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Determination of phenytoin in plasma by molecularly imprinted solid-phase extraction

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Abstract

A molecularly imprinted polymer (MIP) using phenytoin as template and methacrylamide as the functional monomer was prepared. The selectivity was measured by comparing capacity factors of phenytoin and other structurally related compounds. The polymer was evaluated as a selective sorbent in molecularly imprinted solid-phase extraction (MISPE). Several washing solvents were tested to study their ability to disrupt the non-specific interactions occurring between the sample and the polymer matrix and the role of water in the recognition process was also investigated. It was shown that the key step of successful sample extraction is the right choice of the washing solvent. Plasma samples spiked with phenytoin were analyzed by the MISPE methodology developed in this work. Method validation (intra- and inter-day precision, recovery, specificity) was carried out. The calibration curve showed good linearity in the 2.5–40 μg/ml range corresponding to therapeutically relevant plasma levels. The intra- and inter-day precision values were below the 15% limit established for bioanalytical methods. The results showed that the method could be successfully applied for the determination of phenytoin in plasma samples.

Keywords: Molecularly imprinted solid-phase extraction; Phenytoin

1. Introduction

Phenytoin is one of the most commonly prescribed anticonvulsant drugs in the treatment of epilepsy, with a therapeutic concentration of 10–20 μg/ml. To avoid toxicity and ensure efficacy a continuous therapeutic monitoring is needed. The use of standard chromatographic techniques, due to their versatility in measuring drugs and metabolites at the same time, has been established for determination of phenytoin [1–3]. The sample pretreatment in these cases is usually carried out by liquid–liquid extraction or solid-phase extraction (SPE). The selectivity of custom SPE sorbents is limited because they differentiate only by some generic property like hydrophobicity. In order to enhance the selectivity the use of molecularly imprinted polymers (MIPs) as selective sorbents is becoming increasingly popular [4–13]. These are commonly highly reticulated

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polymethacrylates synthesised in presence of a template molecule (usually a close structural analog to the target analyte) and a functional monomer [14]. The resulting polymers exhibit in many cases antibody like affinity and selectivity for the template. Recently the synthesis and chromatographic application of MIPs exhibiting high selectivity for phenytoin or other hydantoins have been described [15–17]. In the present work we have developed a sample preparation method for determination of phenytoin (5,5-diphenylhydantoin) from plasma samples using such polymers as SPE sorbents.

2. Experimental

2.1. Reagents and chemicals

Phenytoin (5,5-diphenylhydantoin), ethylene glycol dimethacrylate (EDMA), methacrylamide (MAAM), diphenylmethane, hydantoin, 5,5-dimethylhydantoin, 5-(4-methylphenyl)-5-phenylhydantoin, and 5-(4-hydroxyphenyl)-5-phenylhydantoin, were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2,2′-Azo-bis-iso-butyronitrile (AIBN) was obtained from Janssen. Ametryne and atrazine were kindly provided by Novartis (Basel, Switzerland) (for structures see Fig. 1). All solvents used [acetonitrile, tetrahydrofuran (THF), methanol (MeOH), dichloromethane (DCM), nitromethane, acetone, chloroform, toluene, hexane] were of HPLC grade. K_2HPO_4 and $NaH_2PO_4 \cdot 12H_2O$ (Reanal, Budapest, Hungary) of analytical grade were used for the preparation of buffer solutions.

2.2. Molecularly imprinted polymer preparation and chromatographic evaluation

The MIP and non-imprinted polymer (NIP) were prepared as described in our previous paper using MAAM as functional monomer and acetonitrile–THF (76:24, v/v) as porogen [17]. The polymer particles were ground and sieved under water, and the 25–40 μm particle size fraction was collected. Stainless steel HPLC columns (125×4 mm) were slurry packed with imprinted and non-imprinted polymers using MeOH–water (80:20, v/v) as pushing solvent at a pressure of 300 bar.

2.2.1. Elution chromatography

For a comprehensive selectivity study 20 μl of 1 m*M* solutions of the phenytoin and reference compounds: 5-(4-hydroxyphenyl)-5-phenylhydantoin, 5-(4-methylphenyl)-5-phenylhydantoin, hydantoin, 5,5-dimethylhydantoin, diphenylmethane, atrazine, ametryne and phenol were injected onto the columns using acetonitrile as mobile phase. The capacity factor was calculated for each analyte as $k' = (t - t_0)/t_0$ where t_0 is the retention time of the void marker (acetone). The imprinting factor (IF) was calculated as the ratio of capacity factors of each analyte on the MIP column and the NIP column.

2.2.2. Frontal chromatography

The columns (NIP and MIP) were equilibrated with acetonitrile (ca. 40 min). Phenytoin solutions of different concentrations ranging from 0.01 to 5 m*M* were prepared in acetonitrile and were used as the mobile phase in frontal chromatographic experiments at a 1 ml/min flow-rate. The breakthrough volume for a non-interactive molecule was measured by flushing the columns with acetonitrile containing 0.5% acetone. The breakthrough volume was expressed as the solvent volume at the maximum of the first derivative of the frontal chromatogram. These data were used to calculate binding site density and affinity constants of the polymer.

2.3. Chromatographic determination of phenytoin

Chromatographic determination of phenytoin, injected in 20 μl, was carried out on an ODS Hypersil analytical column (Hewlett-Packard, Palo Alto, CA, USA) (200×4.6 mm, 5 μm) using an ODS Hypersil pre-column (Bio Separation Technologies, Budapest, Hungary) (20×4.0 mm, 5 μm). The mobile phase consisted of methanol–acetonitrile–phosphate buffer (0.01 *M*, pH 4.8) (30:30:40, v/v) pumped at a flow-rate of 1.0 ml/min by a HPLC pump (Model 420, Kontron, Switzerland). The UV absorbance of phenytoin was measured using a variable-wavelength UV–VIS detector (Jasco FP-970, Jasco, Tokyo, Japan) set at 240 nm. Data acquisition and evaluation were done on a 486 AT IBM compatible computer using Borwin 1.21 chromatography software (JMBS Developpements, Le Fontanil, France). For quantitative evaluation the internal standard method was

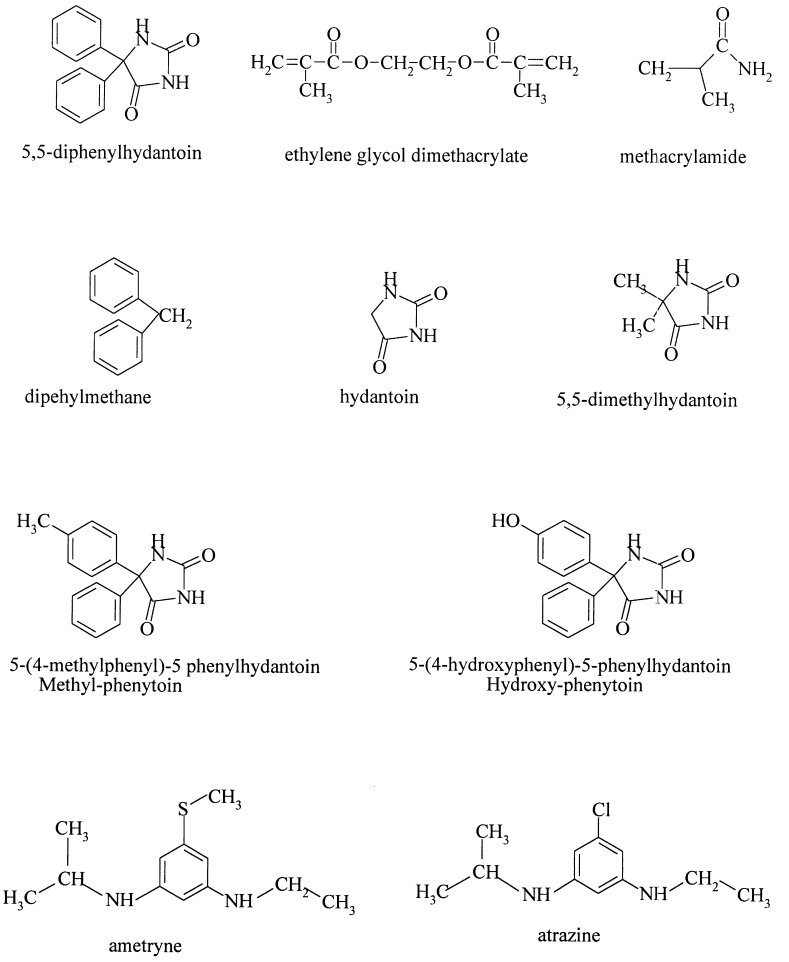

Fig. 1. Names and structures of the compounds used in the measurements.

used, choosing 5-(4-methylphenyl)-5-phenylhydantoin, a compound having a similar chemical structure to that of phenytoin. The peak area ratio of phenytoin and the internal standard was used for quantitation.

2.4. Optimisation of the MISPE procedure

A 50-mg amount of polymer (MIP and NIP, respectively) was slurry packed with methanol into empty polypropylene SPE cartridges. The polymer cartridges were conditioned using 2 ml methanol and 2 ml organic solvent [acetonitrile, THF, acetonitrile–THF (76:24 v/v), nitromethane, dichloromethane, chloroform, toluene, hexane or acetone], which was to be used as washing solvent in a further step. During sample application 25 μg phenytoin in 50 μl washing solvent or 25 μg phenytoin in 1 ml water containing 3% acetonitrile was loaded onto the cartridge. Afterwards a series of selective washing steps was applied: several 500 μl aliquots of washing solvent were successively loaded onto the SPE column. After a 5 min drying step the sample was eluted with 2 ml methanol. The organic solvent was removed from each washing aliquot by drying the sample under nitrogen stream using a TurboVap LV evaporator (Zymark, Hopkinton, MA, USA). The residue was redissolved in 1 ml mobile phase prior to injection into the chromatograph. The amount of phenytoin in each aliquot of the washing solvent and

methanol was calculated. Experiments were carried out in duplicate. Controls, where no phenytoin was added to the solvent, were made in order to check for template bleeding.

2.4.1. Extraction procedure of phenytoin from plasma samples

After conditioning the cartridge with 2 ml methanol, 1 ml distilled water and 1 ml phosphate buffer (pH 7.0, $c=0.025$ *M*), 200 μl plasma sample was applied. The sample application was followed by a washing step (1 ml phosphate buffer (pH 7.0, $c=0.025$ *M*) and 1 ml distilled water) and a 5 min drying step. A 1-ml volume of DCM was used for selective washing, then the cartridge was dried for 5 min and the phenytoin was eluted with 2 ml methanol.

2.5. Preparation of calibration standards and quality control (QC) samples

A 160-mg amount of phenytoin was dissolved in 100 ml of methanol. The standard solutions (0.1, 0.2, 0.4, 0.8 and 1.6 mg/ml) used for spiking calibration samples were prepared from this stock solution by dilution with methanol. During calibration 100 μl of the standard solutions was added to 3.9 ml plasma to obtain 2.5, 5.0, 10.0, 20.0 and 40.0 μg/ml calibration standards. Four separate solutions were used for the preparation of quality control samples. 1.25, 3.75, 12.5 and 15 mg phenytoin was dissolved in 5 ml methanol to obtain 0.25, 0.75, 2.5 and 3.0 μg/ml stock solutions. QC samples were prepared by spiking 8.91 ml plasma with 90 μl of the phenytoin stock solutions so the resulting QC samples contained 2.5, 7.5, 25 and 30 μg/ml phenytoin. The plasma samples were frozen after spiking and kept in a deep freezer at −20°C.

A 10-mg amount of 5-(4-methylphenyl)-5-phenylhydantoin was dissolved in 100 ml methanol and was used as internal standard stock solution. A 20-μl volume of this solution was used to spike plasma samples. For quantitation the peak area ratio of phenytoin relative to the internal standard was plotted against the plasma concentration. A straight line was fitted to the calibration points using the least-squares method without weighting.

3. Results and discussion

3.1. Chromatographic evaluation of the MIP

The phenytoin MIPs were previously evaluated as stationary phase in high-performance liquid chromatography (HPLC) to obtain information on the existence of recognition sites [17]. The retention and the peak shape of phenytoin on the MIP compared to the NIP clearly indicated the presence of templated binding sites in the imprinted polymer. Particularly high selectivity for phenytoin was obtained using MAAM as functional monomer with imprinting factor $IF=k'_{MIP}/k'_{NIP}=5.5$. This is higher than for MIPs prepared using acrylamide as functional monomer [15,16] and shows the influence of subtle structural differences on the quality of the templated binding sites. This selectivity we considered would be sufficient for successful application in SPE.

To investigate the origin of selectivity other structural analogs to phenytoin were used to challenge the HPLC columns packed with the MAAM polymer. In accordance with previous observations, the highest imprinting factor was observed for the template phenytoin. Slightly lower IFs were observed for close structural analogs such as the metabolite of phenytoin, 5-(4-hydroxyphenyl)-5-phenylhydantoin [hydroxy-phenytoin], and 5-(4-methylphenyl)-5-phenyl-hydantoin [methyl-phenytoin] (see Fig. 1) whereas other hydantoins, lacking the two phenyl groups were equally retained on the MIP as on the NIP.

Further compounds containing a hydantoin ring, but less similar to phenytoin than the metabolite or methyl-phenytoin, were also measured, while phenol was chosen to study the interaction between the hydroxyl group and polymer. Compounds with lipophilicities similar to phenytoin (log $P=2.47$), but structurally quite different from it, such as ametryne (log $P=2.98$) and atrazine (log $P=2.61$), were not retained on the columns (Table 1). This is a sign that the separation on the column is not based on differences in the hydrophobicity as in the case of a ''normal'' HPLC sorbent, but rather that highly selective binding sites have been obtained. It is interesting to note that compounds with a hydantoin ring have significantly higher retention on the blank column than compounds not bearing this structural

Table 1
Chromatographic evaluation data of phenytoin and structurally related compounds applied on an MIP and an NIP column using acetonitrile as mobile phase

	NIP k'	MIP k'	IF
Phenytoin	1.30	7.16	5.5
Hydroxy-phenytoin	2.99	10.88	3.6
Methyl-phenytoin	1.22	4.45	3.6
Hydantoin	1.06	1.17	1.1
5,5-Dimethylhydantoin	0.70	1.06	1.5
Diphenylmethane	0.14	0.18	1.2
Ametryne	0.31	0.37	1.2
Atrazine	0.35	0.42	1.2
Phenol	0.38	0.44	1.2

feature. This reflects the intrinsic affinity between the hydantoin structure and the amide group.

Frontal chromatography measurements were carried out to shed light on the strengths and density of the specific binding sites. Scatchard plot of the binding data revealed that two classes of binding sites are present in the imprinted polymer – high affinity binding sites with a binding constant of about 5675 M^{-1} and low affinity binding sites with a binding constant of about 257.8 M^{-1} (Fig. 2.). The number of high affinity binding sites is approx. 2.7 μmol/g polymer, and the number of low affinity binding sites is 29 μmol/g polymer. The obtained

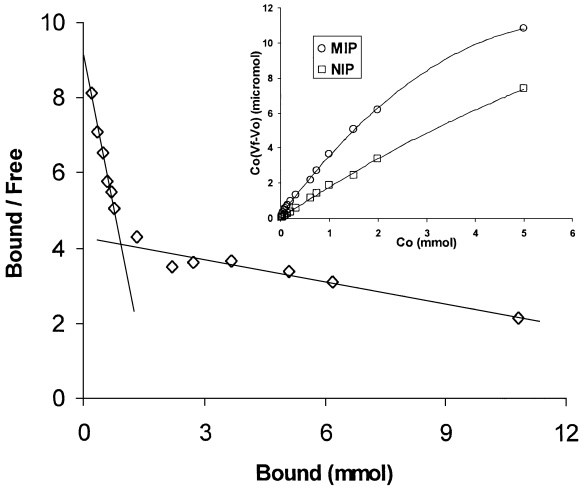

Fig. 2. Scatchard plot of the binding of phenytoin to the MIP. Inset: The adsorption isotherms obtained for NIP and MIP polymers using frontal analysis.

values correspond to 2.5 and 27% of the theoretical total binding sites, respectively, calculated from the amount of the template used for polymerisation. The corresponding numbers on the NIP were 550.7 $M^{-1}$and 6.6 μmol/g polymer. More detailed results will be discussed elsewhere.

3.2. Optimisation of the MISPE procedure

Since we intended to extract and measure phenytoin from biological samples the effect of water content of the acetonitrile mobile phase on the chromatographic retention of phenytoin was first investigated. A 1 m*M* phenytoin solution prepared in the mobile phase was injected onto MIP and NIP columns, using mobile phases with different aqueous contents. In agreement with previous observations, the water content of acetonitrile dramatically reduced the retention time of phenytoin on the MIP column, while having practically no effect on NIP. Thus a decrease in the retention time from 13.1 to 5.9 min and further down to 3.1 min was observed for the MIP column when the water content in the acetonitrile was increased from 0 to 2.5 and 5%, respectively. Therefore, drying the cartridge is necessary before the washing step. However, when phenytoin was loaded in aqueous solution, a 10 min drying step (passing air through the cartridges) followed by washing with 0.5 ml acetonitrile resulted in the removal of 41% of the phenytoin. By comparison, under similar conditions only 7% of phenytoin was removed when loaded in acetonitrile. Thus water is not sufficiently removed in a 10 min drying step, and the phenytoin is weakly retained as was predicted from the HPLC measurements.

The next step in method development was to optimise the washing procedure. Several washing solvents (acetonitrile, acetonitrile–THF, nitromethane, DCM, hexane, toluene, chloroform, acetone) were applied in order to find the solvent resulting in maximum selectivity and recovery of the analytes. The sample application step was performed by loading 25 μg phenytoin dissolved in a small volume (50 μl) of the respective washing solvent. In this way phenytoin was sorbed to the top of the cartridge. Washing with the various solvents was carried out in several steps collecting fractions of the solvent. The percentage of phenytoin relative to the

total eluted amount was calculated in each fraction and the cumulative recovery was plotted against the volume of the washing solvent. The resulting graphs for phenytoin eluted from the NIP and MIP columns using different washing solvents (acetonitrile, acetonitrile–THF, nitromethane and DCM) are presented in Fig. 3a and b.

DCM was the most effective in disrupting non-specific interactions occurring between phenytoin and the polymer (see Fig. 3a), while keeping the specific binding between the analyte and the binding sites in the MIP intact (Fig. 3b). A 2-ml volume of DCM can be used to wash the polymer after sample application without removing the analyte from the cartridge. Only small differences in elution profiles were obtained on NIP and MIP cartridges in the case of acetonitrile, nitromethane and the porogenic solvent acetonitrile–THF (76:24) as washing solvents.

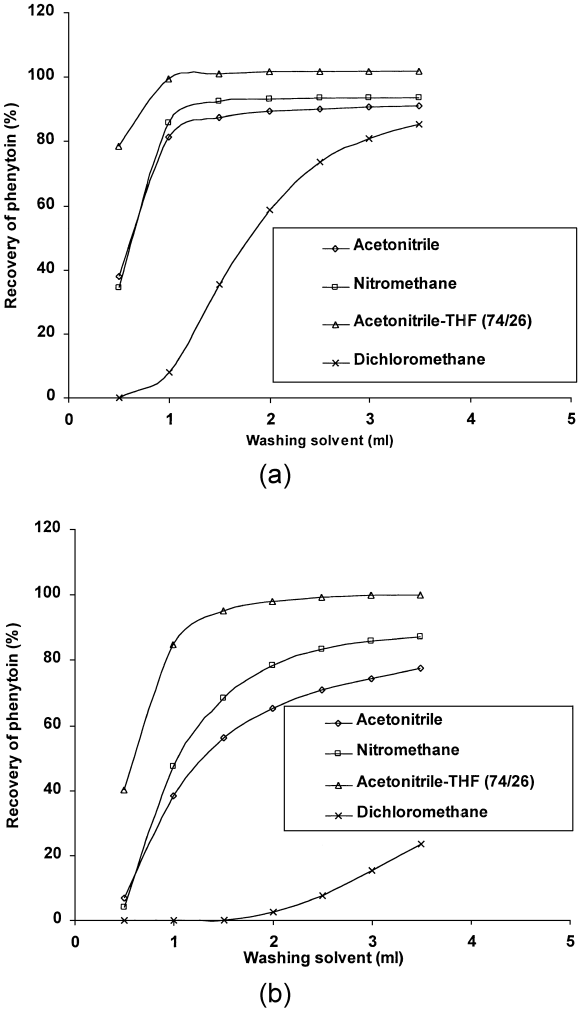

Fig. 3. Recovery of phenytoin after percolation of increasing volumes of different wash solvents through an NIP (a) and an MIP (b) column.

In the case of acetone and chloroform as washing solvents, the recovery of phenytoin versus volume of wash solution on the NIP and MIP were similar. Phenytoin was washed from the cartridges with the first aliquot of the solvents both on NIP and imprinted cartridges (data not shown). Using toluene and hexane washing solvents phenytoin was completely retained on both NIP and imprinted cartridges.

Methanol was chosen to remove any remaining adsorbed material at the end of each MISPE experiment, since we have found that after sample application phenytoin could be quantitatively removed with 2 ml methanol from the cartridge.

To investigate the influence of water residues on the cartridge when DCM (the optimal washing solvent) is used the following experiment was performed. An aqueous phenytoin sample (1 ml, 25 μg/ml) was loaded onto the MISPE column. After drying for 5 min, the cartridge was washed with 1 ml DCM, and then eluted with 2 ml of methanol. The results showed that after addition of 1 ml DCM most of the phenytoin still remained on the MIP column (only 5% was removed), whereas 37% of the applied phenytoin was removed from the NIP column. Again a higher percentage of the phenytoin was removed when the sample application was done in aqueous media. However 5% loss of the analyte during SPE when 1 ml DCM is used is acceptable in the bioanalytical practice. Another question about the application of DCM as washing solvent is whether it removes other components from the sample matrix. This could be found out by loading an ‘‘artificial sample’’ onto the cartridge the components of which mimic specific properties of the analyte. For this purpose 1 ml of an aqueous solution containing 10 μg/ml each of hydantoin, 5,5-dimethylhydantoin, ametryne and atrazine and 3% acetonitrile was loaded on the MIP and NIP cartridges, respectively. The cartridges were then washed with 1 ml DCM. The results obtained showed, that after elution of 1

ml DCM the components of the ''artificial sample'' were eluted up to 90–95% both from the NIP and MIP cartridges. This means that by using 1 ml DCM it is possible to remove organic interferences from the sample even if they have very similar structure or similar lipophilicity as the analyte. Meanwhile phenytoin is retained on the MIP. For this reason 1 ml DCM was selected as the washing solvent for the sample pre-treatment.

Based on these results the sample pre-treatment procedure described in the experimental part was applied to extract phenytoin from plasma samples using the MISPE columns.

3.3. Validation of the MISPE system

To prove the analytical applicability of our MISPE sample preparation procedure, validation of phenytoin determination in blood plasma was carried out.

Linearity was studied by analysing spiked plasma samples at six concentrations in the 0–40 μg/ml concentration range. ($y=0.0798x+0.0373$; $R^2=0.999$). Intra-day precision was determined by measuring five replicate phenytoin quality control samples at different concentration levels. The intra-day precision values are between 1.5 and 2.6% (Table 2). Inter-day precision was determined on 5 different days measuring phenytoin plasma quality control samples at different concentration levels. In this case the precision values fall between 7.4 and 12.8% (Table 2). In both cases the precision was calculated as the relative standard deviation of the parallel results.

Table 2
Intra-day ($n=5$) and inter-day ($n=5$) precision data for phenytoin determination from plasma using the MISPE system

Nominal concentration (μg/ml)	Mean found (μg/ml)	RSD (%)
Intra-day validation data ($n=5$)		
7.5	6.4	1.5
25	22.2	1.7
30	26.6	2.6
Inter-day validation data ($n=5$)		
2.5	2.5	12.2
7.5	7.4	11.3
25	23.8	7.5
30	28.6	12.8

The recovery of the MISPE extraction was determined by comparing the detector response of an extracted sample with that of a directly injected aqueous standard. Recoveries were determined at three concentration levels and fall between 58 and 78%. Specificity of the method elaborated was also investigated. Six different blank plasma samples were tested using the MISPE procedure followed by HPLC measurement. Comparing the chromatograms shown in Fig. 4a–c, it can be concluded that there is no interfering peak at the retention time of the analyte and the internal standard in the blank sample. This indicates that in the concentration range studied, bleeding of the polymer sorbent (i.e., release of residual template from the polymer matrix) is not noticeable.

4. Conclusions

Molecularly imprinted polymer was prepared using phenytoin as template and methacrylamide as functional monomer. The results presented here indicate that the polymer exhibited highly selective binding for phenytoin. The selectivity of the imprinted polymer was investigated using phenytoin and it's metabolite, several structurally related compounds and compounds having similar lipophilicities to phenytoin. Study of the recognition properties of the imprinted polymer in different solvent media was carried out and the role of water in the recognition process investigated. The results indicate that careful choice of the solvents can disrupt non-specific binding, allowing a specific extraction of the template. A procedure for phenytoin determination from plasma samples using the molecularly imprinted polymer as solid-phase extraction sorbent was elaborated. The applicability of the procedure was proved by validation of the method. The results indicate that our MISPE method is applicable for plasma phenytoin level determination in the therapeutic range.

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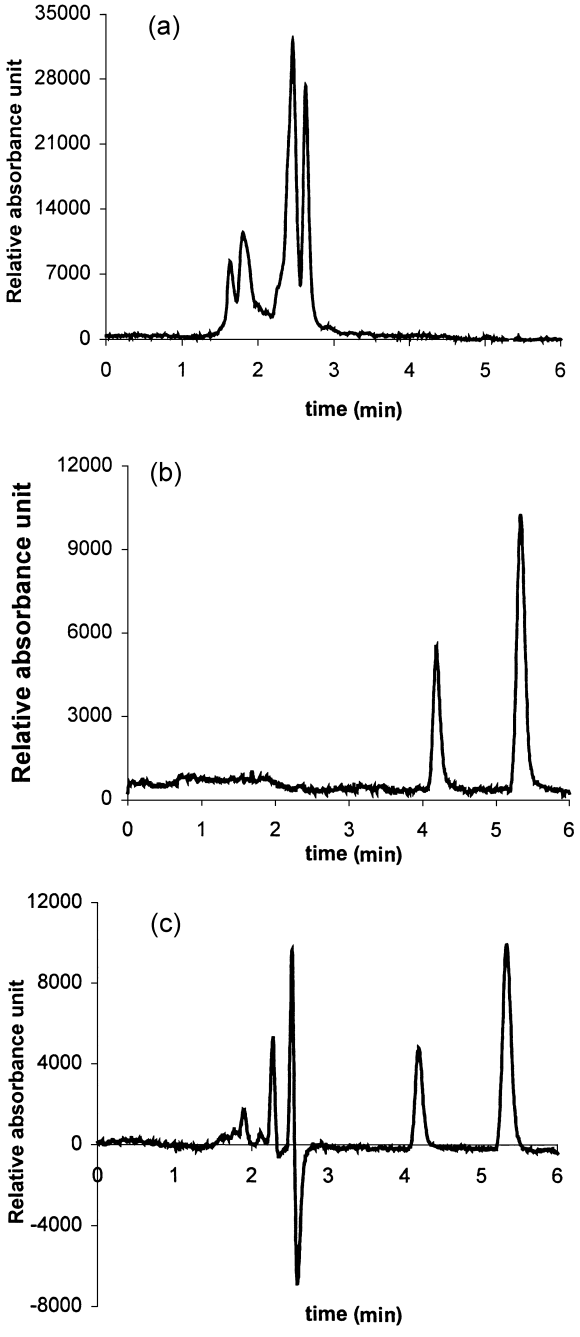

Fig. 4. Chromatogram of a blank plasma sample (a) an aqueous phenytoin solution: 5 μg/ml phenytoin (t_R=4.19 min); 20 μg/ml internal standard (t_R=5.33 min) (b) and a spiked plasma sample: 5 μg/ml phenytoin (t_R=4.19 min); 20 μg/ml internal standard (t_R=5.33 min) (c).

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