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On-line simultaneous removal of human serum albumin and enrichment of doxazosin using a weak cation-exchange monolithic column

Gengliang Yang [a,b,*], Haiyan Liu [b], Yihua Zhang [b], Sumin Wang [b,c], Junfa Yin [a], Boying Yin [b], Yi Chen [a]

[a] *Institute of Chemistry, Chinese Academy of Science, Beijing 100080, China*
[b] *College of Pharmacy, Hebei University, Baoding 071002, China*
[c] *Department of pharmacology, Hebei Medical University, Shijiazhuang 050017, China*

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Abstract

A weak cation-exchange monolithic column was prepared by modifying the GMA-EDMA (glycidyl methacrylate-co-ethylene glycol dimethacrylate) monoliths with ethylenediamine and monochloracetic acid. The properties of the column were investigated; the column exhibited the ability of low backpressure and fast analysis. Using this monolithic column, trace doxazosin in human serum albumin (HSA) solution and plasma samples has been on-line tested, the extraction efficiency and the maximum loading capacity of the monolithic column were obtained. The results showed that the monolithic column could realize deproteinization and trace drug enrichment simultaneously in the HSA solution and human plasma, which provided a simple, cheap, effective and friendly to environment method for assaying drugs in the blood.
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Keywords: Monolithic column; Solid-phase extraction; Deproteinization; Doxazosin; Human plasma; Cation exchange

1. Introduction

The ability to determine accurately the concentration of pharmaceuticals in biological matrix is essential in defining the pharmacokinetics of a pharmaceutical compound [1,2]. Usually macromolecular compounds (e.g. proteins) have to be removed from a sample prior to HPLC analysis. Typically, this has been achieved by techniques such as liquid–liquid extraction, solid-phase extraction (SPE), protein precipitation or column switching [3–7]. Liquid–liquid and solid-phase extraction protocols frequently include an evaporation and reconstitution step and are often time-consuming. Column switching approaches can avoid these problems but often lead to extended chromatography analysis times. Protein precipitation is simple and quick but results in relatively dirty sample solutions and can restrict the injection volume. A promising approach to HPLC with integrated fully automated sample extraction is high speed on-line SPE [8,9]. The technique enables direct injection of plasma samples without prior extraction. Owing to the combination of a small diameter LC column packed with large particles and a high flow rate of the mobile phase, large biomolecules rapidly pass through the column while the small analyte molecules are retained.

Recently, monolithic stationary phase had attracted considerable attention in liquid chromatography due to their large adsorption capacity, various modification methods, simple preparation procedure, unique properties and excellent performance [10,11]. Monoliths consist of single piece of highly porous organic [12,13] or inorganic material [14,15] with pores made up of highly interconnected channel network resulting in high effective porosity and thus enabling efficient flow of the mobile phase. As a result, fast mass transfer between the stationary and mobile phase is possible and, in addition, the pressure drop is much lower than with classic particulate stationary phases.

Doxazosin is a postsynaptic α-1 adrenoreceptor antagonist (Fig. 1). As a potent antihypertensive agent, it is effective when administered either orally or intravenously. It is slowly eliminated in humans and its long half-life provides the basis for once-daily dosing [16]. Several HPLC methods have been employed for the determination of doxazosin. These studies cover the determination of active material in body fluids depending on the pharmacological evaluations [17,18].

* Corresponding author. Tel.: +86 3125079788.
E-mail address: ygl@mail.hbu.edu.cn (G. Yang).

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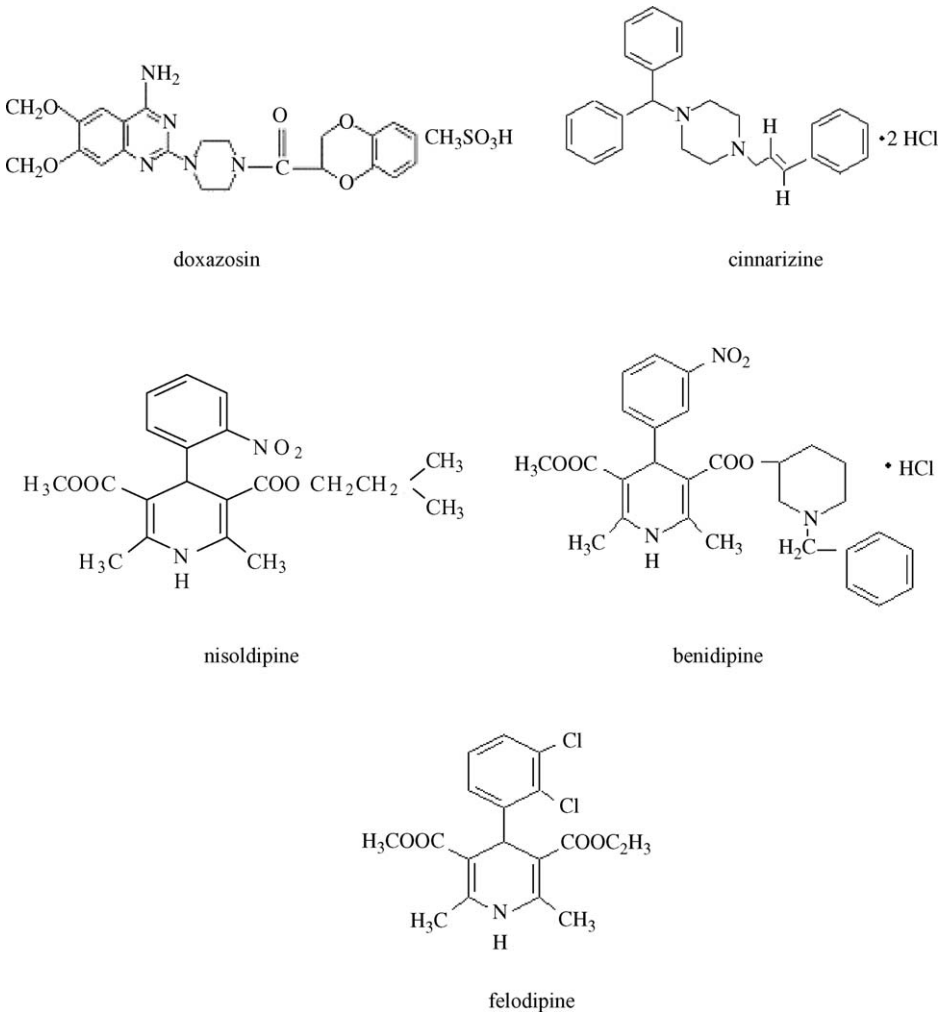

Fig. 1. The chemical structures of drugs.

In this work, a new on-line SPE method was developed to determine trace drugs in human serum albumin (HSA) solution and in human plasma by using a weak cation-exchange monolithic column. For their appropriate retention time, doxazosin and some other related drugs have been chosen as a drug example. Their structures were shown in Fig. 1.

2. Experimental

2.1. Reagents

Glycidyl methacrylate (GMA) and Ethylene glycol dimethacrylate (EDMA) were purchased from Acros (New Jersey, USA). 2, 2′-Azobisisobutyronitrile (AIBN) was produced by Shanghai Chemical Plant (Shanghai, China) and refined before use. 1-Dodecanol and cyclohexanol were from Fuchen Chemical Plant (Tianjin, China). HSA (Haixing Group, Xi'an); doxazosin, nisoldipine, cinnarizine, benidipine and felodipine were obtained from Hebei Medical University. All water was prepared from a Millipore Milli-Q system and solutions were filtered through a 0.45 μm membrane before use.

2.2. Instrumentation and chromatography

Chromatography was performed with a PU-1580 pump and a variable-wave-length UV-1570 detector (Jasco, Japan). Data processing was performed with an HW-2000 chromatography workstation (Nanjing Qianpu Software, China). A CS501-SP thermostat was from Sida Experimentation Apparatus Factory (Chongqing, China). The analytical column was an ODS column (Jiangshen, 50 mm × 4.6 mm I.D.). HPLC column operated at ambient temperature, UV wavelength was set at 276 nm. The mobile phase for enrichment was deionized water; the eluting solution was the mixture of methanol:water (70:30, v/v), the flow rate was 1 ml/min.

2.3. The preparation of the weak cation-exchange monolithic column

The monolithic column was prepared by an in-situ polymerization and chemical modifications according to the procedure described previously [19]. First, a mixture consisting of 2.2 ml glycidyl methacrylate (GMA), 1.5 ml ethylene dimethacylate (EDMA), 0.8 ml dodecanol, 7.0 ml cyclohexanol, 0.09 g azo-

bisisobutyronitrile was purged with nitrogen for 10 min. Then the stainless steel column (10 × 4.6 mm I.D.) sealed at the bottom was filled with the polymerization mixture and then sealed at the top. After the polymerization was allowed to proceed at 55 °C 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 tetrahydrofuran (THF) 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.

The epoxy groups on the surface of monolithic column were targeted for chemical modification. The column was modified on-line with ethylenediamine:tetrahydrofuran (1:1, v/v) and monochloracetic acid (14%, pH 11) in turn. The condition of modification was: the modificatory solution was circulated through the column at 80 °C for 24 h, flow rate was 0.1 ml/min. After that, the column was washed completely by excessive methanol and deionized water, in this way a weak cation-exchange monolithic column was obtained.

2.4. Standard solutions used for method development

Stock solution was prepared by dissolving 1.0 mg of drug in 10 ml of methanol. Blank human plasmas 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 the HSA solution (in 67 mM phosphate buffer, ionic strength 0.17) or human plasma to obtain working solutions with concentration of 1.0×10^{-5}, 1.0×10^{-6} mg/ml.

2.5. On-line SPE

SPE was placed in the sample-loop position of the injection value and used for sample enrichment. In the "load" position, each working solution of 2 ml was passed through the precolumn, respectively, then valve was switched to the "injection" position. Drugs was desorbed by backflushing with methanol-water (70:30, v/v) and transferred on-line to the analytical column.

2.6. Extraction efficiency

The extraction efficiency was experimentally determined at concentrations of 1.0×10^{-5}, 1.0×10^{-6} mg/ml. The absolute extraction recoveries were evaluated by comparing the analyte peak areas obtained from spiked HSA solution or plasma samples ($n = 5$) to those obtained from the corresponding unextracted reference standards prepared at the same concentrations.

3. Results and discussions

3.1. Performance of the chromatographic column

The obvious advantage of monolithic columns was their high permeability. Using deionized water as the mobile phase, the relationship between the flow-rate and backpressure was studied. The results showed that a good linearity was obtained. The backpressure of the monolithic column was about 4.8 MPa at the flow-rate of 4.0 ml/min. It showed that the monolith could be used in analysis at high flow-rate, which would provide a favorable possibility for fast analysis.

3.2. Selection of the solvent of working solution and elution solvent

Through the experiments, it was found that the retention time of HSA and doxazosin was greatly affected by the content of water in the mobile phase. When water was used as mobile phase, the interaction between HSA and the carboxyl groups of the monolithic column was weak; however, the doxazosin with positive charge interacted strongly with the carboxyl groups. So HSA was eluted quickly and doxazosin could not be eluted within 30 min. Thus, the removal of HSA and the enrichment of doxazosin could be realized simultaneously. In order to evaluate the recovery of HSA, different amounts of HSA were added into a quantitative HSA solution. By comparing added quantity and detected quantity, the recoveries of HSA were obtained. The result is shown in Table 1.

Na^+, NH_4^+ and H^+ were often used as elution ions for cation-exchange chromatography. Some salt solutions, such as NaCl, KBr and $(NH_4)_2SO_4$ were all tested. The experiment showed that when NaCl and $(NH_4)_2$ SO_4 solution were used as elution, tailing peaks were detected. So KBr solution could be chosen as eluting solution. Although salt solution could be used as eluting solution, the monolithic column had to be regenerated before next enrichment. This is time consuming.

In order to simplify the operation, methanol–water system was also tested as elution solution. Fig. 2 showed the influence of different proportion of methanol–water on the retention time of drugs. From the experiments, it was also shown that when the concentration of methanol was increased, the drugs also could be eluted quickly. This showed that the interactions between the

Table 1
Recoveries of HAS

	Original quantity of HAS (mg)	Added quantity of HAS (mg)	Determined quantity of HAS (mg)	Recovery (%)	Average recovery (%)
1	3.60	2.88	6.42	97.9	
2	3.60	3.60	7.25	101.4	99.6
3	3.60	4.32	7.90	99.5	

HPLC conditions: mobile phase, deionized water; flow rate, 1.0 mL min^{-1}; detection, 276 nm.

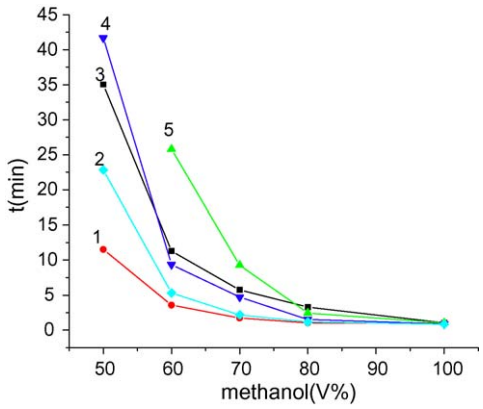

Fig. 2. Effect of methanol content on the retention of drugs. Drugs: 1, nisoldipine; 2, felodipine; 3, doxazosin; 4, benidipine; 5, cinnarizine. Flow rate: 1 ml/min; detection: 276 nm.

drugs and the monolithic column were not only ion-exchange interactions but also hydrophobic interactions. So one can inject the sample solution into the monolithic column first, then wash the column by pure water to elute protein. Finally, the column was eluted by the mixture of methanol:water (70:30, v/v) and the drugs would be pushed into the analytical column for further analysis. By this approach, the deproreinization and sample enrichment could be realized at the same time. The result of enrichment and elution was shown in Fig. 3. From Fig. 3, it could be found that when the column was washed by pure water, no doxazosin was eluted. When the column was eluted by the mixture of methanol:water (70:30, v/v), doxazosin was eluted and no residual HSA was detected.

3.3. The maximum loading capacity

Maximum loading capacity experiment was conducted to investigate the absorption performance of the monolithic column. Firstly, different amounts of doxazosin were injected into the monolithic column when water was used as the mobile phase. Secondly, the column was eluted by the mixture of methanol:water (70:30, v/v). Because the amount of adsorption

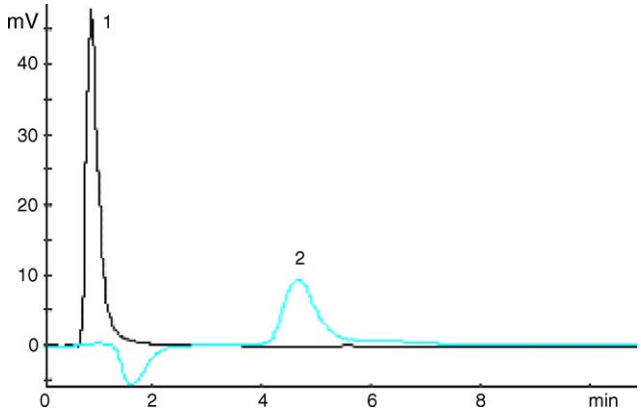

Fig. 3. Chromatogram of enrichment process (1) and elution process (2); The mobile phase for enrichment: deionized water; The mobile phase for elution: methanol:water (70:30, v/v).

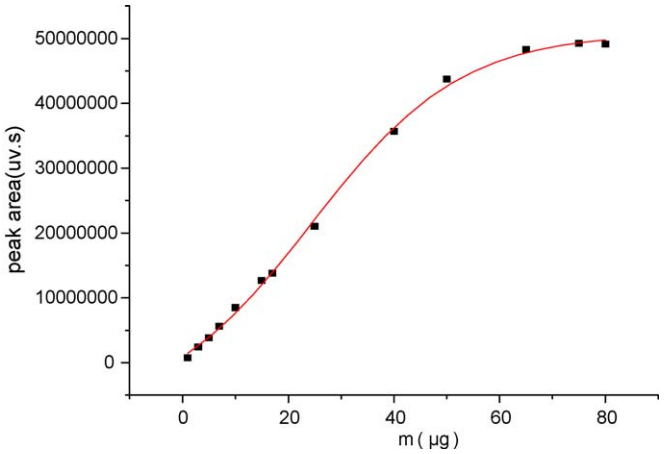

Fig. 4. The effect of inject amount on peak area; the mobile phase for enrichment: deionized water; the mobile phase for elution: methanol:water (70:30, v/v).

was fixed for a given SPE column, the peak area increased when injection amount increased before the pre-concentration column reached saturation. After the pre-concentration column reached saturation, though more doxazosin was added to the column, only part of it was adsorbed on the column. So the peak area kept unchanged when injection amount increased. The result is shown in Fig. 4. The maximum adsorptive amount of doxazosin could be gained from Fig. 4 and it was 75 μg.

3.4. Plasma analysis by on-line SPE

Using the previously determined detection conditions, human plasma samples could be extracted without any other manipulation. For the plasma samples with doxazosin of 1.0×10^{-5}, 1.0×10^{-6} mg/ml, the recoveries were 90% and 91%, respectively. In order to investigate if the method adapt to other drugs or not, four others drugs, nisoldipine, cinnarizine, felodipine and benidipine were tested using the same manner, and the same results were observed. The recoveries of the drugs were shown in Table 2. The results showed that the recoveries were satisfactory.

The stability of the column was also studied. The intra-day reproducibility of the method varied between 0.8 and 1.9%, for inter-day reproducibility relative standard deviations were 2.6%. And no obvious changes in column efficiency and backpressure

Table 2
Recoveries of the drugs

	80% of the background concentration ($n = 3$) (%)	100% of the background concentration ($n = 3$) (%)	120% of the background concentration ($n = 3$) (%)
Doxazosin	92	93	91.5
Nisoldipine	94	92.5	93
Cinnarizine	91	90	91
Felodipine	91.5	92	90
Benidipine	90	91	91

HPLC conditions: the mobile phase for enrichment: deionized water; the mobile phase for elution: methanol-water (70:30, v/v) flow rate, 1.0 ml/min; detection, 276 nm; the analytical column was an ODS column (50 mm × 4.6 mm I.D.).

Table 3
Precision and accuracy of the method for the determination of doxazosin in human plasma

Concentration (ng/mL)	Precision RSD (%)	Accuracy (%)
Intra-day (within batch) ($n = 5$)		
1	1.9	91.8
10	0.8	92.6
Inter-day (between batch) ($n = 2$)		
1	2.6	91.3
10	2.6	92.5

HPLC conditions: the mobile phase for enrichment: deionized water; the mobile phase for elution: methanol-water (70:30, v/v) flow rate, 1.0 ml/min; detection, 276 nm; the analytical column was an ODS column (50 × 4.6 mm I.D.).

were observed within two months. The results were shown in Table 3.

4. Conclusions

A weak cation-exchange monolithic column was successfully used to deproteinize and enrich the drugs simultaneously without tedious pretreatment of samples. The recoveries of doxazosin were more than 90% both in HSA solution and human plasma. The results suggested that such kind of monolithic column could be used as a simple, cheap, effective and friendly to environment method for the assay of plasma sample.

Acknowledgments

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References

[1] A. Berthemy, J. Newton, D. Wu, D. Buhrman, J. Pharm. Biomed. Anal. 19 (1999) 429.
[2] R. Carabias-Martínez, E. Rodríguez-Gonzalo, P. Revilla-Ruiz, J. Hernández-Méndez, J. Chromatogr. A 1089 (2005) 1.
[3] D.F. Hagen, C.G. Markell, G.A. Schmitt, Anal. Chim. Acta 236 (1990) 157.
[4] G. Theodoris, I. Papdoyannis, H. Tsoukali-Papadopoulou, G. Vasilikiotis, J. Liq. Chromatogr. 18 (1995) 1973.
[5] C. Stubbs, M.F. Skinner, I. Kanfer, Chromatographia 29 (1990) 31.
[6] C. Chang-Yun, L. Hsu, R.R. Walters, J. Liq. Chromatogr. 629 (1993) 61.
[7] M.-C. Hennion, J. Chromatogr. A 856 (1999) 3.
[8] J. Ayrton, G.J. Dear, W.J. Leavens, D.N. Mallett, R.S. Plumb, Rapid. Commun. Mass. Spectrom. 11 (1997) 1953.
[9] J. Ayrton, G.J. Dear, W.J. Leavens, D.N. Mallett, R.S. Plumb, J. Chromatogr. A 828 (1998) 199.
[10] H. Hashen, Th. Jira, Chromatographia 61 (2005) 133.
[11] J. Urthaler, R. Schlegl, A. Podgornik, A. Strancar, A. Jungbauer, R. Necina, J. Chromatogr. A 1065 (2005) 93.
[12] K. Andreas, K. Silke, J. Chromatogr. A 1089 (2005) 148.
[13] M. Igor, N. Damjan, P. Aleš, K. Tine, J. Chromatogr. A 1065 (2005) 59.
[14] H.S. Alexander, J. Chromatogr. A 1073 (2005) 377.
[15] M. Al-Bokari, D. Cherrak, G. Guiochon, J. Chromatogr. A 975 (2002) 275.
[16] V.A. Alabaster, M.J. Davey, Br. J. Pharmacol. 21 (1986) 9S.
[17] T. Alebic-Kolbah, A.P. Zavitsanos, J. Chromatogr. A 759 (1997) 65.
[18] P. Sripalakit, P. Nermhom, A. Saraphanchotiwitthaya, J. Chromatogr. Sci. 43 (2005) 63.
[19] Y.M. Wei, X.D. Huang, Q. Chen, X.D. Cheng, Chin. J. Anal. Chem. 28 (2000) 1194.