

# On-Line Solid-Phase Extraction Based on Poly (NIPAAm-MAA-co-EDMA) Monolith Coupled with High-Performance Liquid Chromatography for Determination of Nitrendipine and Nisoldipine in Human Urine

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## Abstract

A solid-phase extraction absorbent material based on poly (N-isopropylacrylamide-co-methacrylic acid-co-ethylene glycol dimethacrylate) [poly (NIPAAm-MAA-co-EDMA)] monolithic column was developed for the simultaneous determination of nitrendipine and nisoldipine from human urine. The morphology of monolithic column and pressure drop across the columns were characterized. The results showed excellent permeability and high selectivity.

Urine samples (0.05 mL) were injected directly onto the prepared Solid-Phase Extraction (SPE) monolithic column. Most biological matrix compounds in urine were removed by on-line SPE technology, while nitrendipine and nisoldipine retained on the SPE column were effectively separated on a C18 analytical column. For all analytes, linear calibration curves were obtained over a range of 50-500 ng/mL with coefficient of correlation > 0.9996. Accuracy and precision for inter- and intra-day assay showed acceptable results for quantitative assay with Relative Standard Deviation (RSD) less than 10%. The recovery was found to be in the range of 89-102%. The results indicated that the prepared monolith was feasible to be used as an on-line SPE sorbent material and the method was especially appropriate for multi-analytes monitoring in urine samples.

**Keywords:** P (NIPAAm-MAA-co-EDMA) monolithic column; On-line solid-phase extraction; Nitrendipine; Nisoldipine; Human urine

**Abbreviation:** AIBN: 2, 2'-azobisisobutyronitrile; AR: analytical reagent; EDMA: ethyleneglycol dimethacrylate; GC: gas chromatograph; HPLC: high-performance liquid chromatography; LOD: limit of detection; LOQ: limit of quantification; MAA: methacrylic acid; MS: mass spectrometry; NIPAAm: N-isopropylacrylamide; QC: Quality control; RSD: relative standard deviation; SEM: scanning electron microscope; SPE: solid-phase extraction

## Introduction

In analytical laboratory work, one of the most important factors for the successful quantitative analysis of biological samples prior to the high-performance liquid chromatography (HPLC) would have to be proper sample pretreatment [1]. As well known to all, sample preparation is one of the most laborious, time-consuming, and error-prone steps for the traditional methods [2]. In the recent years, SPE has been paying much attention for sample preparation [3-4], especially on-line SPE, because it not only overcomes many disadvantages in traditional methods e.g. protein precipitation [5-7], liquid-liquid extraction [8], but also incorporates the sample pretreatment and enrichment of organic analytes in the extraction and analysis steps, and there are minimal sample preparation steps required, only need sample mixing with the urine. Meanwhile, on-line SPE technique has high sensitivity, selectivity and cost-effective as well as environmental protection [9-11]. In view of these features, SPE column and analytical column (e.g. C18 commercial column) have been combined to apply in HPLC [12-14]. This method has already been successfully used for analysis of similar compounds [12-14]. Therefore, highly selective sorbents in SPE column have to become an important factor. Monoliths based on organic polymers and those based on silica have been developed intensively in liquid chromatography. Monoliths not only offer a relatively large surface area arising from its highly interconnected porous structure, but also have the unique properties of

easy preparation and high permeability for liquid biological samples at high flow rates [15,16]. For these advantages, monoliths are becoming increasingly popular as SPE sorbents [17].

Nitrendipine and nisoldipine (Figure 1) are dihydropyridine-type calcium antagonists. They are widely used in the treatment of cardiac dysrhythmias and hypertension [18]. However, these compounds containing of the nitro group can lead to reduction of the nitro group to the nitroso group under daylight [19]. In addition, they also involve the oxidation of the dihydropyridine ring to a pyridine ring [20]. These structural changes will result in decreasing of therapeutical activity and even some toxic effects. Therefore, a screening procedure is necessary for the detection of these drugs in biological fluids before quantization by HPLC. Many of analytical methods by using common sample pretreatment methods combined with HPLC, GC, GC-MS, LC-MS have been reported [21,22].

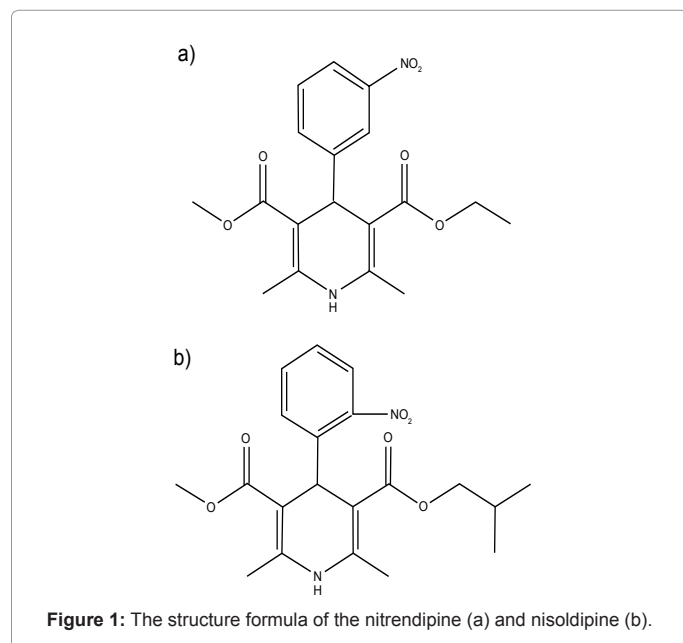
In this analytical work, a new poly (NIPAAm-MAA-co-EDMA) monolithic column was synthesized via in-situ free-radical polymerization. The micro morphology of monolithic column and pressure drop across the columns were characterized. Moreover, the new monolithic column was applied as SPE column combined with HPLC for simultaneous enrichment and determination of in urine.

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## Materials and Methods

### Chemicals and reagents

NIPAAm was purchased from Kohjin (Tokyo, Japan) and was purified by recrystallization in a benzene/ n-hexane mixture (3:7, v/v) and dried in vacuum. EDMA and MAA were purchased from Acros (New Jersey, USA). EDMA was purified by extraction with 10% aqueous sodium hydroxide to remove inhibitor before use. MAA was distilled under vacuum prior to use. 2, 2'-azobisisobutyronitrile (AIBN) was produced by Shanghai Chemical Plant (Shanghai, China) and refined before use. Dodecanol and methanol were purchased from Tianjin Kemiou Com (Tianjin, China). Nitrendipine and nisoldipine were purchased from Hebei Medical University. All reagents were of analytical reagent (AR) grade. Triply distilled water was used throughout all experiments. All media were filtered through a 0.45  $\mu$ m membrane before injection for LC analysis.

Human urine obtained from the Hospital of Hebei University were centrifuged at 5000 r/min for 10 min, and then stored under  $-20^{\circ}\text{C}$  before use.

### Instruments

The Jasco HPLC system (Jasco Co., Japan) equipped with a PU-1580 pump a variable wave-length UV-1570 detector and an HW-2000 chromatography data system (Nanjing Qianpu Software, China). The synthetic monolithic column (50  $\times$  4.6 mm i.d.) was tested as the SPE column and a C18 Dikma column (150  $\times$  4.6 mm i.d., 5  $\mu$ m, Dikma, NY, USA) was used as the analytical column. The mobile phase for enrichment was deionized water; the mobile phase for separation and analysis was methanol-water, which the proportion was 65:35 (v/v) for nitrendipine and nisoldipine. The detection wavelength was 237 nm. The flow rate was set at 1 mL/min. The system was operated at room temperature.

### Standard solutions

Stock solutions of nitrendipine and nisoldipine were prepared separately in methanol at 1 mg/mL. Then, the mixed stock solution

of nitrendipine and nisoldipine were also prepared in methanol at 1 mg/mL. The mixed stock solution was further diluted with methanol to yield intermediate solutions of 10  $\mu$ g/mL, 5  $\mu$ g/mL, 2  $\mu$ g/mL, 1  $\mu$ g/mL, 0.5  $\mu$ g/mL, 0.25  $\mu$ g/mL. Urine calibration standards containing 1000 ng/mL, 500 ng/mL, 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL were prepared by adding 20  $\mu$ L intermediate solutions into 180  $\mu$ L of blank urine. Quality control (QC) samples were prepared by spiking blank urine with prepared standard stock solutions to give final concentrations of 500 ng/mL, 200 ng/mL, 50 ng/mL for the evaluation of precision, accuracy and recovery in analysis of urine samples. The calibration standards and QC samples were stored at  $-20^{\circ}\text{C}$  and kept at 4 h until use, thawed and filtered before injection on the on-line SPE-HPLC system.

### Preparation and characterization of monolithic material

The monolithic material was prepared by an in-situ polymerization according to the following procedure. First, 0.05 g NIPAAm, 0.05 mL MAA, 0.005 g AIBN and 0.4 mL EDMA were dissolved in the mixture of 0.5 mL dodecanol and 1.2 mL methanol. Second, the mixture was shook for 1 min, sonicated and degassed briefly for 30 min. Finally, the mixture was poured into the 50  $\times$  4.6 mm i.d. stainless steel column sealed at one end and then sealed at the other end. After the mixture was left to polymerize at  $60^{\circ}\text{C}$  in a water bath for 24 h, the seals were removed from the tube and the column was flushed with methanol and water to remove unreacted monomers and porogens in the polymer rod for 1 h at a flow rate of 1 mL/min, respectively.

The prepared monolith was cut into small pieces and dried under vacuum at  $60^{\circ}\text{C}$  overnight. Then, the prepared monolith was characterized with regards to its macroporous and mesoporous structures as well as permeability. The morphological properties of this monolith was photographed using scanning electron microscope (SEM) by Hitachi (Hitachi High Technologies, Tokyo, Japan) S-4300 SEM instrument and the pressure drop across the column at different flow rates was also analyzed.

### Investigation of the pretreatment ability on the polymer monolith

The deproteinization ability of the monolithic column was also tested by directly injecting blank urine into empty column and the monolithic column and eluted with deionized water at 280 nm. Then, ability of drug enrichment on the monolith was also investigated by injecting 1  $\mu$ L of 1mg/mL nitrendipine and nisoldipine solution into the monolithic column and eluted with deionized water and methanol at the same condition as above.

### On-line SPE procedure

First, the monolith should be equilibrated with deionized water for 5 min. Second, 0.05 mL urine samples which no prior treatment was done before loading onto the monolith were directly injected into the SPE, and then washed with 10 mL water to remove protein and retain analytes. Finally, elution with methanol-water 65:35 (v/v) was carried out to elute the retained analytes into the analytical column for HPLC analysis. The flow rate was always 1 mL/min in the elution process. For consecutive use, the monolith was washed with methanol and finally with deionized water before re-using the monolith for the subsequent SPE.

## Results and Discussions

### Characteristics of the monolithic column

SEM and back pressure were carried out to characterize the

monolithic column. As shown in Figure Supplementary-1, the morphological properties of the monolith are apparent. From the SEM image, it can be seen that many macropores and mesopores exhibited in the network skeleton of the monolith. The macropores offered a large number of channels, which allowed the mobile phase to flow through with low column backpressure.

Figure Supplementary-2 showed the effect of flow-rate on the back pressure when deionized water and methanol were used as the mobile phase. When methanol and deionized water were used as mobile phases at the flow rate of 8.0 mL/min, the pressure was 36 and 43 bar, respectively. Meanwhile, an excellent linear relationship was obtained. The results showed the monolithic column has high permeability and could be used for rapid analysis at high flow rate, which was favorable for liquid biological samples at high flow rates.

### Efficiency of the pretreatment on the monolith

It can be seen that nearly identical peak area of blank urine samples on the empty column (1) and the monolithic column (2) with deionized water as elution solution (Figure Supplementary-3A). The results suggested that most biological matrix compounds in human urine could be cleaned up with 2 mL of deionized water. Therefore, this condition was adopted for selective washing of biological matrix compounds. Figure Supplementary-3B showed that nitrendipine and nisoldipine could not be eluted when deionized water (a) was used as the mobile phase. However, when methanol was used as mobile phase, nitrendipine(b) and nisoldipine(c) were eluted quickly from the monolithic column. In view of this result, we could draw a conclusion that the monolithic column could be used as SPE column to eliminate matrix interferences completely and retain the analytes.

### SPE-HPLC

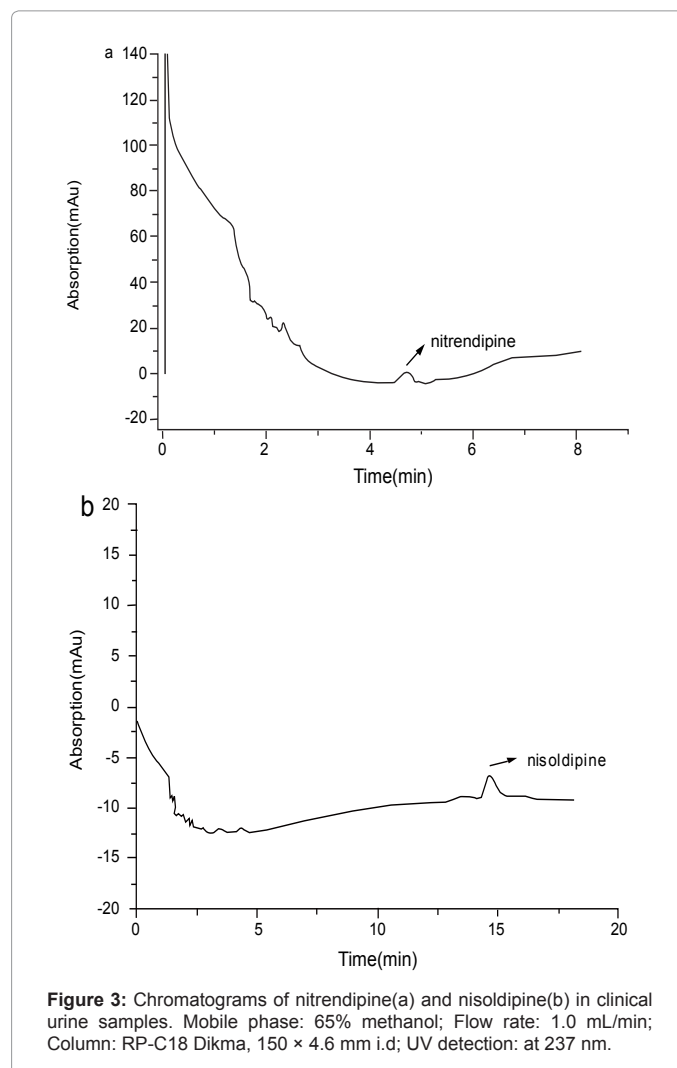
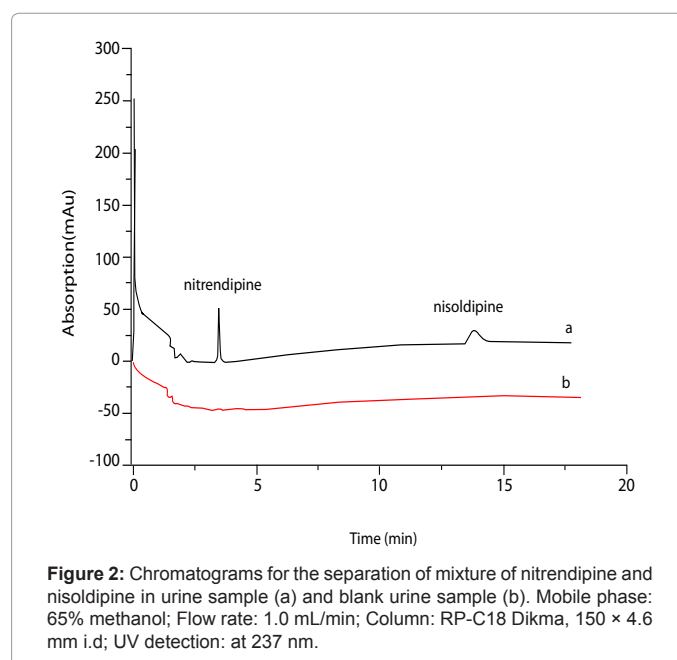
According to the On-line SPE procedure, the on-line SPE-HPLC of urine samples containing nitrendipine and nisoldipine was completed. Typical chromatogram was depicted in Figure 2. From Figure 2, nitrendipine and nisoldipine were well separated from interfering matrix components. These results demonstrated on-line SPE technique was a highly selective and efficient samples pretreatment method for proteins removal.

### Method validation

**Selectivity:** The selectivity of the method was evaluated by comparing the chromatograms obtained from the spiked samples containing nitrendipine and nisoldipine with those obtained from blank urine samples. As shown in Figure 2, they were free from significant interfering endogenous substances at the retention times for the selected drugs. These results showed that the developed method is selective and specific.

**Linearity:** Calibration curve was constructed from the concentrations of 1000 ng/mL, 500 ng/mL, 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL for urine samples containing nitrendipine and nisoldipine. Each calibration sample of different concentrations was injected at least three times. Limit of detection (LOD) and limit of quantification (LOQ) were defined as the concentrations which yielded measure peaks with signal-to-noise ratio equal to 3 and 10, respectively. The peak area showed a linear relationship over the range of 25-1000 ng/mL. Calibration equation, correlation coefficient, LOD and LOQ were represented in Table 1.

**Precision and accuracy:** The precision and accuracy of the method was determined using QC samples at low, medium and high levels.



Analytes (urine samples)	Calibration equations	Correlation coefficient	LOQ (ng/mL)	LOD (ng/mL)
Nitrendipine	$Y=5.02 \times 10^4 x + 1.05 \times 10^2$	$r^2=0.9998$	30	10
Nisoldipine	$Y=4.25 \times 10^4 x + 1.46 \times 10^2$	$r^2=0.9996$	30	10

**Table 1:** Calibration curve, LOD and LOQ of nitrendipine, nisoldipine from urine samples.

Analyte (urine samples)	Concentration (ng/mL)	Precision RSD (%)		Accuracy (%)
		Intra-day	Inter-day	
Nitrendipine	50	6.83	6.74	99.6
	200	5.85	4.26	98.8
	500	5.35	5.84	100.4
Nisoldipine	50	8.62	9.35	95.7
	200	7.13	6.52	101.1
	500	4.82	7.36	89.8

**Table 2:** Intra- and inter-day precisions and accuracies of nitrendipine and nisoldipine in human urine samples at three different concentrations.

Analyte (urine samples)	Concentration (ng/mL)	Absolute recovery (%)	Method recovery (%)
Nitrendipine	50	100.3±3.2	97.6±4.8
	200	101.5±5.4	99.9±5.6
	500	98.4±7.5	89.5±6.9
Nisoldipine	50	92.6±6.8	99.8±5.6
	200	95.1±3.8	92.4±6.9
	500	101.3±8.7	98.7±4.4

**Table 3:** Recovery of nitrendipine and nisoldipine in human urine samples.

Sample	Analytes	Dosage (mg)	Sampling time (h)	Concentration (ng/mL)
Sample 1	nitrendipine	10	1.5	25.21
Sample 2	nisoldipine	5	1.5	22.68

**Table 4:** Concentrations of the target analytes in the clinical urine samples.

The precision was determined by measuring inter- and intra -day RSD. The intra-day precision was accessed with spiked samples at three different concentrations (50, 200 and 500 ng/mL) for nitrendipine and nisoldipine on a single day; while the inter-day precision was accessed with spiked samples at three different concentrations for nitrendipine and nisoldipine on five consecutive days. The accuracy of this method was obtained by the measured concentrations of nitrendipine and nisoldipine in urine samples according to the on-line SPE to those targeted concentrations. All the results were given in Table 2. The intra- and inter-day precision was also within the acceptable range of 10% for all samples. The accuracy was also near to 100%. These could show that the reproducibility of method was excellent.

**Recovery:** The extraction recovery was determined by analysis the spiked urine sample at three different concentrations, low, medium, and high concentrations (50, 200, 500 ng/mL). The absolute recovery was measured by comparing the peak area measured after SPE-LC analysis of spiked urine samples to the peak area obtained by direct injection of nitrendipine and nisoldipine dissolved in methanol without SPE pretreatment. To check the reliability of this method, the method recovery was measured by comparing the concentration of analytes obtained from the calibration curve to the initial concentration of analytes in the spiked urine standard. All analyses were carried out five times. The results of recovery which were shown in Table 3 demonstrated that the recoveries were satisfactory and the method was acceptable for the analysis of urine samples.

**Reproducibility:** In order to evaluate the reproducibility of the prepared monolithic column as SPE column, four monolithic columns were prepared with the same polymerization process as described in

preparation and characterization of monolithic material and used for extraction of nitrendipine and nisoldipine from urine samples. The reproducibility of the SPE monolithic column could be expressed through the RSD of the retention time and the peak area of two dipine series in urine samples. The RSD of the retention time and the peak area were 2.82%, 3.05%, respectively, and RSD for peak area were all less than 5%. These data revealed that the prepared monolithic column could provide excellent reproducibility and implied that the prepared monolith was feasible to be used as on-line SPE sorbent material.

### Clinical application

The proposed on-line SPE-HPLC method was used to analyze real urine samples. Samples were obtained from anonymous hypertensive patients who had taken orally 5 or 10 mg doses of nitrendipine or nisoldipine tablets, respectively. The contents of each analyte in clinical urine samples were determined by interpolating the peak area on the calibration curve. The results were listed in Table 4, and the chromatograms were shown in Figure 3.

### Conclusions

In this study, a new synthesized poly(NIPAAm-MAA-EDMA) monolithic column as SPE cartridge coupled with HPLC was applied to analysis of nitrendipine and nisoldipine in urine samples. The prepared monolith enabled advantages of easy preparation and simple pretreatment of sample as well as excellent extraction efficiency through the on-line SPE procedure. Good linearity, accuracy and precision as well as recovery were also achieved. The results provided a re-confirmation in the application of monolith in bioanalysis, particularly

for the sample pretreatment of complex biological matrices like urine. Therefore, the new monolithic column as SPE cartridge is appropriate for the analysis of urine samples. Meanwhile, the established method is also proved to be a simple, rapid and reliable procedure.

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