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In-tube solid phase microextraction using a β-cyclodextrin coated capillary coupled to high performance liquid chromatography for determination of non-steroidal anti-inflammatory drugs in urine samples

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Abstract

A configuration of in-tube solid-phase microextraction (SPME) coupled to HPLC was constructed by using a pump and a six-port valve combined with a PEEK tube as the pre-extraction segment. The extraction capillary was fixed directly on the HPLC six-port valve to substitute for the sample loop. The whole system could be handled easily to perform accurate on-line extraction, and the possible inaccurate quantification caused by sample/mobile phase mixing when using an autosampler could be eliminated.

A β-cyclodextrin coated capillary, prepared by sol–gel method, was used as the extraction capillary for in-tube SPME. Three non-steroidal anti-inflammatory drugs, ketoprofen, fenbufen and ibuprofen, were employed to evaluate the extraction performance of the capillary. After optimizing the extraction conditions, satisfactory extraction efficiency was obtained and detection limits for ketoprofen, fenbufen and ibuprofen in diluted urine samples were 38, 18 and 28 ng/mL, respectively. The extraction reproducibility was evaluated with intra-day and inter-day precision, and the R.S.D.s obtained were lower than 4.9 and 6.9%, respectively. The capillary was proved to be reusable and the extraction efficiency did not decrease after 250 extractions.

Keywords: In-tube solid-phase microextraction; β-Cyclodextrin coated capillary; Non-steroidal anti-inflammatory drugs; SPME-HPLC

1. Introduction

The main purpose of sample pretreatment methods are sample clean-up and analyte enrichment, which will lead to higher sensitivity and better accuracy of analysis. In coping with samples in complicated matrices or analytes at low concentrations, such as biological and environmental samples, traditional treatment methods such as liquid–liquid extraction (LLE), protein precipitation and so on, cannot satisfy all the analytical requirements. This situation has propelled the development of new sample pretreatment techniques, such as solid-phase extraction (SPE), solid-phase microextraction (SPME), membrane extraction, microwave assisted extraction, etc.

SPME was introduced by Arthur and Pawliszyn in the beginning of 1990s [1] and was combined with HPLC in 1995 [2], providing an alternative choice for sample clean up and enrichment for the analysis of non-volatile analytes. SPME has gained wide acceptance due to its better sensitivity compared to direct injection, adequate extraction reproducibility and commercial availability of SPME devices for combining with GC, LC, and CE [3,4]. The original forms of SPME-HPLC and the automated form of in-tube SPME-HPLC, introduced at 1997 [5], have been widely used since then in the analysis of environmental and biological samples and determination of food and drug samples. Its applications were reviewed recently by Kataoka [6].

In-tube SPME-HPLC can be automated by the modification of a commercial LC autosampler into an automated extraction device [5]. It provides shorter sample analysis time, more accurate quantification and better reproducibility and thus has good potential for routine analysis. However, little improvement was made since the device was first described. An inherent systematic error of this configuration was demonstrated when coping with analytes in given matrix by Raghani and Schultz recently [7]. In that system, the

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six-port valve is allowed to be situated at the LOAD position during extraction, and there is residual mobile phase contained in both the pre-extraction, including the sample loop, metering valve, tube prior to the metering valve, and the extraction segment. When the sample solution is drawn, traces of analyte migrate to these segments causing contamination of the mobile phase that resides inside the segments. Thus the concentration of analyte cumulatively increases as a result of sample/mobile phase mixing as the number of draw/eject steps increase [7]. This will result in inaccurate quantitative information and overestimation of the limit of detection. Raghani and Schultz demonstrated this by substituting the extraction capillary with an inert stainless steel tube and obvious analyte enrichment was found when increasing the draw/eject cycles. As to the solution of the problem, they suggested the insertion of an air plug into the extraction step to minimize the sample/mobile phase mixing. However, the attempt to construct other forms of the in-tube SPME-HPLC configurations that satisfy the same extraction requirement could be taken.

Common coatings applied in SPME are PDMS, PDMS-DVB, PA and CAR-TPR, which are mainly transplanted from GC capillary coatings. In the past several years, the polypyrrole coating, introduced by Pawliszyn and co-workers, has been employed in a wide range of research works [8–14]. Besides, biocompatible restricted-access materials (RAMs) [15,16], and chemical bonded silica monolith [17] were also introduced. However, the development of further coatings for SPME would be useful.

β-Cyclodextrin has been widely used in separation and analytical chemistry due to its abilities to form inclusion complexes with certain analytes. As to non-steroidal anti-inflammatory drugs (NSAIDs), the research on complex formation [18,19] and chromatographic separations [20,21] involving β-cyclodextrin have been reported, which provides the possibility of using β-cyclodextrin coating for extraction. However, the use of β-cyclodextrin in SPME-HPLC has not been reported as yet. It is expected that satisfactory extraction efficiency towards these analytes could be obtained with β-cyclodextrin contained capillary coating.

In the present paper, we describe a configuration of in-tube SPME-HPLC. A capillary coated with β-cyclodextrin using a sol–gel method was employed for the determination of three non-steroidal anti-inflammatory drugs. The results indicate that this system can be used for quantitative analysis and has the potentiality for extraction of real biological samples.

2. Experimental

2.1. Chemicals and reagent

The three investigated NSAIDs were ketoprofen (KEP) [2-(3-benzoylphenyl)propionic acid], fenbufen (FEP) [3-(4-biphenylylcarbonyl)propionic acid], and ibuprofen (IBP) [2-(4-isobutylphenyl)propionic acid], obtained from Pharmacy Administration of Hubei Province (Wuhan, China). The stock solution of the drugs was 1 mg/mL, prepared in methanol, with which the sample solution was spiked to a certain concentration. Sodium acetate (NaAc), sodium chloride (NaCl), methanol and other solvent were purchased from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China), and were of analytical grade.

β-Cyclodextrin was purchased from Sigma (St. Louis, MO, USA); 3-glycidoxypropyltrimethoxysilane (KH-560) and *n*-tetraethoxylsilane (TEOS) (95%) were obtained from Chemical Plant of Wuhan University (Wuhan, China).

2.2. In-tube SPME-HPLC instrument

The configuration of the in-tube SPME-HPLC is shown in Fig. 1. The whole system consisted of the pre-extraction segment, which included a Shimadzu LC-4A six-port valve (valve 1), a Shimadzu LC-6A pump (pump A) (Shimadzu, Tokyo, Japan) and a PEEK tube (0.03 in. i.d., 575 μL total volume), and the analytical segment, which included a Shimadzu LC-10AT pump (pump B) (Shimadzu, Tokyo, Japan), Rheodyne 7125 six-port valve (valve 2) with a 20 μL loop (Cotati, CA, USA) and a Shimadzu SPD-10A UV detector (Shimadzu, Tokyo, Japan). Valve 1 and valve 2 were connected by a stainless steel tube.

The analytical column was 150 mm × 4.6 mm i.d., packed with Kromasil ODS (5 μm),which was purchased from Eka Chemicals (Bohus, Sweden). The optimized mobile phase was 70% methanol and 30% 0.025 mol/L NaAc buffer solution with the pH adjusted to 5.0. The flow rate of the mobile phase was kept at 1 mL/min, and detection was performed at 223 nm with the UV detector for all the analytes.

2.3. Preparation of β-cyclodextrin coated capillary

The inner surface of the capillary was coated with β-cyclodextrin by a sol–gel method in order to obtain relatively high β-cyclodextrin content. The fused silica capillary (60 cm × 0.25 mm, i.d.), obtained from Yongnian Fiber Plant (Hebei, China), was first activated by 1 mol/L NaOH and then 1 mol/L HCl. After rinsing with double distilled water, it was dried at 160 °C under N_2 flow for several hours.

0.1 mL of TEOS was added to 0.1 mL of 0.01 mol/L HCl and the mixture was allowed to stir at 60 °C in water bath until a homogenous solution (A) was obtained. KH-560 derived β-cyclodextrin (synthesized according to reference [22]) 0.05 g was dissolved in a solution of 0.5 mL of 0.01 mol/L HCl and 0.3 mL of acetonitrile, and the whole mixture (B) was stirred to thoroughly dissolve the materials. Then A and B were mixed, and the whole mixture was stirred at room temperature for 5 min before centrifugation. The supernatant was used for coating. The solution was first allowed to fill capillary and left static for 20 min, and then it was driven out slowly with the aid of N_2, followed

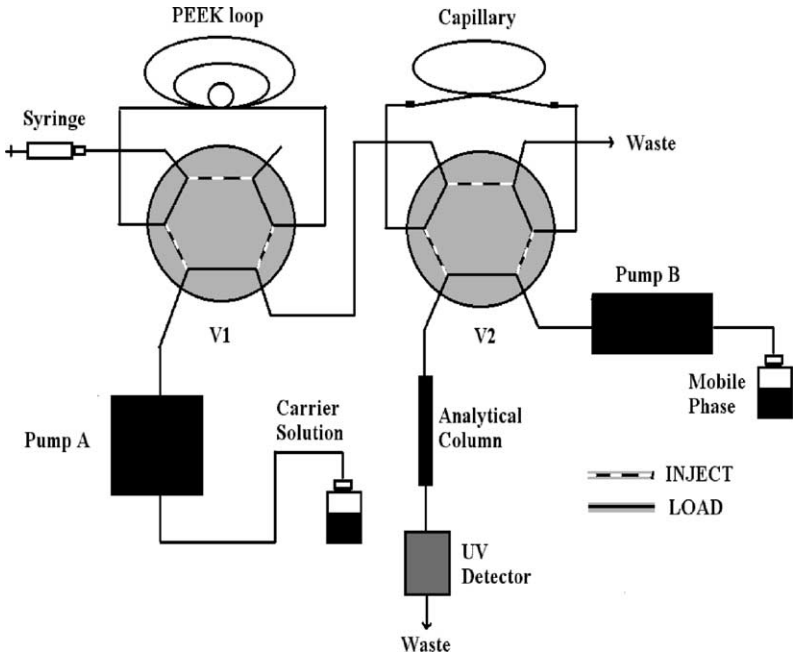

Fig. 1. Configuration of in-tube SPME-HPLC system.

by a N_2 to flow through the capillary for 2 h. The coating procedure was repeated three times to obtain a proper coating thickness. The capillary was then aged at 40 °C for at least 48 h. Finally, it was coupled to SPME-HPLC system and conditioned with methanol before use.

2.4. In-tube SPME procedure

A schematic diagram of the in-tube SPME-HPLC system is illustrated in Fig. 1 and the procedure for extraction was listed in Table 1.The mobile phase was driven by pump B directly through the analytical column to obtain a stable baseline in preparation for chromatographic separation and pump A was used to drive the carrier solution (double distilled water). For comparison, an uncoated capillary was also tested in the same way.

2.5. Sample preparation

The urine samples were collected from drug-free healthy volunteers. Any precipitated material in the sample was removed by giving centrifugation at 4500 rpm for 10 min, then the supernatant of the urine was first diluted 10 times with double distilled water and spiked with the analytes for investigation.

3. Results and discussion

3.1. Configuration of in-tube SPME-HPLC

In the configuration of in-tube SPME-HPLC described herein, the coated capillary is directly fixed on the six-port valve (valve 2 in Fig. 1) and none of the additional connecting tube is necessary. The function of the autosampler is achieved by using a pump and a six-port valve with a PEEK tube as the sample loop. The sample volume can be easily and accurately controlled by controlling the valve switch-reswitch time interval and the flow rate of the carrier solution, which guarantees the precision of the extraction. Besides, the extraction segment is independent of the analysis segment, thus the sequential runs can be operated simultaneously with the separation process of the previous sample and the time for the whole analysis can be shortened.

Table 1
Program for in-tube SPME process

Event	Pump A	Valve 1	Valve 2	Time
Fill the PEEK tube with methanol	Run	Load	Load	
Condition the capillary with methanol	Run	Inject	Load	1 min
Condition the capillary with H_2O and fill the PEEK tube with sample	Run	Load	Load	2 min
Extraction begin	Run	Inject	Load	50 s
Extraction end	Run	Load	Load	
Wash the capillary with H_2O	Run	Load	Load	1 min
Desorption and separation	Stop	Load	Inject	

For the traditional system with autosampler, the residual mobile phase in the extraction segment was thought to bring the inaccurate results in quantification [7,16]. It is noteworthy that with the present system, the carrier solution, double distilled water for instance, is allowed to flow through the whole extraction segment to substitute all the residual solution before the following extraction. In the case, the extraction segment is independent of the analytical segment and the inaccurate quantification caused by mixing of sample/residual mobile phase will be avoided. The validation of this system can be performed by substituting the coated capillary with an uncoated capillary or an inert stainless steel tube, as that was done in reference [7]. No analyte was found after extracting 250 μL of spiked samples. While for extraction using the traditional one, an inner stainless steel capillary showed similar extraction ability to that of the Omegawax-250 capillary in extracting ranitidine after 15 draw/eject cycles of extraction [7].

3.2. Optimization of separation and desorption

After extraction completed, on line desorption of the analytes was simply accomplished by directing the mobile phase through the capillary. Thus the mobile phase should provides complete desorption of the extracted analytes, while still providing the proper separation of the three analytes in the analytical column. Methanol mixed with different content (10–30 vol.%) of NaAc buffer solution at different pH (varied from 4 to 5.5) was evaluated as mobile phase. As a result, 70% methanol mixed with 30% NaAc buffer solution at pH 5.0 was selected in respect that it could provide proper retention time and separation factor while at the same time, carryover was not detected by performing blank analysis.

3.3. Extraction profile

The extraction profile for KEP, FEP and IBP was obtained by extracting 1 μg/mL sample solution for progressively longer periods of sampling time. The flow rate of the carrier solution was kept at 500 μL/min, and the valve switching time interval was increased from 6 to 69 s, corresponding to 50–575 μL of sample volume. As shown in Fig. 2, the amount of extracted analyte increased greatly with the increasing sample volume and the extraction equilibrium had not been reached even after 69 s sampling time for FEP and IBP. However, it seems that equilibrium has been reached for KEP. This result might be ascribed to the weak interaction between KEP and β-cyclodextrin, because the stability constant, $K_{1:1}$, of inclusion complexes formed between the NSAIDs and β-cyclodextrin is related to the hydrophobility of the NSAIDs, and was found to be in the order of IBP > FEP > KEP [18]. Though it is recognized the sample volume required to reach equilibrium value would ensure the highest possible sensitivity, further increasing the sampling time, i.e. the sample solution volume will increase the analysis time, which dose not favor routine analysis. Thus, 250 μL of sample solution was selected to achieve sufficient extraction efficiency within a shorter period of time.

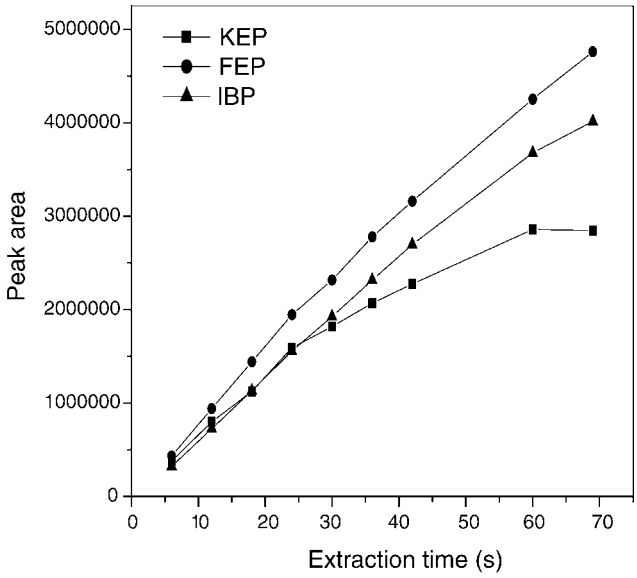

Fig. 2. In-tube SPME-HPLC extraction time profile of KEP, FEP and IBP. Sample solution consisted of KEP, FEP and IBP, all spiked at 1 μg/mL. Extraction flow rate was 500 μL/min; desorption mobile phase was methanol–sodium acetate buffer solution (pH 5.0) (70:30, v:v). Detection wavelength was set at 223 nm.

3.4. Optimization of in-tube SPME conditions

3.4.1. Extraction flow rate

It is known that the time required to obtain extraction equilibrium is proportional to the length of the capillary, the analyte distribution constant and the volume of the coating, while it is inversely proportional to the extraction flow rate [3,5]. Thus for the capillary used here, the extraction flow rate was optimized first to obtain high extraction efficiency while still offering a reasonable analyzing time. Keeping the total sample solution volume for extraction constant at 250 μL, different extraction times were employed (150, 50, 30, and 15 s), corresponding to flow rate at 100, 300, 500 and 1000 μL/min, respectively. It can be seen from Fig. 3 that the extraction efficiency was higher with a lower flow rate, suggesting that the mass transfer of the analytes to the active sites was improved with longer extraction time, which was different from observations reported [12]. Further increasing the extraction time to beyond 50 s just leads to small enhance of the extraction efficiency, thus from a analytical point of view, the flow rate of 300 μL/min was selected for the following experiments, which will offer both relatively short analysis time and adequate extraction efficiency.

3.4.2. Salt concentration and pH of the matrix

Further enhancement of the extraction efficiency of the in-tube SPME-HPLC system was achieved by optimizing

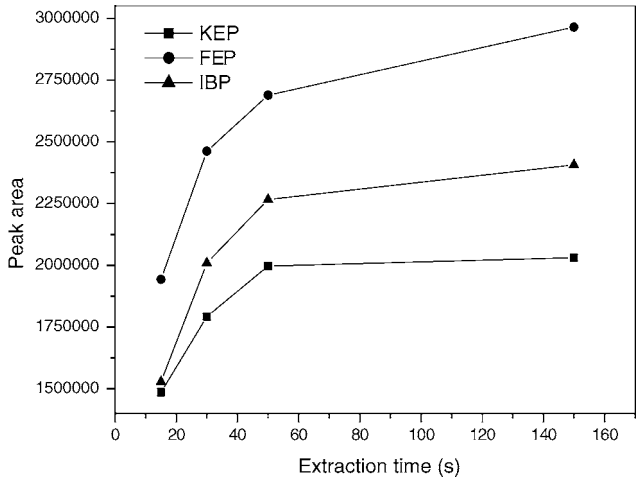

Fig. 3. Relationship between extraction time and extraction efficiency (represented as peak area). Sample solution consisted of KEP, FEP and IBP, all spiked at 1 μg/mL. Extraction sample volume was 250 μL; desorption mobile phase was methanol–sodium acetate buffer solution (pH 5.0) (70:30, v:v). Detection wavelength was set at 223 nm.

the sample matrix, including the inorganic salt concentration and pH.

Some authors thought that addition of salt to the sample matrix for SPME was useful to reduce the amount of water molecules effectively available for solvation of the analytes, which could result in an increase in the extraction efficiency [15]. However, we have found the opposite in performing the extraction by adding 100 mg/L to 10 g/L NaCl to the sample matrix. A decrease in extraction efficiency was found when increasing the salt concentration, especially for KEP. The reasons might lie on that the presence of the salt might act as competitive subject for the analytes to form inclusion complexes with β-cyclodextrin [23,24] and affected the distribution equilibrium of the analytes [25,26]. KEP seems to be affected by the salt most strongly for it interacts with β-cyclodextrin most weakly of the three, as discussed in Section 3.3. However, IBP still maintained 71% extraction efficiency in the presence of 10 g/L of salt in comparison to that obtained from a sample matrix of double distilled water, suggesting that IBP interacted more strongly with β-cyclodextrin than other two analytes.

The NaAc buffer solution in pH range of 3–7, within which the coating could maintain stable, was used to investigate the influence of the matrix pH on the extraction efficiency. When pH increased from 3 to 7, the extraction efficiency for the drugs increased. What we found was not in accordance with the observation that β-cyclodextrin cavity has a preferential affinity for the neutral acid form [27] since KEP, FEP and IBP are likely to exist in their deprotonated forms when the pH increases to 7 [28]. It could be suspected that other factors besides inclusion complexation are affecting the interaction between the drugs and β-cyclodextrin. And the coating matrix was supposed to be partly responsible for the results since the unreacted silane coupling agent might remained and have influence on the retention of the drugs in the coating. Further investigation on the property of β-cyclodextrin-containing coating obtained by different synthesis method is undertaken.

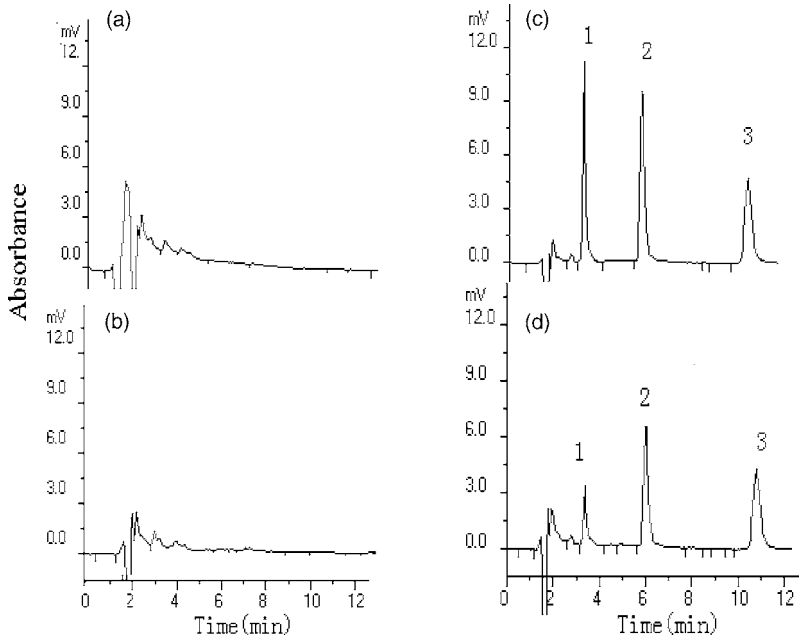

Fig. 4. Chromatograms obtained from the in-tube SPME-HPLC system. (a) Extraction of a urine sample with an uncoated capillary, (b) extraction of a blank urine sample with a coated capillary, (c) extraction of sample with double distilled water as the matrix, and (d) extraction of urine sample spiked with KEP, FEP and IBP at 1 μg/mL with coated capillary. The sample volume for extraction was 250 μL. Extraction flow rate was 300 μL/min; desorption mobile phase was methanol–sodium acetate buffer solution (pH 5.0) (70:30, v:v). Detection wavelength was set at 223 nm. (1) KEP, (2) FEP, and (3) IBP.

Table 2
Extraction efficiency and method reproducibility for SPME of KEP, FEP and IBP

Compound	Standard sample			Urine sample		
	Extraction efficiency (ng)	Intra-day precision (%)	Inter-day precision (%)	Extraction efficiency (ng)	Intra-day precision (%)	Inter-day precision (%)
Ketoprofen	139.3	2.8	3.6	41.8	4.9	6.9
Fenbufen	250.5	1.9	1.6	170.3	3.3	2.9
Ibuprofen	286.7	1.5	1.9	255.2	2.3	2.5

The extraction efficiency was calculated after extraction of 575 μL of sample solution. The intra-day and inter-day precisions were determined with repeated analysis of a sample that has been independently prepared over 1 day ($n = 5$) or for continuous 5 days.

3.5. In-tube SPME of urine samples

SPME provides an alternative approach for extracting analytes from complex sample matrices for it combines the preconcentration and removal of the interfering component as a whole. Using the previously described in-tube SPME configuration and the optimized conditions for extraction and separation, urine samples were investigated.

For unspiked samples or uncoated capillary in extracting spiked samples, no peaks were found in the chromatograms, as shown in Fig. 4(a,b). When extracting urine samples, a decrease in extraction efficiency was observed for the coated capillary, as shown in Table 2; the co-existence of inorganic salt and protein are thought to be responsible for it. However, it is also noteworthy that the extraction performance of IBP is affected by the matrix to the smallest extent, which was consistent with what we found in Section 3.4.2. The conclusion could be drawn that IBP was strongly interacting with β-cyclodextrin without being too much affected by the sample matrix, and thus was more suitable for extraction with the β-cyclodextrin coatings. The matrix peak in the chromatogram of spiked real sample was not obviously larger than that of standard samples, indicating that it is effective to allow the carrier solution to continue to flow through the capillary after extraction to wash off the matrix to a considerable extent. Besides, it could be expected that components having weak interaction with β-cyclodextrin would be removed as well as the sample matrix by the thorough washing step and thus the interference to the analysis of three drugs could be depressed remarkably. Our previous research work also demonstrated it [24,25].

Calibration curves for the standard samples and urine samples were constructed with the results listed in Table 3: excellent linearity was observed for all the analytes. The detection limit (DL) was established by calculating the lowest analyte concentration that produced a detectable signal three times above the value of the baseline noise. The DL for urine sample was not so well as that of standard sample due to the influence of the sample matrix but was still adequate for support of clinical studies. The precision of the developed method was proved to be satisfactory, as could be seen from Table 2, indicating the configuration of in-tube SPME-HPLC system works efficiently and reliably in extracting KEP, FEP and IBP.

To compare the sensitivity of the SPME and direct injection, the calibration curves were also constructed for the latter. It is found that the slope of the calibration curve obtained by SPME was about 10 times larger than that for direct injection method, indicating that the great increase in sensitivity was achieved with this in-tube SPME-HPLC system.

The preparation of the β-cyclodextrin coated capillary was easily performed by sol–gel method. The reproducibility of the preparation was evaluated by comparing the extraction efficiency of three independently prepared capillaries under the same conditions, and no obvious difference of their extraction ability was found with the R.S.D. less than 6% for the extraction efficiency. In our experiment, the coated capillary was shown to be functional after 250 extractions without any decrease in the extracting efficiency, suggesting its reusability in routine practical analysis, es-

Table 3
Linear regression and detection limits data for calibration curves of KEP, FEP and IBP

Compound	Standard samples				Urine samples			
	Regression line			DL (ng/mL)	Regression line			DL (ng/mL)
	Slope	Intercept	r		Slope	Intercept	r	
Ketoprofen	2.4E6	43040	0.9999	1.7	3.9E6	202288	0.9989	38
Fenbufen	4.2E6	−12734	0.9998	9.2	1.6E6	68882	0.9999	18
Ibuprofen	3.4E6	29682	0.9999	10	1.7E6	49633	0.9999	28

Concentration range for calibration was 0.05–1 μg/mL for standard samples and 0.1–2.0 μg/mL for urine samples; sample solution consisted of KEP, FEP and IBP spiked in double distilled water for standard samples or diluted urine for urine samples at 1 μg/mL. The sample volume for extraction was 250 μL; extraction flow rate was 300 μL/min; desorption mobile phase was methanol–sodium acetate buffer solution (pH 5.0) (70:30, v:v). Detection wavelength was set at 223 nm.

pecially when biological and environmental samples are employed.

4. Conclusions

The in-tube SPME-HPLC system provided a simple on-line approach for performing microextraction. In comparison to the configuration of the most popular in-tube SPME-HPLC system, this new configuration can be constructed easily with a pump and a switching valve while still providing on-line analysis with accurate and reproducible quantitative results. Therefore, the system was convenient for common laboratory use. A capillary with the inner surface coated with β-cyclodextrin by sol–gel method was used in this in-tube SPME system to extract three NSAIDs, KEP, FEP and IBP, from urine samples. Satisfying detection limits and good precision were obtained with optimized in-tube SPME conditions, especially for IBP. Since the inclusion complex between β-cyclodextrin and certain molecules have been investigated a lot, it is expected that many more analytes, not only drugs in biological samples but also pollutants in environmental samples, could be managed using this coating. And additional experiments in our laboratory are underway to further facilitate this configuration as useful tool for determination of a wide range of analytes in complex biological and environmental samples.

Acknowledgements

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