min [11–13].

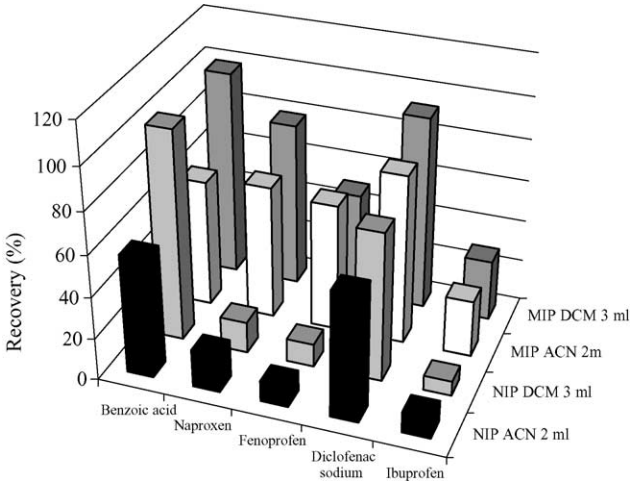

Fig. 2. Selectivity of the molecularly imprinted polymer (MIP) and the non-imprinted polymer (NIP) after a washing step with 2 ml of ACN or, alternatively, with 3 ml of DCM.

The results arising from the optimisation of the elution solvent (Table 1) showed us that ACN was potentially a good washing solvent, thus 2 ml of ACN was used to reveal the imprinting effect (Fig. 2). After this clean-up procedure was applied, while all the compounds were still retained on the MIP with recoveries close to 60% except for ibuprofen which was only 30% recovered, they were successfully stripped off the NIP (except for benzoic acid and diclofenac sodium).

After the clean-up with ACN, benzoic acid and diclofenac sodium were still retained on the MIP and the NIP. As a result, it is necessary to use a greater volume of ACN to disrupt the non-specific interactions than was used for either ibuprofen or fenoprofen.

DCM was also tested in the clean-up step. Thus, when 1 ml of DCM was applied, the recoveries measured for all compounds were nearly the same as those obtained in the absence of a washing step. The volume of DCM was therefore increased to 3 ml and the imprinting effect was then revealed. Fig. 2 shows the difference in behaviour between the MIP and

Table 1
Recoveries (%) obtained with the molecularly imprinted polymer (MIP) and the non-imprinted polymer (NIP) using acetonitrile or acetonitrile containing 1% of acetic acid as the elution solvent when 25 ml of a standard solution spiked at 55 μg l^{-1} for naproxen and 110 μg l^{-1} for the other analytes was pre-concentrated[a]

Analyte	Volume ACN (ml)						Volume ACN/AcOH (99:1) (ml)[b]		
	MIP	NIP	MIP	NIP	MIP	NIP	MIP	MIP	MIP
	1		2		3		1	2	3
Benzoic acid	–	13	39	50	95	90	30	80	104
Naproxen	–	14	36	82	60	105	50	70	99
Fenoprofen	20	22	39	90	98	99	60	90	101
Diclofenac sodium	20	17	19	40	49	53	70	80	99
Ibuprofen	30	20	73	90	103	97	100	102	105

[a] R.S.D.s were lower than 8% in all instances ($n = 3$).
[b] NIP values are not included because the recoveries were 100% in all instances.

the NIP. As can be clearly seen, the effect of using DCM was similar to that of ACN.

To probe the selectivity of the MIP in the solvent used originally as the porogen (solvent memory effect) [11–14], a number of experiments were performed where several milliliters of toluene were applied. However, no imprinting effect was observed even after a clean-up step involving 6 ml of toluene, because toluene is insufficiently polar to elute analytes from such polymers.

It has been shown that ACN and DCM have similar effects as washing solvents on the MIP and the NIP. Both solvents allow us to demonstrate that the polymer was imprinted as was expected given the earlier chromatographic evaluation results. Moreover, when 3 ml of DCM or 2 ml of ACN was applied, other aromatic compounds (3 mg l^{-1}) such as phenols and naphthalene sulfonates (previously described in Section 2) were also completely removed from the MIP.

The performance of the MIP in selectively extracting naproxen from real samples was also evaluated with the polymer being applied to the extraction of naproxen from human urine.

3.3. Analysis of urine samples

Drug free urine samples, obtained from healthy volunteers, were used in this study. To determine NSAIDs at the levels normally found in humans, a urine volume of 25 ml was chosen because there were no differences, in terms of recovery, between percolating 10 ml samples and 25 ml samples. Matrix interferences affecting molecular recognition and the use of ACN or DCM in the clean-up step are discussed below.

The urine samples could be loaded directly onto the MIP cartridge because binding of naproxen to the MIP was complete in water. For this reason the urine was not diluted using organic solvents or buffers [15,16], but was acidified to pH 3 using HCl.

Twenty-five milliliters of urine spiked with 55 μg l^{-1} of naproxen and 110 μg l^{-1} of the other NSAIDs and benzoic acid was percolated through the MIP after conditioning of the polymer.

After drying the cartridge for 15 min, the effect of using DCM as washing solvent was first checked. When 3 ml of this solvent was used, the broad band at the beginning of the chromatogram was only slightly reduced; for this reason a more polar solvent (ACN) was then tested. When experiments with 2 ml of ACN as washing solvent were performed, it was demonstrated that not only was the broad band at the beginning of the chromatogram clearly reduced, but also that naproxen was selectively extracted from a mixture of NSAIDs in urine (Fig. 3). The recovery of naproxen was 60%, whereas fenoprofen, diclofenac and ibuprofen were completely stripped off the MIP. Benzoic acid was only slightly retained on the MIP but it could not be quantified because it appears at the beginning of the chromatogram

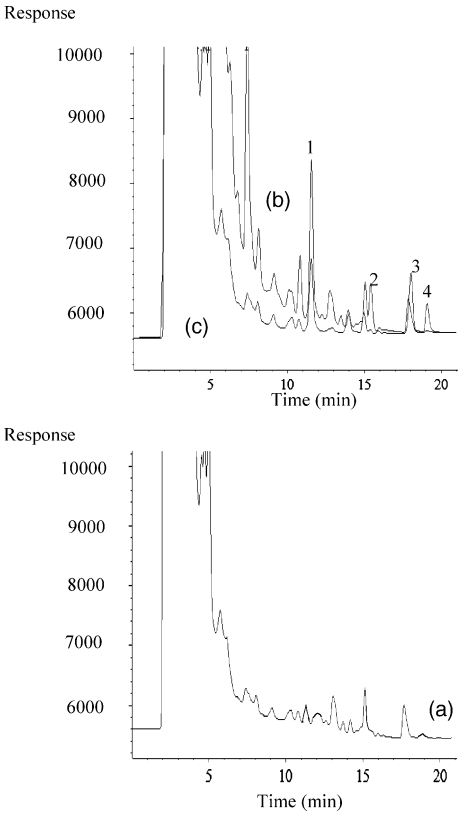

Fig. 3. Chromatograms obtained upon percolating 25 ml of a urine sample (pH 3) spiked at 55 μg l^{-1} with naproxen and 110 μg l^{-1} with a mixture of the other compounds through the MIP cartridge: (a) blank of urine after a clean-up step with 2 ml of ACN, (b) without a washing step, and (c) with a washing step involving 2 ml of ACN: (1) naproxen, (2) fenoprofen, (3) diclofenac sodium, (4) ibuprofen.

with other interferent peaks. The decrease in recoveries observed for the NSAIDs in urine can be explained because although urine is mainly water (95%), other compounds can, in principle, interfere with the MISPE process. Nevertheless, we were able to selectively extract naproxen from the mixture of NSAIDs. ACN was then used as the basis for further investigation.

For comparative purposes experiments were also performed with a 200 mg commercial C_{18} Bakerbond (Deventer, Holland) SPE catridge. When 25 ml of urine spiked with 55 μg l^{-1} of naproxen and 110 μg l^{-1} of the other NSAIDs was loaded, all compounds were retained on the cartridges and the recoveries were found to be nearly the same as those on the MIP. Fig. 4 shows that after a clean-up step involving 2 ml of ACN, all the compounds were completely eluted from the C_{18} sorbent, consistent with the fact that no selective interactions can be established between naproxen and this class of sorbent. This behaviour is completely different to that shown by the MIP in Fig. 3.

In order to decrease the time of analysis, the gradient profile was optimised, from 50 to 60% B in 10 min, to 100% B in 4 min and then isocratic elution for a further 2 min.

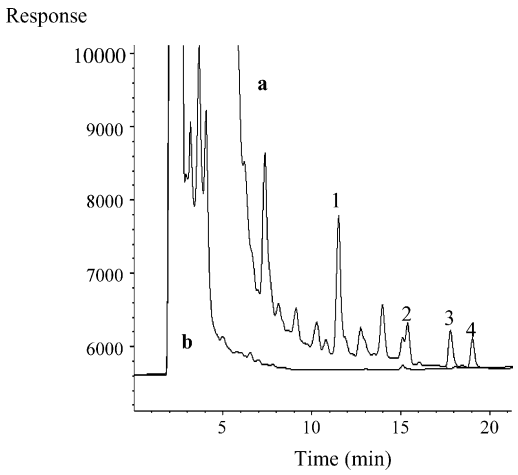

Fig. 4. Chromatograms obtained upon percolating 25 ml of a urine sample (pH 3) spiked at 55 $\mu g\,l^{-1}$ with naproxen and 110 $\mu g\,l^{-1}$ with a mixture of the other compounds through a commercial C_{18} solid-phase extraction cartridge: (a) without a washing step and (b) with a washing step involving 2 ml of ACN. Peak designation as per Fig. 3.

Thus, after passing 25 ml of urine spiked with 55 $\mu g\,l^{-1}$ of naproxen through the MIP and applying 2 ml of ACN for the clean-up step, naproxen was eluted after only 7 min (Fig. 5).

Under the optimised analysis conditions and with the shortened analysis time the linearity of the method was evaluated for naproxen. To check the linear range, 25 ml of urine, which did not contain any NSAIDs, was spiked with naproxen at concentrations between 110 and 3 $\mu g\,l^{-1}$.

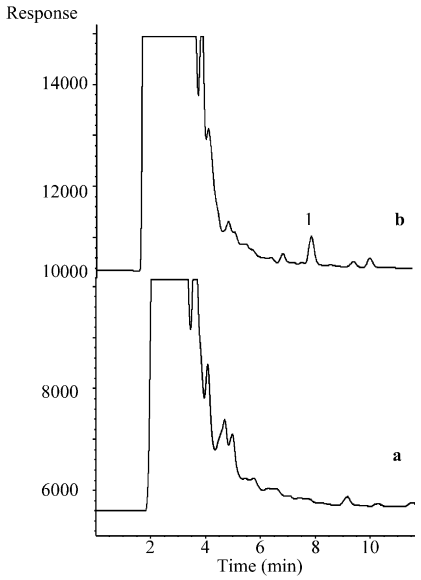

Fig. 5. Chromatograms obtained upon decreasing the time of analysis. Preconcentration of 25 ml of a urine sample (pH 3): (a) without addition of naproxen and (b) spiked at 25 $\mu g\,l^{-1}$ with naproxen and with a washing step involving 2 ml of ACN. Peak designation as per Fig. 3.

A washing step with 2 ml of ACN was then applied. Good linearity was obtained with a determination coefficient (r^2) greater than 0.990. The repeatability for 25 ml of spiked (25 $\mu g\,l^{-1}$ of naproxen) urine, expressed as R.S.D. ($n = 3$), was lower than 5%. The limit of detection of the method was established according to the signal-to-noise relation rule equal to 3 and was 3 $\mu g\,l^{-1}$. The application of the imprinted polymer to the MISPE of urine samples has therefore been successfully demonstrated. Significantly, not only can naproxen be selectively extracted from urine samples, but it can also be reliably and accurately quantified at low, biologically relevant levels (9–110 $\mu g\,l^{-1}$).

4. Conclusions

This study shows, for the first time, the synthesis and the application in SPE of a polymer imprinted following a conventional non-covalent molecular imprinting protocol using (*S*)-naproxen as a template. The MIP was successfully applied as a selective sorbent in SPE, and it has been demonstrated that the MIP is able to selectively extract naproxen from human urine samples after a clean-up step involving 2 ml of ACN. Moreover, this work also demonstrates the feasibility of using a naproxen MIP, prepared using a straight-forward non-covalent synthetic procedure, for the direct determination of naproxen in urine. Due to the minimal sample preparation required and short time of analysis, this method appears to be very well-suited for the control of naproxen in human urine.

Acknowledgements

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