



## Synthesis and evaluation of a selective molecularly imprinted polymer for the contraceptive drug levonorgestrel

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### ABSTRACT

A molecularly imprinted polymer (MIP) has been prepared using levonorgestrel (LEV) as template. The polymer was synthesised in a non-covalent approach using methacrylic acid (MAA) as functional monomer and ethylene glycol dimethacrylate (EGDMA) as cross-linking monomer via a free radical polymerization. An equivalent blank polymer was also synthesised in the absence of the template compound. Batch adsorption experiments were used to evaluate the binding affinity of the imprinted polymer. After packing MIP into a stainless steel column (150 mm × 4.6 mm i.d.), retention and elution of the template and related compounds were evaluated by high-performance liquid chromatography (HPLC). This LEV imprinted polymer was further applied for selective solid phase extraction (SPE) of LEV from human serum. It was confirmed that the binding ability of the prepared MIP for LEV was essentially sufficient in the presence of other compounds coexisting in serum sample. Therefore, as a selective and efficient solid phase material, LEV imprinted polymer has a high potential application in analysis of this steroidal hormone in clinical purposes.

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### 1. Introduction

Steroids which act as hormones such as estrogens and progestagens have great importance to living organisms. Progestagens (also called progestogens or gestagens) are hormones, which produce effects similar to progesterone (PRO), the only natural progestagen. All other progestogens are synthetic and are often referred to as progestins. Levonorgestrel (LEV), (–)-13 $\beta$ -ethyl-17 $\beta$ -hydroxy-18,19-dinor-17 $\alpha$ -pregn-4-en-20-yn-3-one, with the molecular structure given in Fig. 1, is a synthetic progestogen female sex hormone which is used in pregnancy prevention in humans [1]. Several analytical techniques including radioimmunoassay (RIA) [2], gas chromatography/mass spectrometry (GC/MS or GC/MS/MS) [3], and liquid chromatography/tandem mass spectrometry (LC/MS) [4], have been used to determine these steroidal compounds in biological samples. Although RIA methods have detection limits in the low pg ml<sup>-1</sup> range, they suffer from health hazards and are cumbersome to handle. In nearly all other 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. SPE has received much attention in recent years for the sample preparation and analysis of trace concentra-

tions in samples. 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) [5] have been developed. Molecular imprinting is a known method to produce tailor-made polymers with high affinity towards the specific template molecule. High selectivity, ease of preparation and economical synthesis of molecularly imprinted polymers make them competitive with other molecular recognition materials [6]. Because of their molecular recognition abilities, MIPs are extensively used in many technologies, such as separation of enantiomers [7], SPE [8], biochemical sensors [9], simulating enzyme-catalyzed reactions [10], drug delivery systems [11], and membrane separations [12].

Because of almost similar structures and different functional groups of steroidal compounds, using one of them, as a template to synthesis MIP can be helpful to realize the effects of functional groups in the recognition process. In the recent years, some efforts have been dedicated to prepare imprinted polymers with recognition properties towards steroids [13–26].

In this work, we study the development of an MIP using the non-covalent approach, able to bind LEV selectively with respect to other concomitants, e.g. similar steroids for its applications in the selective clean up and quantification of this compound in complex matrices such as human serum. Binding characteristics and selectivity of the imprinted polymer have been evaluated using batch rebinding and chromatographic studies. To the best of our knowl-

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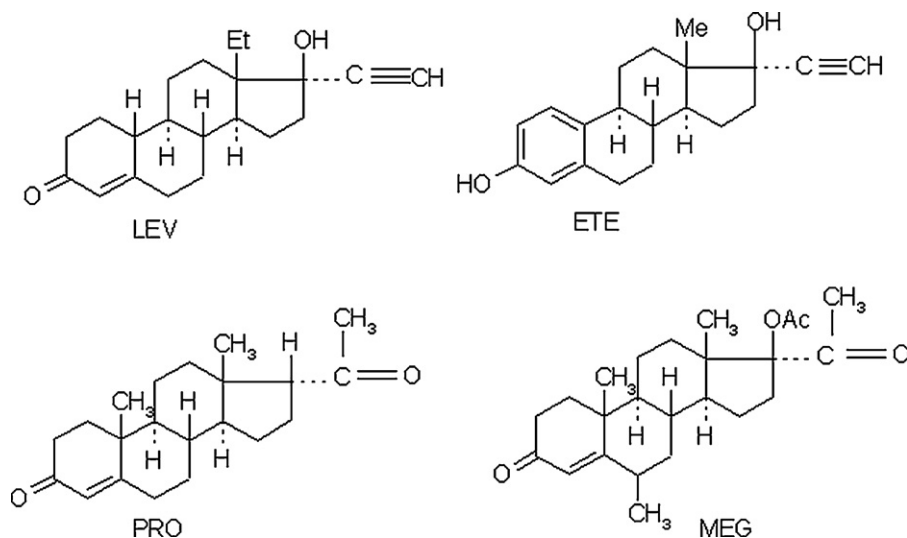


Fig. 1. Chemical structures of levonorgestrel (LEV), ethinylestradiol (ETE), progesterone (PRO), megestrol (MEG).

edge this is the first time an MIP synthesised by a non-covalent imprinting procedure, using LEV as the template molecule, has been used as a sorbent in MIP-SPE for selective extraction of LEV from biological samples.

## 2. Experimental

### 2.1. Reagents and standards

Levonorgestrel, ethinylestradiol (ETE), progesterone, megestrol (MEG) were obtained from Iran Hormone Co. (Tehran, Iran) and used without further purification. All solvents and reagents were of analytical grade and purchased from Merck (Darmstadt, Germany). Chloroform used to prepare the polymer was dried by distillation on 4.5 Å, molecular traps. Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were distilled under reduced pressure immediately before use. 2,2'-Azobisisobutyronitrile (AIBN) as the initiator of polymerization was used as received from Acros Organics (Geel, Belgium).

### 2.2. Apparatus

A chromatographic system (PerkinElmer, USA) consisting of binary pump Model 200, Rheodyne injector with a 20  $\mu$ l loop and ultraviolet detector Model 200 was used. The TotalChrom software was used to acquire and process spectral and chromatographic data from the detector. All separations were achieved using an analytical reversed-phase column (Spherisorb ODS, 5  $\mu$ m, 4.6 mm i.d.  $\times$  22 cm length, PerkinElmer) at room temperature under isocratic conditions. The column temperature was adjusted by a column thermostat (Knauer, Germany). For LEV determination, the detector was set at 235 nm for peak area measurements and the mobile phase was composed of acetonitrile/water (49/51, v/v) at a flow rate of 1 ml min<sup>-1</sup>. Temperature of the column was set at 25 °C.

### 2.3. Synthesis of LEV-imprinted polymer

For the preparation of LEV-imprinted polymer, 0.312 mg (1 mmol) template LEV and 0.34 g (4 mmol) functional monomer MAA were dissolved in 8 ml chloroform in a 20 ml thick-walled glass tube, 3.88 g (20 mmol) cross-linking monomer EGDMA and 39 mg AIBN as initiator were added. After oxygen-free nitrogen gas bubbling into the solution for 5 min, the tube was sealed

under vacuum, and the mixture kept in an oil bath at 60 °C for 24 h. The resulting bulk rigid polymers were crushed and ground manually with a mortar and pestle and sieved, under water, through 100, 50 and 25  $\mu$ m sieves. Particles between sieves were collected for further experiments. Then, template molecule was extracted in a Soxhlet apparatus by repeated extraction with water, methanol/trifluoroacetic acid (90/10, v/v) and methanol, until template was not detected in effluents using proposed HPLC method. A non-imprinted polymer that did not contain any template was prepared simultaneously using the same protocol.

### 2.4. Batch rebinding experiments

Batch adsorption experiments were used to evaluate the binding affinity of the imprinted polymer. MIP particles between 100 and 50  $\mu$ m sieves (150 mg) were mixed with 5 ml acetonitrile solution containing LEV at different concentrations (15–95  $\mu$ mol l<sup>-1</sup>). The mixtures were incubated for 18 h at room temperature accompanied by continuous shaking with a magnetic stirrer. After incubation the mixtures were centrifuged and the concentration of free LEV was determined by analyzing the supernatant solutions by RP-HPLC according to Section 2.2. The amount of LEV bound to the polymers, *B*, was calculated by subtracting the free amount of LEV, *C*, from the total amount of LEV added in the mixture. The unbounded amount of LEV was determined using an appropriate calibration curve.

### 2.5. Liquid chromatography experiments

Chromatographic separation of the LEV and its structural analogs depicted in Fig. 1 was performed to study the selectivity of the prepared MIP. Chromatographic experiments were performed with a PerkinElmer Model 200 HPLC system. MIP particles between 50 and 25  $\mu$ m sieves after removing their fine particles by sedimentation in acetone were slurry packed into a 150 mm  $\times$  4.6 mm HPLC stainless steel column. The ultraviolet detector wavelength was set at 235 nm and the analysis performed at room temperature. The mobile phase was acetonitrile/acetic acid (99.5/0.5, v/v) at a flow rate of 0.5 ml min<sup>-1</sup> in isocratic mode. Before the evaluation of the polymer, the packed columns were washed with a mixture of methanol/acetic acid (95/5, v/v) for about 10 h to remove template and unreacted monomers and establish a constant base line. The NIP was evaluated under identical chromatographic con-

ditions. The capacity factor was calculated as  $k = (t - t_0)/t_0$ , where the  $t$  is the retention time of the analyte and  $t_0$  is the retention time of acetone, which was used as the void marker. The imprinting factor, IF, was calculated as  $IF = k_xMIP/k_xNIP$ , where  $k_xMIP$  and  $k_xNIP$  are the capacity factors of a compound on MIP and NIP columns, respectively. The selectivity factor,  $\alpha$ , was calculated as  $\alpha = k_{LEV}/k_x$ , where  $k_{LEV}$  and  $k_x$  are the capacity factors of the LEV and its analogs, respectively.

## 2.6. MIP-SPE conditions

MIP column was prepared by packing 200 mg of the polymer into a 4 ml empty SPE cartridge. The polymer in the cartridge was secured by polyethylene frits at the top and the bottom. Cartridge was conditioned sequentially with 3 ml of methanol/trifluoroacetic acid (90/10, v/v), 3 ml of methanol and 5 ml of deionized water. Extraction experiments entailed loading the cartridge with 1 ml of water/acetonitrile (90/10, v/v) solution containing  $10 \text{ ng ml}^{-1}$  LEV. After loading, column was washed with 1 ml of toluene. Finally, the elution was performed by passing 3 ml of methanol/trifluoroacetic acid (90/10, v/v). All the fractions from the sample loading, washing, and elution steps were collected and then evaporated to dryness at  $40^\circ\text{C}$  under a stream of  $\text{N}_2$  gas. The residues were reconstituted by dissolution in  $200 \mu\text{l}$  acetonitrile. Then,  $20 \mu\text{l}$  of each sample was injected onto the analytical column of HPLC. Recoveries were calculated by using the constructed calibration plot.

## 2.7. Preparation of human serum sample

Serum samples were collected from a healthy female volunteer and stored frozen at  $-20^\circ\text{C}$  until assay. After gentle thawing,  $100 \mu\text{l}$  of serum samples were introduced into two centrifugation tubes, then successively spiked with different amounts of LEV (10 and 100 ng). In order to precipitate proteins,  $100 \mu\text{l}$  acetonitrile was added to each tube and mixed well. The tubes were centrifuged for 15 min at 12,000 rpm for separation of the precipitated proteins. The clear supernatants were then filtered through a  $0.45 \mu\text{m}$  filter. The filtered supernatants were diluted with deionized water to 1 ml and then loaded into the MIP-SPE cartridges as described in Section 2.6. Blank serum sample was prepared with the same method in the absence of LEV for comparison.

## 3. Results and discussion

### 3.1. Batch rebinding studies

Rebinding ability of the MIP was evaluated by a batch adsorption method. It is found that the incubation time of the polymer with LEV and the amounts of MIP taken greatly affected the rebinding results. Experiments showed that 18 h incubation time was necessary to complete the rebinding of LEV to MIP. Fig. 2 shows the effect of the amounts of MIP on the adsorption percentage in 5 ml acetonitrile solution containing  $100 \mu\text{mol l}^{-1}$  LEV. According to the obtained results, in our following experiments, 18 h of incubation and 150 mg of MIP were taken for the adsorption studies.

In order to characterize binding sites of the prepared MIP, experimental data were fitted to the Langmuir adsorption isotherm model, which is a model of discrete distribution in which homogeneous surface is supposed. Hence, Eq. (1) was used for binding site characterization at equilibrium:

$$\frac{C(N_t - B)}{B} = K_d \quad (1)$$

where  $N_t$  means the number of available active centers in the MIP per unit of volume and  $K_d$  is the equilibrium desorption constant

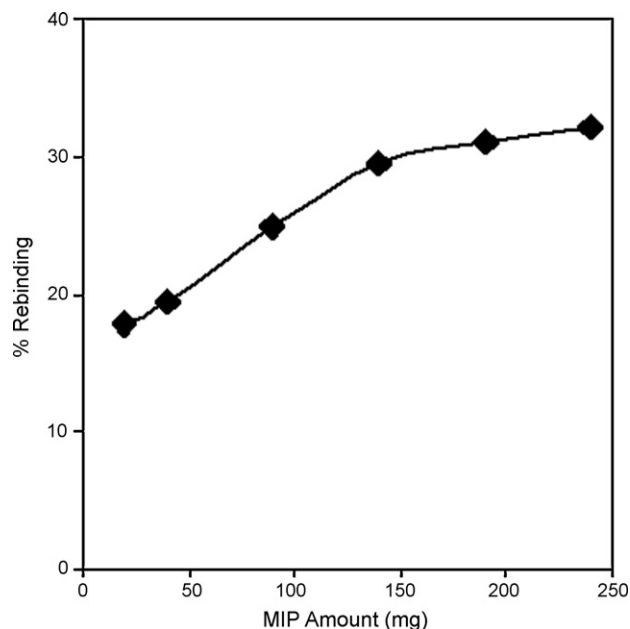


Fig. 2. Effects of the amounts of MIP on the adsorption.

[27]. This equation can be rearranged to Eq. (2):

$$\frac{B}{N_t} = \frac{C}{C + K_d} \quad (2)$$

which is the equation for a rectangular hyperbola with horizontal asymptote corresponding to 100% saturation of  $N_t$  such that  $[\text{bound}] = [\text{binding sites}]$ . As the polymer is solid, the ratio  $f = B/N_t$ , also referred as the fractional occupancy, gives the amount of bound template per gram of imprinted polymer. A satisfactory regression coefficient, 0.9993, was achieved by fitting experimental data to this adsorption isotherm model. Eq. (2) shows that  $K_d$  can be also defined as the concentration of free template at which 50% of the imprinted binding sites are occupied (i.e.,  $f = 0.5$ ). In Fig. 3, each calculated  $f$ -value and its corresponding  $B$ -value were plotted to establish a binding isotherm for the imprinted polymer. The calculated dissociation constant,  $K_d$ , was  $55 \mu\text{M}$  and the maximum number of available active centers,  $N_t$ , was  $66 \mu\text{mol g}^{-1}$ .

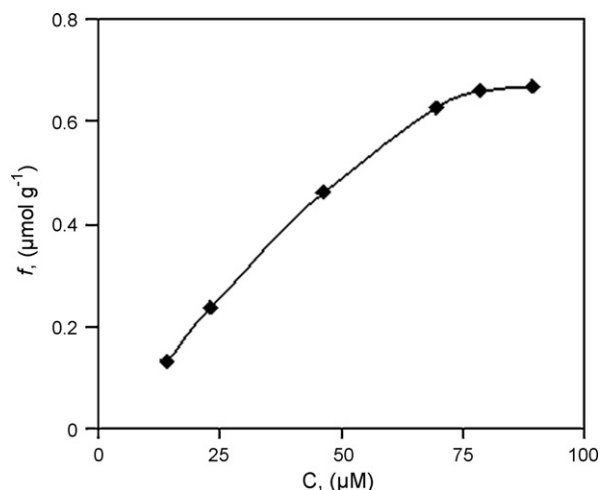
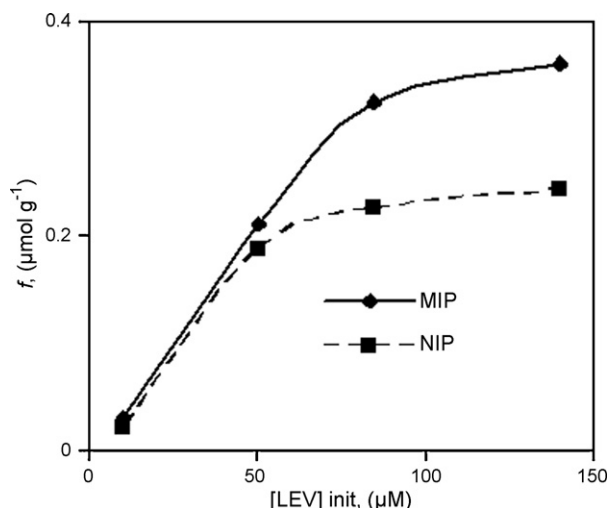


Fig. 3. Fractional occupancy,  $f$ , of the imprinted polymer as a function of free amount of LEV,  $C$ .



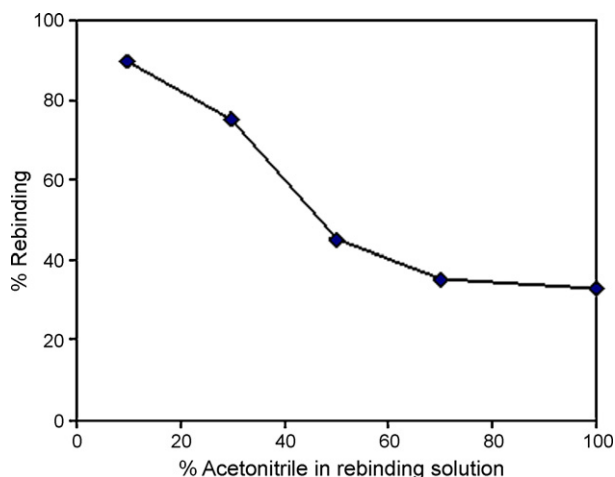
**Fig. 4.** Binding isotherms of LEV on MIP and NIP.  $f$ , represents the fractional occupancy of MIP bound to the polymers and  $[LEV]_{init}$  represents the initial concentration of LEV.

Equilibrium adsorption experiments were performed on MIP and NIP particles to evaluate the binding affinity of the MIP. The results showed that the MIP has higher binding capacity than NIP when the initial concentration of LEV was higher than  $50 \mu\text{M}$  (Fig. 4). Results proved that the binding affinity of the MIP was from the specific sites formed by the imprinting effect.

Additional batch experiments were performed to evaluate the solution composition effect on the rebinding of LEV. As it can be seen from Fig. 5, the recovery percent of LEV increased by increasing water content in the solution. Approximately, more than 90% of LEV was in the bounded form within 18 h when the solution contained more than 90% of water in mixtures with acetonitrile. Furthermore, higher recoveries were obtained at higher water content in the rebinding solution. It can be concluded that, under aqueous conditions, the hydrogen bonding interactions between LEV and the polymer sites, decreases and that the analyte is strongly retained on the MIP because of additional non-specific hydrophobic interactions.

### 3.2. Chromatographic study of LEV imprinted polymer

To confirm the imprinting effect and selectivity of the LEV imprinted polymer, its ability to resolve structural analogs was



**Fig. 5.** Percent rebinding of LEV on the LEV imprinted polymer by using different contents of water in rebinding solution.

**Table 1**  
Capacity and imprinting factors for LEV and its analogs on MIP and NIP

	Capacity factor		Imprinting factor
	MIP	NIP	
LEV	2.79	0.66	4.23
PRO	2.01	0.60	3.35
ETE	1.71	0.79	2.16
MEG	1.22	0.85	1.43

MIP column:  $150 \text{ mm} \times 4.6 \text{ mm}$  stainless-steel. Mobile phase: Acetonitrile/acetic acid (99.5/0.5, v/v) at a flow rate of  $0.5 \text{ ml min}^{-1}$ . UV detector was set at 235 nm. Data from triplicate measurements were averaged.

**Table 2**  
Selectivity factors for LEV and its analogs on MIP

Selectivity factor <sup>a</sup>		
$k_{LEV}/k_{ETE}$	$k_{LEV}/k_{PRO}$	$k_{LEV}/k_{MEG}$
1.63	1.38	2.29

MIP column:  $150 \text{ mm} \times 4.6 \text{ mm}$  stainless-steel. Mobile phase: Acetonitrile/acetic acid (99.5/0.5, v/v) at a flow rate of  $0.5 \text{ ml min}^{-1}$ . UV detector was set at 235 nm. Data from triplicate measurements were averaged.

<sup>a</sup> Selectivity factor for LEV relative to the analogs.  $k_{LEV}$ ,  $k_{ETE}$ ,  $k_{PRO}$  and  $k_{MEG}$  are the retention factors of levonorgestrel, ethinylestradiol, progesterone and megestrol, respectively.

measured chromatographically. LEV and its analogs ETE, PRO and MEG, which have almost similar molecular structures, were used for this study. The capacity and imprinting factor values obtained from the experiments are listed in Table 1. MIP obviously exhibited high binding affinity for LEV, while the structurally related compounds used showed less binding capacity. As for NIP, it showed considerably less binding for most of the analytes although some of them seem to show slight binding, but the binding on NIP is lower than that on MIP. These values, taken together, demonstrated that the MIP showed higher affinity for LEV than the NIP and that the MIP was imprinted. From these results, the selectivity factors of LEV in the MIP column could be calculated (Table 2). The MIP had pronounced selectivity for LEV. For compounds whose structural relationship is more similar, for example PRO, some recognition was observed but it was much less than that for the template molecule. Results obviously showed that the functional groups on the four-ring backbone of the steroid molecules play important roles in selective recognition of LEV by the LEV imprinted polymer.

### 3.3. SPE using LEV imprinted polymer

The prepared LEV imprinted polymer was used to specific MIP-SPE by means of cartridges, which had been conditioned previously (Table 3). The procedure for the MIP-SPE consisted of sample loading, washing (clean-up) and elution. After the sample loading, the effluent from the MIP cartridge was analyzed by HPLC. LEV was not detected considerably (5%), showing that the loaded LEV was completely retained. During the sample loading, the MIP can be predictable to run in the reversed-phase mode, because in aque-

**Table 3**  
Percent recoveries of LEV on MIP by using loading, washing, and elution

Fractions	Recovery <sup>a</sup> (%)
Loading (1 ml of water/acetonitrile (90/10, v/v) containing $10 \text{ ng ml}^{-1}$ )	$5.0 \pm 3$
Washing (1 ml of toluene)	$3.2 \pm 4$
Elution (3 ml of methanol/trifluoroacetic acid (90/10, v/v))	$90 \pm 5$

Conditions for SPE: Conditioning with 3 ml of methanol/trifluoroacetic acid (90/10, v/v), 3 ml of methanol and 5 ml of deionized water.

<sup>a</sup>  $\bar{x} \pm \text{R.S.D.}$  ( $n = 3$ ).

**Table 4**  
Recovery test of LEV from human serum using LEV imprinted polymer

Added value (ng)	Determined concentration (ng)	Recovery <sup>a</sup> (%)
–	N.D. <sup>b</sup>	–
10	8	80 ± 4
100	88	88 ± 3

Conditions for SPE: Conditioning with 3 ml of methanol/trifluoroacetic acid (90/10, v/v), 3 ml of methanol and 5 ml of deionized water. Washing with 1 ml of toluene, and elution with 3 ml of methanol/trifluoroacetic acid (90/10, v/v).

<sup>a</sup>  $\bar{x} \pm R.S.D.$  ( $n = 3$ ).

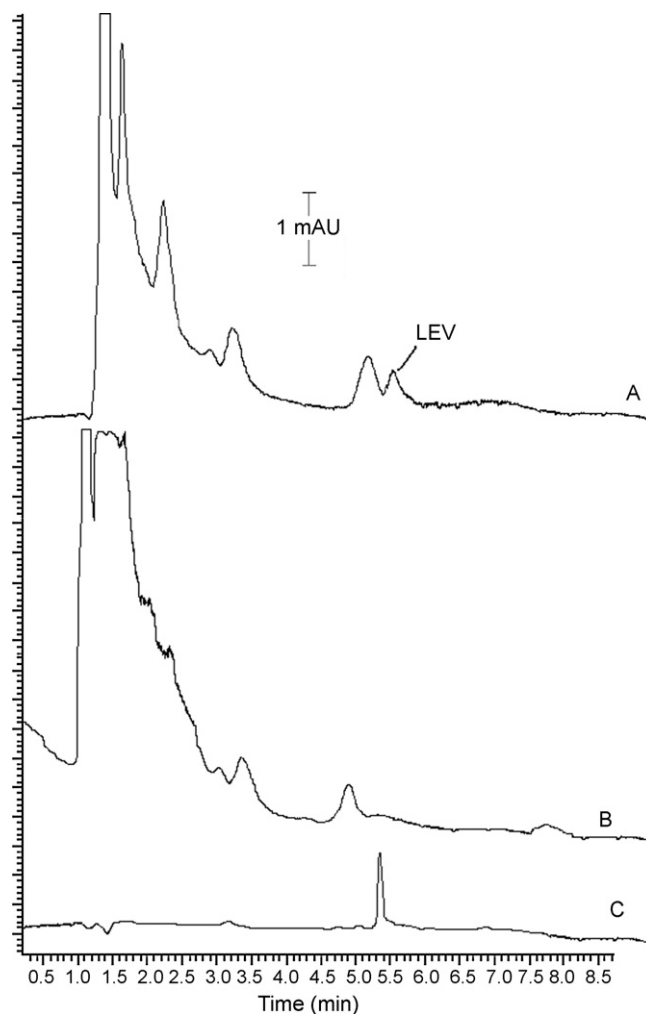
<sup>b</sup> N.D.: Not determined.

ous media large non-specific hydrophobic interactions dominate. After the cartridge was carefully dried by applying a vacuum for 15 min, the washing step was carried out. A washing step with an organic solvent was included to remove the undesired non-specifically bound compounds from the MIP, such that only LEV remained selectively bound through specific interactions with the MIP binding sites. Toluene was selected as the washing solvent due to its low polarity and aprotic properties. The MIP can be run in the affinity mode under the conditions, because a hydrogen bond could be a dominant interaction in the specific retention. The HPLC analysis of the wash waste showed that LEV was not washed out significantly (~3%). The subsequent recovery step was carried out with 3 ml of polar organic solvent methanol/trifluoroacetic acid (90/10, v/v). HPLC analysis of the extract showed that LEV was specifically recovered (90%).

#### 3.4. Analysis of standard spiked serum sample

In order to examine the performance of the imprinted polymer in complicated matrix, the prepared MIP was used as a SPE stationary phase (MIP-SPE) to extract of LEV from human serum sample. Extraction of LEV from the prepared serum samples was performed under the same conditions as extractions of the standard solutions discussed in Section 2.6. The fractions from the wash step by toluene were found to be free of the analyte. In blank serum sample, no signal could be detected. In spiked serum samples of 10 and 100 ng (see Table 4), the adsorption recoveries for LEV were above 80% and relative standard deviation values were less than 4%. The limit of detection and limit of quantification based on three and ten times of the noise of HPLC profile were 0.08 and 0.26 ng ml<sup>-1</sup>, respectively.

The results suggested that the LEV imprinted polymer exhibited acceptable adsorption ability towards LEV even in the presence of other matrix compounds in serum sample and the matrix of the tested samples caused no difficulty in the analysis. Chromatograms obtained with washing and elution fractions obtained by SPE of serum sample spiked with LEV are shown in Fig. 6. The chromatograms clearly show the difference between the elution profiles obtained by RP-HPLC analysis before and after extraction on LEV-imprinted polymer. Direct injection of the spiked serum sample after protein precipitation gave matrix peaks co-eluting with LEV, hindering accurate quantification. Also, HPLC column lifetime because of remained matrix components will be short. Previous MIP-SPE, however, resulted in a cleaner elution profile with almost quantitative recovery (>80%) of LEV. According to Maier et al. [28] there can be a probable “deactivation” of selective binding cavities of the MIP by matrix components, which are almost similar to template molecule. In order to examine the possibility of this phenomenon, the SPE experiment was repeated by non-imprinted polymer under the same conditions. The LEV of the spiked samples treated with non-imprinted polymer was not retained satisfactorily and a poor quantitative recovery (~34%) of LEV from serum sample was achieved. Although, it was observed that recovery decreased



**Fig. 6.** Chromatograms obtained by injecting human serum sample (1 ml containing 100 ng LEV) without preceding MIP-SPE (A), washing fraction by 1 ml of toluene after percolating the spiked sample through MIP cartridge (B), and elution fraction by 3 ml of methanol/trifluoroacetic acid (90/10, v/v) (C). HPLC conditions are illustrated in Section 2.2. MIP-SPE were performed as described in Section 2.6 and serum samples were prepared as described in Section 2.7.

if more than five successive extractions of serum samples were performed with the same MIP cartridge. This was attributed to accumulation of matrix components non-selectively retained by the MIP [29]. Adsorption of these components could block the active sites of the polymer and could even modify its retention properties. In such circumstances, washing the cartridge with excess elution solvent sufficed for its “reactivation”.

#### 4. Conclusion

To the best of our knowledge, this is the first time that an MIP is synthesised following a non-covalent approach using LEV as the template molecule. According to the batch rebinding studies and chromatographic evaluations, the LEV imprinted polymer showed good selectivity and affinity for LEV. The MIP obtained was successfully applied as a sorbent in SPE for selective extraction of LEV from human serum samples prior to RP-HPLC. Because of minimal sample preparation and a short time were required for the analysis, this method seems very well suited for direct determination of LEV in human serum samples.

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