

On-line SPE of Three Steroids with a Novel Polymeric Monolith

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Abstract

In this report, a novel poly (N-isopropyl acrylamide-glycidyl methacrylate-ethylene glycol dimethacrylate) monolithic column was introduced for solid phase extraction. The monolith was prepared using N-isopropyl acrylamide and glycidyl methacrylate as the functional monomers, ethylene glycol dimethacrylate as the cross linker. In order to evaluate the mechanical stability and permeability of the material, the pressure drop across the column was measured in different flow-rates. Moreover, the monolith was used as on-line solid phase extraction material and coupled to high performance liquid chromatography to determine dexamethasone acetate, norgestrel and halcinonide in urine samples.

Keywords: Poly (NIPAM-GMA-EDMA); Monolithic column; SPE; Urine samples

Introduction

Analysis of pharmaceuticals in biological matrix is becoming increasingly important. Usually macromolecular compounds (e.g., proteins) which can damage conventional HPLC supports have to be removed from a sample prior to analysis. Among the biological fluids, urine has become the preferred specimen not only because of longer time periods in urine than in blood for many compounds but also because of easy collection. Recently, the use of monoliths for solid-phase extraction (SPE) has become popular for the analysis of drugs in body fluids. This was because that the monoliths have the ability to prevent the access of matrix components such as proteins while retaining the analytes of interest [1,2]. Alvarez et al. [3] have developed an automatic solid phase extraction and liquid chromatography mass spectrometry method by using a monolithic column for the analysis of cyclosporin A in human plasma. Namera et al. [4] have developed a device to overcome the limitations of solid-phase extraction, which comprised a spin column packed with octadecyl silane-bonded monolithic silica for extracting amphetamines and methylenedioxyamphetamines from urine. Feng et al. [5,6] have investigated the extraction of drugs using a monolith as solid phase microextraction materials coupled to high performance liquid chromatography and analysis of drugs in human plasma and urine. Ho et al. [7] have applied a silica-based monolith as solid phase extraction cartridge for extracting polar compounds from urine.

NIPAM is one of the monomers that has appealed to a great deal of investigation because poly (N-isopropylacrylamide) (PNIPAM) is an important smart hydrogel that responds to temperature changes [8,9]. For this reason, PNIPAM has been studied extensively for decades. The PNIPAM chains are in the expanded conformation in the aqueous mobile phase because of the strong hydration below the lower critical solution temperature (LCST). So NIPAM can be used as a hydrophilic monomer at room temperature. Moreover, hydrogen bonding could be formed easily between the NH group and the carbonyl group of NIPAM and such groups of other compounds.

This paper describes the synthesis of a new monolithic chromatographic material using NIPAM and GMA as the functional monomers, EDMA as the crosslinker. Furthermore, the permeability and the ability of deproteinization and enrichment of three steroids from urine samples have been studied. The structures of steroids were shown in Figure 1.

Experimental

Reagents

NIPAAm was purchased from Kohjin (Tokyo, Japan). GMA and EDMA were purchased from Acros (New Jersey, USA). 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). Dexamethasone acetate, norgestrel and halcinonide were obtained from Hebei Medical University. Triply distilled water was used throughout all experiments. All media were filtered through a 0.45 µm membrane before injection for LC analysis.

Instrumentation and chromatography

Chromatography was performed with a Binary P3000 High Pressure Gradient HPLC System. Data processing was performed with a CXTH-3000 chromatography workstation (Beijing Chuangxintongheng Software, China). A CS501-SP thermostat was from Sida Experimentation Apparatus factory (Chongqing, China). The morphological property of the monolith was photographed using scanning electron microscope (SEM) by Hitachi (Hitachi High Technologies, Tokyo, Japan) S-4300 SEM instrument. The analytical column was an ODS column (Jiangshen, 150 mm × 4.6 mm i.d.). HPLC column operated at ambient temperature, UV wavelength was set at 240 nm. The mobile phase for deproteinization and enrichment of drugs was deionized water; the eluting solution was the mixture of methanol: water (60:40, v/v), the flow rate was 1 mL/min.

The preparation of the monolithic column

Monolith was prepared as follows: 0.1 g NIPAAm, 0.5 mL GMA,

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0.004 g AIBN and 0.4 mL EDMA were dissolved in the mixture of 0.8 mL dodecanol and 0.6 mL methanol. The mixture was shook for 1 min, sonicated and degassed briefly for 10 min. Then, the mixture was poured into the 50 mm × 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°C in a water bath for 24 h, the seals were removed from the tube and the column was provided with fittings, attached to the HPLC system and washed with methanol at a flow-rate of 1 mL/min for 60 min to remove the alcohols and other soluble compounds present in the polymer rod after the polymerization was completed.

Standard solutions used for method development

Stock solution was prepared by dissolving 1.0 mg of drug in 10 mL of methanol. Blank urine from healthy volunteers were stored at -20°C and kept at -4°C before use. Any precipitated material was removed by centrifuging the sample at 5000 rpm for 15 min. Different amount of stock solutions were spiked in an appropriate volume of urine to obtain working solutions with concentration of 0.1 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL and 2 µg/mL.

On-line SPE and extraction efficiency

The process for deproteinization and SPE was performed according to the procedure described previously [10]. During the experiment, the monolith should be equilibrated with deionized water for 5 min. Then, 20 µL working solution was passed through the monolith and the column was washed for 10 min at the flow of 1 mL/min to remove protein. Finally, the retained analytes on the SPE column were eluted into the analytical column for HPLC analysis with mixture of methanol-water.

Results and Discussion

Characteristic of the monolith

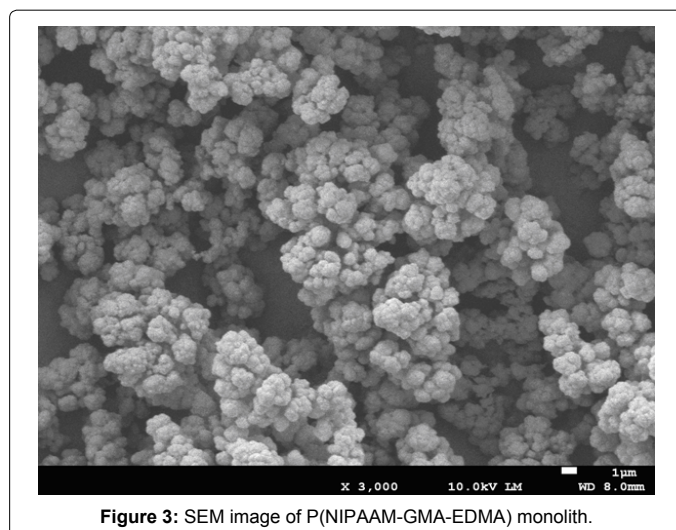
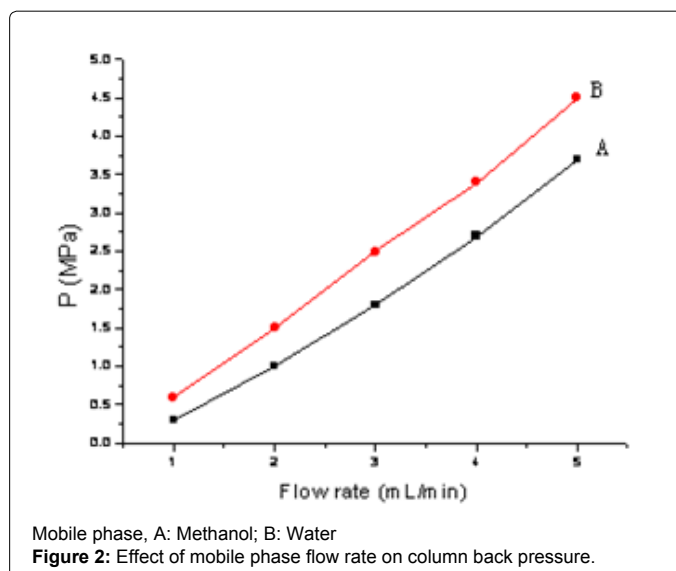
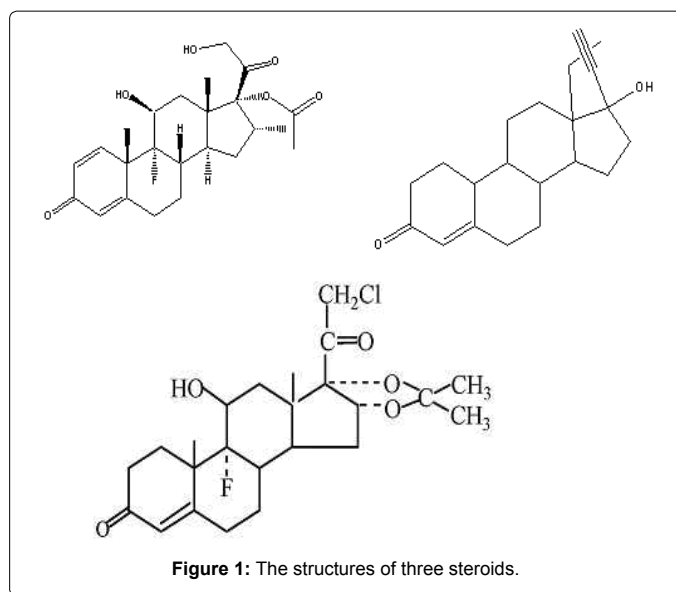
The obvious advantage of monolithic columns was their high permeability. In order to evaluate the permeability and the mechanical stability of the column material, the pressure drop across the column was measured at different flow-rates. Using deionized water and methanol as the mobile phase, the relationship between the flow-rate and backpressure was studied. An excellent linear dependence of the column pressure on the flow-rate was obtained Figure 2. The lower column backpressure was due to the macropores of the monolith shown in Figure 3.

Selection of the solvent of working solution and eluting solvent

To investigate the ability of the monolith for deproteinization and enrichment of drugs, water was first selected as the mobile phase to elute urine and three steroids. The results showed that nearly all substances in urine were eluted, which was proved by comparing peak area of blank urine samples on the empty column and the monolithic column. On the contrary, three steroids could not be eluted entirely. So water was suitable for both deproteinization and enrichment of drugs. When methanol was used as mobile phase, three steroids could be eluted quickly from the monolithic column. Therefore, three steroids could be eluted in an optimum retention time by adjusting the ratio of methanol and water.

Urine analysis by on-line SPE

Firstly, the urine samples passed through the SPE column with pure water as the mobile phase, and then the column was eluted by



the mixture of methanol: water (60:40, v/v) and the drugs would be pushed into the RP-C18 column to analysis. The result shown in Figure 4 indicated that there was no interfering peak around the retention time of three steroids.

Method validation

Linearity, recovery, selectivity, and reproducibility were assessed for the on-line SPE-HPLC method. **5.4.1 Linearity:** The extraction procedure was validated by analyzing urine samples spiked with dexamethasone acetate, norgestrel and halcinonide at different concentration levels of 0.1 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL and 2 µg/mL. Successful extraction was accomplished with the satisfactory regression coefficient for the calibration curves obtained (Table 1). The 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.

Recovery: 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 dexamethasone acetate, norgestrel and halcinonide without SPE pretreatment. The results were shown in Table 2.

Selectivity: The selectivity of this method was evaluated by comparing the chromatograms obtained from the sample with that obtained from blank urine sample. As shown in Figure 4, they were free from significant interfering endogenous substances at the retention

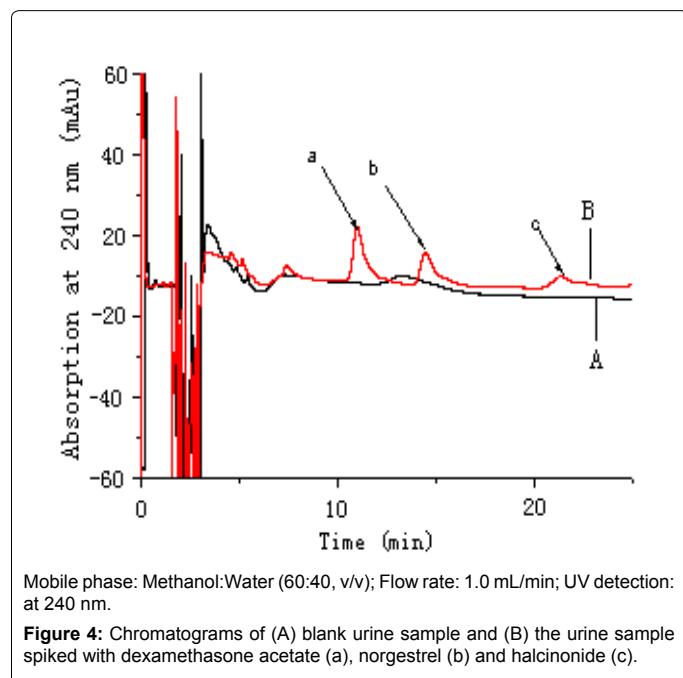


Figure 4: Chromatograms of (A) blank urine sample and (B) the urine sample spiked with dexamethasone acetate (a), norgestrel (b) and halcinonide (c).

Samples	Calibration equations	Correlation coefficient (r)	LOQ (ng/mL)	LOD (ng/mL)
Dexamethasone acetate	y=54937.1x-315	0.997	4.6	1.9
Norgestrel	y=39723.1x-1284.5	0.999	4.3	1.6
Halcinonide	y=30160.5x-426	0.998	4.5	1.8

Table 1: Regression equations, LOD, and LOQ of three steroids spiked in human urine.

Recovery	Dexamethasone acetate (%)	Norgestrel (%)	Halcinonide (%)
0.25 µg/mL	79.8	87.0	83.9
0.5 µg/mL	79.9	85.9	80.0
1 µg/mL	80.9	87.3	84.1

Table 2: Recovery of dexamethasone acetate, norgestrel and halcinonide in urine samples.

times for the selected drugs. The result showed that the developed method is selective and specific.

Reproducibility: Seven monolithic columns were prepared following the process described in Section 2.3 and used for extraction of the three drugs from urine sample to evaluate the reproducibility of the monolith. RSDs of three drugs (dexamethasone acetate, norgestrel and halcinonide, in order) retention time and peak area were 0.16%, 0.19%, 0.18% and 1.55%, 1.62%, 2.01%, (n=7) respectively. The results suggested that the prepared monolithic column provided excellent reproducibility.

Conclusion

In this paper, a novel poly (NIPAAm-GMA-EDMA) monolithic column was prepared and used as the SPE material to extract dexamethasone acetate, norgestrel and halcinonide from urine samples. The application of the monolith could realize not only deproteinization but also enrichment of the drugs simultaneously without tedious pretreatment of samples. The results suggested that such kind of monolithic column was simple, cheap, effective and friendly to environment for the assay of urine samples.

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