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# Preparation of a Novel Emulsion-Templated MIP Monolith and its Application for on Line Assay of Nifedipine in Human Plasma

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## Abstract

A novel nifedipine molecularly imprinted polymer (MIP) monolith was prepared by radical polymerization of the water in oil (W/O) emulsions, using vinyl ester resin and methacrylic acid (MAA) as the monomers and nonionic surfactants such as Polyethylene glycol 400 (PEG 400) and Pluronic F68 (PF 68) as the emulsifying agents. The properties of the material were investigated. The MIP monolithic column was installed in the high performance liquid chromatography (HPLC) system and used as the selective sorbent for on line solid-phase extraction of nifedipine in human plasma due to the good selectivity to nifedipine. For this method, the calibration curve was linear in the concentration range of 5-150 ng/mL ($r^2$=0.998) for nifedipine in human plasma and the limit of detection (LOD) was 2 ng/mL. Accuracy, precisions and recovery was acceptable for screening nifedipine in plasma samples. The results indicated that the novel material could be used as pre-column for on line clear-up and screening of nifedipine in plasma samples, which provided a simple and rapid assay of the drugs in plasma.

**Keywords:** Emulsion; MIPs; Monolithic column; Solid-phase extraction; Nifedipine

**Abbreviation:** W/O: Water in oil; MAA: Methacrylic Acid; PEG 400: Poly Ethylene Glycol 400; PF 68: Pluronic F68; MIPs: Molecularly Imprinted Polymers; SEM: Scanning Electron Microscopy; HPLC: High Performance Liquid Chromatography; LOD: Limit of detection; GMA: Glycidyl Methacrylate; EGDMA: Ethylene Glycol Dimethacrylate; DEA: diethylamine; MISPE: Molecularly Imprinted Solid-Phase Extraction; BADE: Bisphenol A Diglycidyl Ether

## Introduction

Nowadays, polymeric monoliths hold an impressively strong position due to excellent properties in comparison with conventional chromatographic supports and extensive applications such as biological tissues caffolds [1], catalysis supports [2], ion-exchange resin [3] and separation media [4]. In recent years, emulsion templating polymerization technology has been introduced in the preparation of monolith due to well defined open porous material that has good mechanical strength and possess favorable stability. Emulsions are colloidal systems made of liquid droplets dispersed in another liquid phase, which are produced by shearing these two immiscible liquids to reach a metastable state through fragmentation of one phase into the other with various surfactants [5]. Moreover, the preparation of water-in-oil emulsions containing polar monomers requires a careful selection of the surfactant and the formulation of the continuous phase. Krajnc P et al. [6] synthesized high internal phase emulsion templated monolith using glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EGDMA) and surfactant PEL121. Then the column was modified with diethylamine (DEA) to separate four standard proteins successfully.

The molecular imprinting technique is an effective strategy for preparing stationary phases with special molecular recognition properties. Wulff G et al. [7] first synthesized imprinted polymers by means of free radical copolymerization. After that, molecular imprinting technique has undergone rapid development in 1990s. Because of the advantages over biopolymers, such as low cost, good physical and chemical stability and tailor-made selectivity for a target molecule, the MIPs materials have been widely applied in solid-phase extraction [8], biosensor [9], chromatographic separation and analysis [10-12].

Nifedipine (Figure 1), a dihydropyridine calcium channel antagonist, is widely used in the treatment of hypertension and other cardiovascular disorders [13]. With the development of modern technology, new preparation formulation of nifedipine, such as solid dispersion [14], sustained-release or controlled-release formulation [15], gradually hold leading position in clinical medication and expand the clinical application of nifedipine. Consequently, efficient and fast quantification of nifedipine plays an important part in analysis of the drug in blood. Numerous methods have been reported for the quantitative determination of nifedipine in plasma, including gas chromatography combined with different detectors, HPLC coupled with varied detectors and recently combined with solid

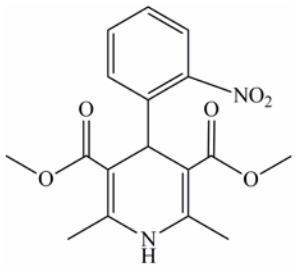


**Figure 1:** The structure of nifedipine.

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phase extraction [16-18]. Given that nifedipine is a highly unstable compound, which is rapidly photo-degraded to its nitroso analogue when exposed to daylight [19] and its plasma protein binding rate reached 98%, on line solid-phase extraction is suitable for the pre-treatment of plasma sample instead of liquid–liquid extraction and traditional off-line SPE. But the common material for on line SPE is a commercially available pre-column, which is not cost-effective and selective enough to detect nifedipine. Molecularly imprinted solid-phase extraction (MISPE) offers a higher degree of selectivity for clean-up and enrichment steps. Nevertheless, Lanza et al. [20] synthesized nifedipine MIPs, but failed to adopt it in the assay of nifedipine in plasma.

In this work, an alternative method has been developed for preparing molecularly imprinted monolithic materials by polymerization of the W/O emulsions. In the polymerization, the vinyl ester resin and methacrylic acid were used as monomers. The properties of the material were investigated and the column exhibited good stability and selectivity to nifedipine, then the monolith was used as the SPE pre-column for on line assay of nifedipine in human plasma coupled to a HPLC-UV system, which overcame the disadvantages in traditional off -line SPE and enabled clean, fast, efficient analysis for plasma samples.

## Materials and Methods

### Reagents and material

Bisphenol A diglycidyl ether (BADE) and tetrabutyl ammonium bromide were purchased from Tianjin Chemicals Co., Lmt. (Tianjin, China). EGDMA was purchased from Acros (New Jersey, USA). MAA and 1, 4-dioxane was purchased from Tianjin Jinli chemicals Co., Lmt. (Tianjin, china). PF 68 was obtained from Shenzhen Youpuhui Chemical Co., Ltd. (Shenzhen, China). PEG 400 was purchased from Beijing Huaboyuan Science and Technology Development Center (Beijing, China). Potassium persulfate was purchased from Huadong Chemical Reagents Factory (Tianjin, China). Anhydrous calcium chloride was purchased from Tianjin Beifangtianyi Chemical Reagents Factory (Tianjin, China). Nifedipine reference substance (purity 99.6%) was obtained from CSPC ZhongNuo Pharmaceutical Co., LTD (Shijiazhuang, China). Methanol (analytical grade) was obtained from Kermel Chemical Co., Ltd. (Tianjin, China). Water was purified by a Millipore system. All solutions were filtered through 0.45 $\mu$m membrane filters (Millipore) before use.

Human plasma, donated by volunteers, was obtained from the Hospital of Hebei University and was centrifuged at 4500 rpm for 10 min, then stored under -18°C before use. The institutional review board approval from Hospital of Hebei University has already been obtained for the acquisition of plasma from human subjects.

### Synthesis of vinyl ester resin

Vinyl ester resin was used as one of the monomer. First, it was prepared according to the procedure described in literature [21]. The process was as follows: 10 g of BADE, 0.2 g of tetrabutyl ammonium bromide and 10 milliliters of 1, 4-dioxane was put into a three-necked flask which was heated in a hot up set. 4.3 milliliters of methacrylic acid was dropped in when the temperature was up to 80°C. Then the temperature was raised up to 90°C and kept for 4.5 h. The vinyl ester resin was synthesized. The synthesis scheme was shown in Figure 2.

### Preparation of molecularly imprinted monolith

Five hundred microliters of vinyl ester resin, one hundred microliters of MAA, eight hundred microliters of EDMA and nine hundred microliters of PEG 400 were placed in a round-bottomed flask and the mixture was stirred with an overhead stirrer at 400 rpm to form a homogeneous phase. Then, three hundred microliters of methanol containing 0.006 g nifedipine was added as the template dropwise with stir. After that, the aqueous phase consisting 1.5 milliliters of initiator (0.4 % potassium persulfate in deionised water), 375 milliliters of electrolyte (2% anhydrous calcium chloride in deionised water) and 0.06 g of surfactant PF 68 was added dropwise in the oil phase slowly. Stirring was continued for another 45 minutes and a white-milky emulsion was formed. All of the above steps were conducted in the absence of light.

The emulsion was transferred to a stainless-steel tube of chromatographic column of 50 × 4.6 mm i.d. and baked at 55°C for 36 h. After cooling to room temperature, the column was connected with the HPLC system to remove the surfactant and soluble compounds by pumping deionized water (60 mL) and methanol (30 mL) through the column. Then, methanol- acetic acid (4:1, v/v) was used to wash the template for 12 hours and methanol was used to wash the acetic acid subsequently. Thus a novel MIP monolithic column was obtained.

For comparison, blank polymer (NIP) was prepared as mentioned above with the same procedure but in the absence of the template nifedipine.

### Characterizations of the MIP monoliths

The monoliths, synthesized and washed in the former steps, were cut into small pieces followed by drying at 60°C overnight. Morphology of the dried monolith samples was observed by scanning electron microscopy (SEM). FT-IR spectra were recorded on a FT-IR-8400 spectrometer (Shimadzu, Japan).

### Chromatography

**Instruments and conditions:** Jasco HPLC system (Jasco Co., Japan) equipped with a PU-1580 pump and a variable-wavelength UV-1570 detector was used for analyses. Data processing was performed with an HW-2000 chromatography workstation (Nanjing Qianpu Software, China).

The pre-column was uesd as a pre-column and a $C_{18}$ diamonsil™ column (150 × 4.6 mm i.d., 5μm, Dikma, NY, USA) was used as the analytical column to detect nifedipine at the temperature 25°C.

The mobile phase for separation and analysis was methanol-water (80:20, v/v) at a flow-rate of 1.0 mL/min. The analytes were monitored at a wavelength of 235 nm, which was the maximum absorption wavelength of nifedipine.

**Standard solutions preparation:** First, nifedipine was dissolved with methanol to prepare stock solution of 150 ng/mL. Working solutions of concentration 2.5, 5, 12.5, 25, 37.5, 50, 75μg/mL were prepared from the stock solution. These working solutions were then

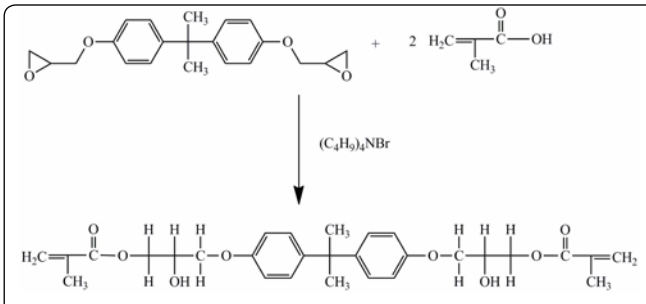


**Figure 2:** Synthesis of vinyl ester resin.

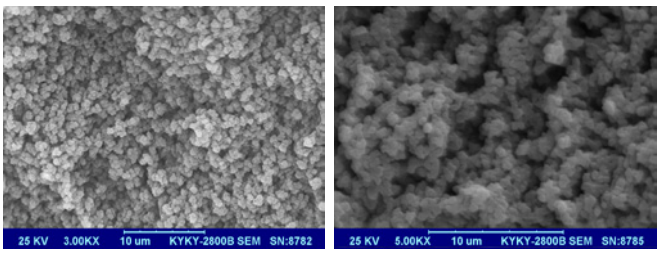


**Figure 3:** SEM of the MIPs.

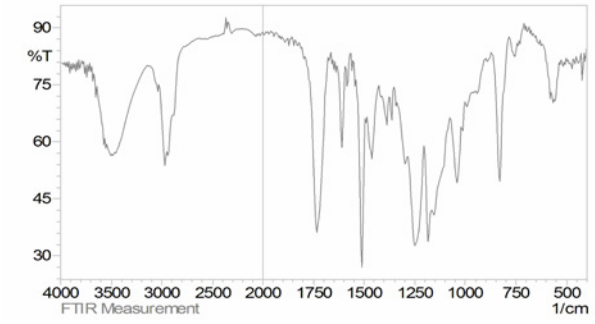


**Figure 4:** FT-IR spectrum of the monolithic material.

diluted with blank human plasma to obtain standard solutions with concentration of 5, 10, 25, 50, 75, 100, 150 ng/mL, respectively.If not used immediately, the stock solution and other samples were all stored in the absence of light and at -20°C before use.

**On-line SPE:** The monolithic column was used as an SPE column, which was placed in the sample-loop position of the injection valve and used for deproteinization and retaining nifedipine in plasma. In the "load" position, 5μL of plasma samples were directly injected into the SPE column and were washed with 3 mL of water. Then the valve was switched to the "injection" position and nifedipine was desorbed by backflushing with methanol-water (80:20, v/v) and transferred to the analytical column ($C_{18}$).

**Precision, accuracy and recovery studies:** Quanlity control samples at three different concentration levels of 5, 50, 150 ng/mL (low, medium and high) were prepared for the evaluation of precision, accuracy and recovery.

Precision of the method could be expressed as intra-day and inter-day variability in the concentration ranges of nifedipine in plasma samples and was evaluated by the relative standard deviation (RSD).

The accuracy of this method was expressed as relative error (RE). Relative error = [(found concentration - nominal concentration) / nominal concentration] × 100%. Found concentration was determined by calibration curve according to the procedure on-line SPE. The nominal concentration was the spiked concentration.

Intra-day precision and accuracy were determined by five replicated injections of each three nifedipine plasma samples (5, 50 and 150 ng/mL) within the same day. Inter-day precision and accuracy were tested over five consecutive days for the same samples in intra-day precision test.

The absolute recoveries of nifedipine were determined by comparing nifedipine peak area obtained by HPLC analysis of spiked plasma samples after SPE pretreatment to that obtained by direct injection of nifedipine dissolved in methanol without SPE pretreatment.

Relative value of the concentrations determined by calibration curve according to the procedure on-line SPE to the real concentrations was calculated and its percentage form was set as the relative recovery. All analyses were performed five times.

### Stability of nifedipine samples

The stability of nifedipine stock solution and plasma samples at experimental conditions were carried out, including freeze and thaw stability and long-term stability.

First, stock solution and spiked plasma samples of 5, 50, 150 ng/mL nifedipine were analyzed by HPLC before storing. Then they were freezed at -20°C and thawed at room temperature for three cycles. Long-term stability was tested after one month stored at -20°C.

## Results and Discussion

### Preparation of MIP monolith

The formation of stable emulsion was extremely essential in the whole polymerization process. The factor played important part in formation of stable emulsion included ratio of oil phase and water phase, sequence and rate to add liquid, agitating rate and kind of surfactant. Multiple experiments confirmed the optimal condition were as followed: 400 rpm agitating, PEG 400 and PF 68 as surfactant. The emulsion could keep stable for at least 36 hours without delamination before the polymerization completed.

In the polymerization of MIPs, nifedipine was used as the template molecule, vinyl ester resin and MAA were used as the dual monomers, among which MAA was the functional monomer to provide carboxyl group to form hydrogen bonding interactions with NH group of nifedipine and vinyl ester resin was the functional monomer to form hydrogen bond with ester group of nifedipine. However, the recognition ability of nifedipine was low when a single monomer was used in the preparation of MIPs, but it was improved when dual monomers were used to the polymerization process, as reported in [22].

In the conventional bulk polymerization of MIPs, the porogenic solvent governs the strength of non-covalent interactions and non-polar solvents are favorable for most polymerization of MIPs. Aqueous solutions can interfere with the imprinting process and the selectivity is still not of completely satisfactory. Moreover, if the water content during preparation is further increased, the monolith would become flexible and provide poor mechanical strength [23]. In this experiment, emulsion templating polymerization could overcome the problem. Once the ratio to form a stable milky emulsion was established, the nifedipine molecularly imprinted monolith could be synthesized in water containing system with surfactant PEG 400 and PF 68 and the selectivity and mechanical strength were not weaken correspondingly, which made the MIP monolith capable of purification in aqueous solution.

### Characterizations of the MIP monolithic column

**Morphology and permeability:** The morphology of the monolith was studied by SEM and pictures of different magnifications were shown in Figure 3.

The SEM micrographs showed the multiple micro-pores and through-pores formed by emulsion templating polymerization and pores were well-distributed in the monolith.The average pore diameter of MIPs was 0.65μm. The large pores in monolith facilitated the polymeric ligand, such as the recognition sites for template, to access to the surface of the media and might relate to the good permeability of the monolithic column.

The monolith was further characterized using FT-IR spectroscopy on all samples to confirm their chemical structure (Figure 4). The broad peak around 3500 $cm^{-1}$ indicated the stretching vibration of -OH belonging to poly (vinyl ester resin) and poly MAA. The strong absorption observed at 1720 $cm^{-1}$ was due to the C=O of carboxyl group. The existing groups of –OH and–COOH provided the hydrogen bond binding sites for nifedipine in plasma samples.

The permeability of the material was tested by connecting it to the HPLC pump. The backpressure at different flow-rates was recorded in water and methanol and their relation was studied, as illustrated in Figure 5. Good linearity ($r_1$=0.9992, $r_2$=0.9996) between the pressure drop and the velocity of mobile phase proved that the porous monolith was stable and not compressed at high flow-rate, which manifested the potential for fast analysis.

**The tailor-made selectivity of MIPs for nifedipine:** The tailor-made selectivity of the MIPs to nifedipine was studied using the mixture sample of nifedipine and its analogue nisoldipine at the wavelength of 235nm.With the increasing of water content, retention time of nifedipine was prolonged while that of nisoldipine kept invariably at dead time. When the mobile phase was methanol-water (80:20, v/v), the baseline separation between nifedipine and nisoldipine could be achieved and the resolution ($R_s$) was 2.68 under the condition. Figure 6 was the separation chromatogram of nifedipine and nisoldipine. At the same time, the retention of the compounds on blank NIP was also studied. The results showed that both nifedipine and nisoldipine were eluted quickly and simultaneously and no separation ability was observed. So the MIPs had significant molecular imprinting effect and had high affinity and selectivity for nifedipine in water-containing systems, thus could be used as SPE pre-column.

**Stability and reproducibility of the material:** In methanol-water mobile phase system, multiple injections operated on the column seemed to affect the performance scarcely. At the same condition, the migration time and peak area of nifedipine after multiple injections manifested reproducible data for the same column (0.5%, 0.8% RSD, respectively, n=11), which indicated the good ruggedness of the monolith.

For different columns synthesized by the same processes, the data, % RSD of migration time and peak area of nifedipine were 1.1 % and 1.8 % respectively (n=11), showed fine reproducibility between batches of the material.

## On line assay of nifedipine in plasma sample

**Study of plasma sample stability after pretreatment and chromatography:** Firstly, deproteinization ability was tested by directly injecting 5μL of blank plasma into the SPE column and eluted with pure water at the wavelength of 280 nm (Figure 7 a). The elimination of the biological matrix could be considered completely eluted when the detector signal reached the baseline. The experiment showed that a washing volume of 3 mL was sufficient for sample clean-up. Then, 5μL of 50 ng/ml nifedipine solution (nifedipine dissolved in methanol) was injected directly into the column at the same condition as above. The chromatogram (Figure 7b) showed that nifedipine could not be eluted at all when pure water was used as mobile phase. However, when methanol was used as mobile phase, nifedipine was eluted quickly from the monolithic column in two minutes. Therefore, during the washing process (washing liquid was water), biological matrix was simply removed and nifedipine was well retained on the SPE column, which illustrated the ability of the column for plasma sample pretreatment.

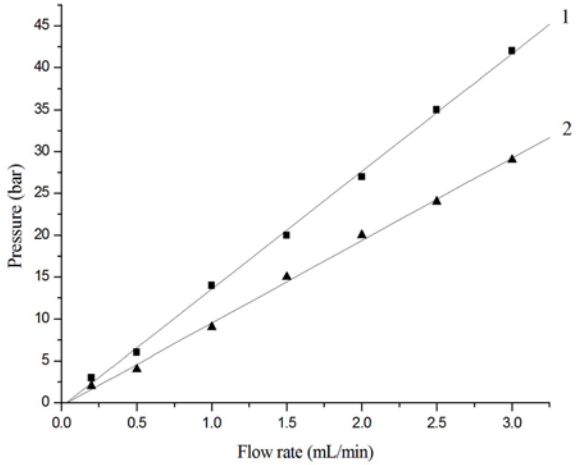


**Figure 5:** Dependence of the back pressure on the flow-rate.

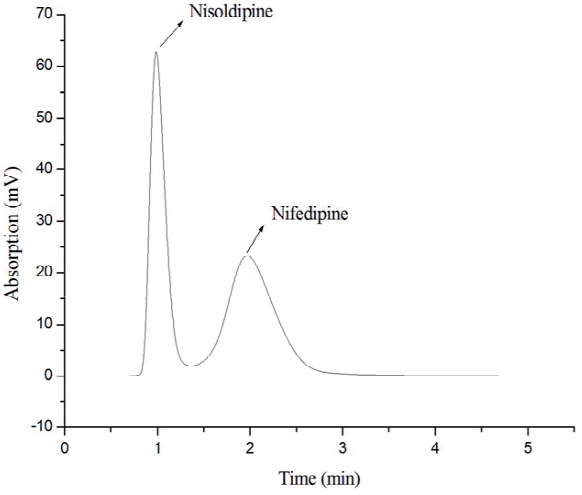


**Figure 6:** Separation Chromatogram of nifedipine and nisoldipine

The chromatographic conditions were also investigated. Satisfactory separation could be achieved by the use of mixed solution of methanol and water as mobile phase. When the proportion of methanol was increased, the drug could be eluted more quickly. Considering the retention time and separation effect, methanol-water (80:20, v/v) at a flow-rate of 1.0 mL/min was selected for analysis. Typical chromatogram resulting from the HPLC-UV analysis of plasma sample after on line SPE was depicted in Figure 8b. Compared with chromatogram of standard solution of nifedipine in Figure 8c, the migration time of nifedipine was approximately 5.118 minutes and the peak of nifedipine showed good separation from matrix peaks. Chromatogram of blank plasma sample (Figure 8a) was used to determine whether there were any interfering peaks around the migration time of nifedipine. The result showed that there was no interference from endogenous compounds around the peak of nifedipine under the optimized conditions, reflecting the high specificity and selectivity of the described method.

**Calibration:** Calibration curve was calculated by linear regression analysis of the peak area of nifedipine versus its concentrations (ng/mL) in plasma. Standard plasma samples with seven different concentrations (5, 10, 25, 50, 75, 100 and 150 ng/mL) of nifedipine were analyzed to obtain the intra-day calibration curve. Each sample

was injected for three times. Each level of the calibration curve was analyzed in triplicate in the following four days. The peak area-concentration showed a linear relationship over the range of 5-150 ng/mL for plasma samples. The mean equation of the calibration curve obtained from seven points was y = 734.7 x - 745.2 (n = 5 days) with a determination coefficient of $r^2 = 0.998$. The RSD for the slope, intercept and $r^2$ for linear regression model obtained on five different days was 2.45, 1.98 and 1.35%, respectively.The limits of detection (LOD) and quantitation (LOQ) calculated at a signal-to-noise ratio of 3 and 10 were determined as 2 and 5 ng/mL, respectively. The back-calculated concentrations of the different levels of samples by the intra-day calibration curve were within the acceptance criteria.

## Precision, accuracy and recovery

The intra-day (n = 5) and inter-day (n = 5) precision and accuracy were evaluated from the calibration curve results. The results of intra-day and inter-day precision and accuracy were given in Table 1 and Table 2, respectively. For plasma quality control samples at low, medium and high levels of nifedipine investigated, RSD for intra- and inter-day precision was found to be 4.29-6.16%, indicating good repeatability of this method; Relative error for intra- and inter-day accuracy was less than 4.83% and it was obvious that the method was remarkably accurate which ensures obtaining of reliable results.

The recoveries determined at three different concentrations (5, 50 and 150 ng/mL) were shown in Table 3.The recoveries included the relative recovery and absolute recovery, among which the relative recovery was also viewed as method recovery and the absolute recovery was regarded as extraction recovery. The result for relative recovery of nifedipine at three concentrations was 95.17, 96.62 and 94.83%, respectively, while that for absolute recovery was 83.92, 85.63 and 88.26%, respectively.

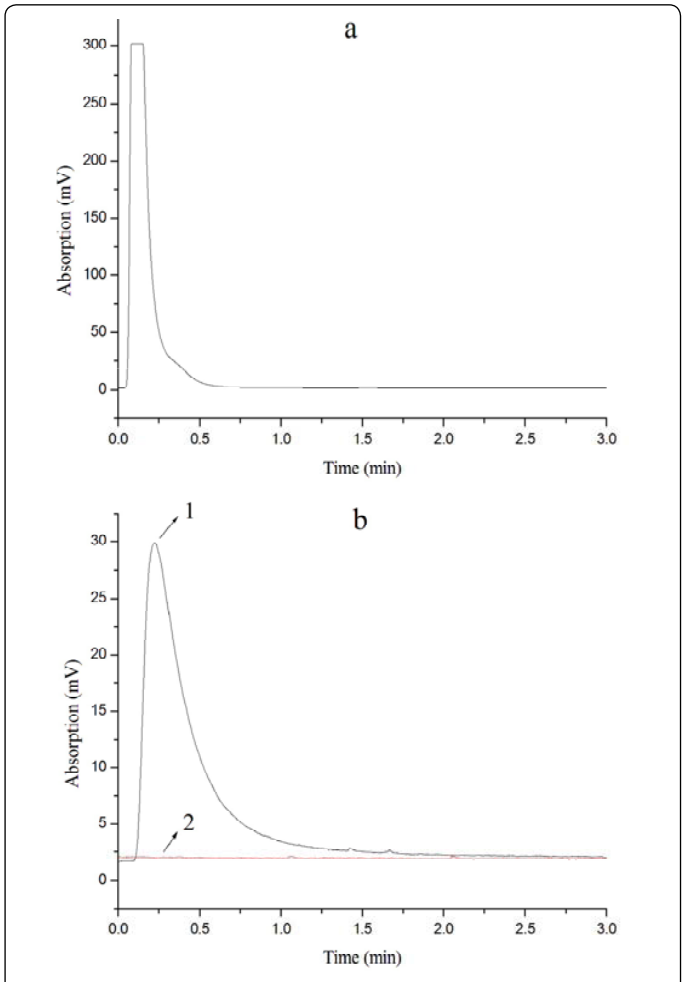


**Figure 7:** Chromatograms of the deproteinization and enrichment of nifedipine with the monolithic column.

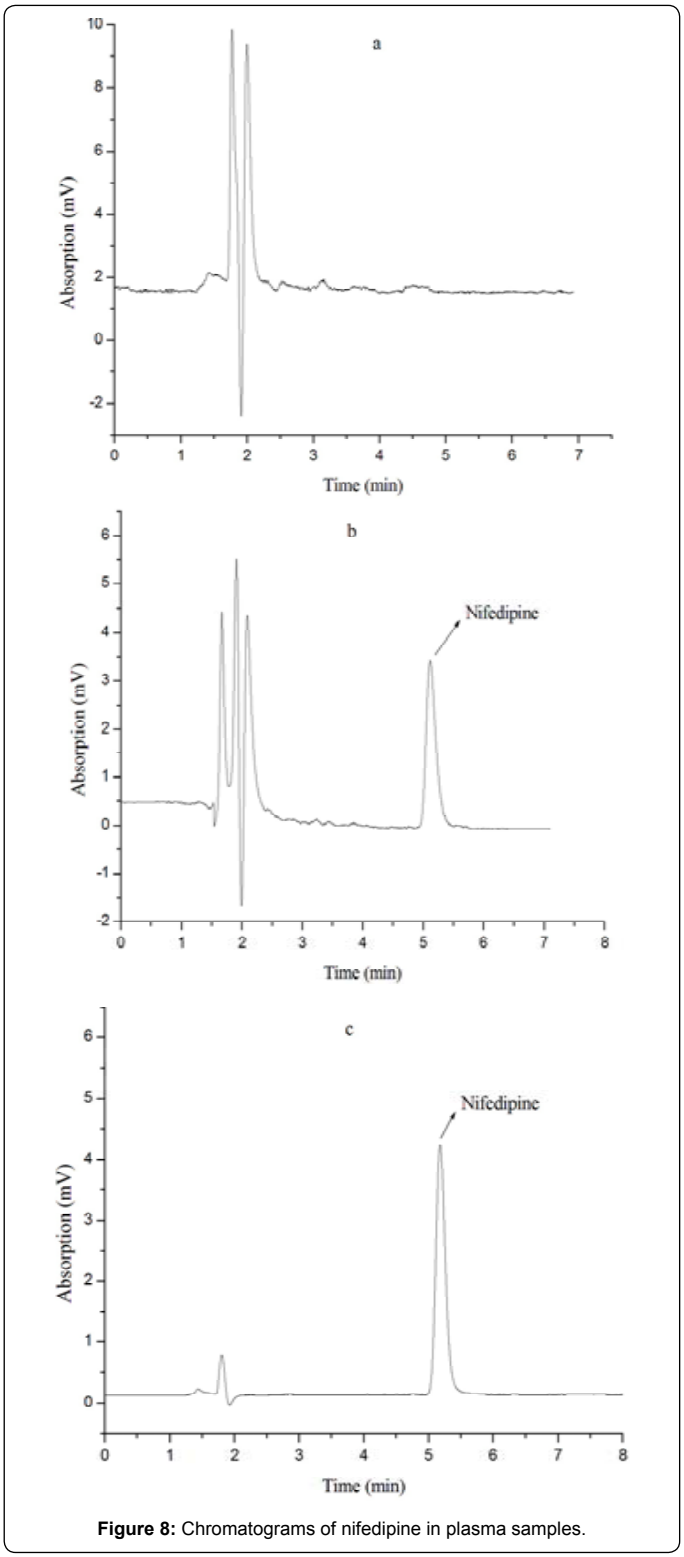


**Figure 8:** Chromatograms of nifedipine in plasma samples.

| Nominal Concentration (ng/mL) | Found concentration (ng/mL) | Precision RSD (%) | Accuracy RE (%) |
|---|---|---|---|
| 5 | 5.24 ± 0.23 | 4.48 | 4.83 |
| 50 | 51.69 ± 2.84 | 5.50 | 3.38 |
| 150 | 153.2 ± 6.57 | 4.29 | 2.13 |

Data were expressed as mean ± SD (n = 5)

**Table 1:** The intra-day precision and accuracy of nifedipine in human plasma samples for validation.

| Nominal Concentration (ng/mL) | Found concentration (ng/mL) | Precision RSD (%) | Accuracy RE (%) |
|---|---|---|---|
| 5 | 5.21 ± 0.28 | 5.54 | 4.12 |
| 50 | 51.16 ± 3.15 | 6.16 | 2.32 |
| 150 | 147.2 ± 7.90 | 5.37 | -1.84 |

Data were expressed as mean ± SD (n = 5)

**Table 2:** The inter-day precision and accuracy of nifedipine in human plasma samples for validation.

| Spiked concentration (ng/mL) | Relative recovery (%) | Absolute recovery (%) |
|---|---|---|
| 5 | 95.17 ± 3.62 | 83.92 ± 4.31 |
| 50 | 96.62 ± 1.17 | 85.53 ± 2.26 |
| 150 | 94.83 ± 1.89 | 88.26 ± 1.87 |

Data were expressed as mean ± SD (n = 5)

**Table 3:** The recovery of nifedipine in human plasma samples.

## Stability of the samples

Stability was determined by comparing the nominal concentration of nifedipine in plasma samples and the test samples. The result showed that the recovery for tested samples at different concentrations decreased less than 6.6%, but there was no significant degradation observed after three freeze/thaw cycles and one month stored at -20°C, indicating that nifedipine added to plasma were stable in different storage conditions.

## Conclusions

In summary, an alternative method to prepare molecularly imprinted monolithic materials was developed. The permeability of the column was high and the column could be used for rapid analysis at high flow rate. The MIPs had significant molecular imprinting effect and had high affinity and selectivity for nifedipine in water-containing system. It was successfully used as on-line SPE material to deproteinization and screening nifedipine in human plasma. The method required microamount of samples, which could be directly injected into chromatography system without tedious pretreatment of samples. The results suggested that on line SPE applying the MIP monolith as pre-column could be considered as a simple, cheap, effective and friendly to environment method for assaying drug in plasma sample.

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