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Pretreatment of plasma samples by a novel hollow fiber centrifugal ultrafiltrate device for the determination of cefaclor concentrations in human plasma

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ABSTRACT

A simple sample preparation method was developed by using a centrifugal ultrafiltration (CF-UF) device with hollow fiber (HF) for the determination of cefaclor in plasma by HPLC. Samples were placed into a homemade device, which was consisted of a glass tube and a U-shaped hollow fiber. The filtrate was withdrawn from the hollow fiber into a syringe after centrifugation and 20 μL was directly injected into the HPLC for analysis. The HPLC method had a linear calibration curve in the concentration range of 6.00×10^{-2}–30.7 μg mL^{-1} ($r = 0.9996$). The limit of detection (LOD) and limit of quantitation (LOQ) were 0.02 and 0.06 μg mL^{-1}, respectively. The intra and inter-day precisions (RSD) were 1.7%, 1.2%, 1.0% and 3.6%, 2.5%, 1.9%, respectively, for three concentrations. Assay accuracy was higher than 99.2% and the absolute recovery was 86.8–92.5%. It is feasible to use this novel and low cost device for sample pretreatment for the analysis of cefaclor in plasma.

1. Introduction

Determination of drug concentration in blood samples is a routine task in the field of biopharmaceutical analysis and therapeutic drug monitoring. It is well known that removing the proteins is the key step for separation and analysis of drugs in blood samples. Currently, protein precipitation (PPT) [1], liquid–liquid extraction (LLE) [2,3], solid-phase extraction (SPE) [4] and ultrafiltration (UF) [5] are the techniques frequently used for sample preparation.

In PPT process, a variety of reagents, such as acids, salts and organic solvents, are added to plasma or serum to remove proteins as well as to solublize hydrophobic analytes [6]. After centrifugation, the upper clear layer is analyzed to determine the total drug concentration. However, to precipitate the proteins completely, the sample has to be diluted 4–8-fold by the addition of reagents [7]. As a result, the sensitivity is greatly reduced. LLE is another widely used method to isolate the analytes from plasma, in which the organic phase containing the analytes is evaporated to dryness after extraction and the residue is re-dissolved in a small-volume of liquid to increase the concentration [8]. This method is simple and the extracts are clean, but it is generally unsuitable for hydrophilic compounds [9]. In recent years, SPE has become popular and the most often used sample pretreatment technique. It is simple and has good specificity. At the same time, a significant degree of analyte enrichment (between 10 and 100-fold) can be achieved. Unfortunately SPE is an expensive and complicated process, which requires protein precipitation first. UF separates fluid and proteins by allowing only small molecules to pass through the membrane. Recently, UF has been widely used a method preferred for protein enrichment and the separation of small molecules from proteins [10–15]. It is also used in other areas for the separation of small molecules and macromolecules [16–20]. Recently, UF devices for the treatment of small volume samples have been commercially available, such as the Amicon Ultra-15 centrifugal filter units [11], the Centricon-10 (Amicon, Beverly, MA, USA) [12], Amicon Ultra-4 centrifugal filter devices (Millipore, Billerica, MA) [15,16], the Ultrafree-MC centrifugal filter units (Millipore, USA) [21,22], and Nanosep® 10 K centrifugal filter device (PALL Corporation, USA) [10,23–25]. Even though a flat membrane is used, there is still concentration polarization [26] because the centrifugal force is perpendicular to the membrane. Moreover, the size of membrane pore can change and the membrane can even break off when the centrifugal force exceeds the limit. And these commercial devices are usually expensive.

In the present work, a homemade device, which contain a slim glass tube and a U-shaped hollow fiber, was used to overcome the concentration polarization phenomenon because the direction of centrifugal force is parallel to the hollow fiber [27]. In addition, this device avoids sample dilution of protein precipitation and only 0.2 mL of sample was used for sample preparation. The device used in this paper is an improved version of our previously studied devices [27,28]. For the previous device, about 10 mL sample solution is needed. The small volume of new device seems more suitable for the analysis of biological specimen. Therefore, it is possible to

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use this method for the direct measurement of the concentration of small molecule drugs in plasma.

Cefaclor is one of the second generation cephalosporin antibiotics, which is prepared by a semi-synthetic method. Cefaclor is water-soluble and orally administered [29]. It has a broad antibacterial spectrum against gram-positive bacteria and gram-negative bacteria [30]. The sample preparation for the determination of cefaclor in human plasma was mainly carried out by the protein precipitation method [29,31–33]. In this report, we present an improved method using a novel UF device without sample dilution. Although other sample preparation method could obtain the same level of LOD using the HPLC system with UV detection [31], the proposed method is more convenient and does not need any preconcentration step. Samples can also be batch processed to save time. The method is inexpensive because the hollow fiber can be reused after washing.

2. Experimental

2.1. Materials

Cefaclor standard and internal standard cefradine (IS) were obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Test preparation was offered by Bokang Pharmaceutical Co. Ltd. (Shanxi, China), and reference preparation was purchased from Eli Lilly and Company (USA). Acetonitrile (HPLC grade) was purchased from Kangside Scientific (Tianjin, China), and tetrahydrofuran (THF) was from Fisher Scientific (USA). Deionized water (HPLC grade) was prepared using the Milli-Q50 water purification system (Millipore, Bedford, MA). All other chemicals were of analytical grade. The polyvinylidene difluoride (PVDF) hollow fiber membrane was from FoShan, China. The wall thickness of this fiber was 200 μm, the inner diameter was 1000 μm, and the molecular weight cut-off was 10,000 Da.

2.2. Apparatus and HPLC conditions

Analysis was performed on a HPLC system consisting of an L-6200A ternary pump (Hitachi, Japan) and a 785A UV detector (Applied Biosystems, USA). The chromatogram was monitored at 265 nm, and data were collected by a HW chromatograph data workstation (Qianpu. Corp., Nanjing, China). Separations were accomplished on a Diamonsil C18 column (250 mm × 4.6 mm, 5 μm, Dikma, China) at room temperature. The separations were performed under isocratic elution using a mobile phase containing of acetonitrile (6%), tetrahydrofuran (5%) and 10 mmol L^{-1} sodium dihydrogen phosphate (pH 2.9, 89%). The flow rate was 1 mL min^{-1}.

2.3. Standard solution preparation

Stock solutions of cefaclor and IS cefradine were prepared with deionized water and diluted with mobile phase to obtain solutions containing 614 μg mL^{-1} cefaclor and 19.0 μg mL^{-1} internal standard, respectively. A series of working solutions containing compounds at appropriate concentrations were prepared by diluting stock solutions with mobile phase. The final solution containing cefaclor (1.92 μg mL^{-1}) and cefradine (1.90 μg mL^{-1}) was prepared to investigate the optimum conditions of centrifugal ultrafiltration just before the start of the experiment. The stock solutions were stored at 4 °C until use.

2.4. Sample preparation

Human blood in a 2 mL centrifuge tube was centrifuged at 10,000 rpm for 10 min to collect the plasma. 800 μL of plasma was spiked immediately with 100 μL of phosphate buffer solution (2.0 mol L^{-1}, adjusted to pH 2.0). The solutions were vortex mixed for 5 s, and stored at −20 °C.

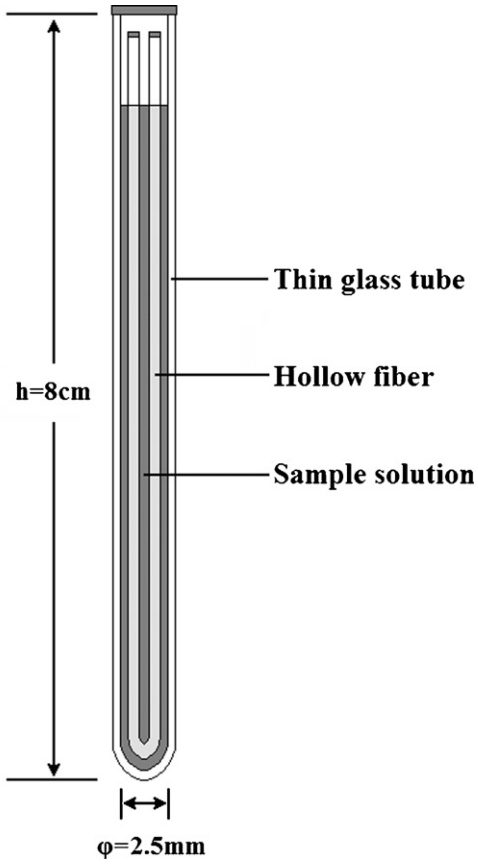

Fig. 1. The schematic diagram of the device used for sample pretreatment.

The buffered plasma (225 μL) and 25 μL IS cefradine solution (19.00 μg mL^{-1}) were mixed and placed into a homemade device, which was consisted of a glass tube and a hollow fiber. The hollow fiber was bent to form a U-shape (15cm) and put into the slim glass tube with both of the fiber's ends above the liquid level as shown in Fig. 1. The length of the glass tube is 8 cm and the i.d is 2.5 mm.After centrifugation for 20 min at 10,000 rpm, the filtrate from the hollow fiber was withdrawn with a syringe and 20 μL was injected for HPLC analysis.

3. Results and discussion

3.1. About concentration polarization

In our experiments, a hollow fiber centrifugal ultrafiltration (HF-CF-UF) technique was introduced, which simplified the plasma sample preparation process. The basic principle is similar to that of traditional ultrafiltration, where small molecules can pass through the membrane whereas macromolecules cannot do so in the centrifugal process.

For ultrafiltration the direction of the mobile fluid movement is perpendicular to the ultrafiltration membrane, and therefore sample solution is always affected by concentration polarization. A pycnotic macromolecular layer is formed during concentration polarization, and this layer further interferes with the filtration of small molecular substances, rapidly decreases filtering speed, and even blocks the membrane. To overcome concentration polarization, dilution is often used. But dilution could lead to a decrease of the concentration.

For the homemade HF-CF-UF device, separation of macromolecules and small molecules was easily achieved and concentration polarization was avoided [27]. Under this condition, small molecules can move freely so that the concentration of analyte in the interior is the same as in exterior.

3.2. Optimization of sample pretreatment procedure

3.2.1. Optimization of centrifugation

The efficiency of ultrafiltration is correlated with the centrifugal force, which was optimized in order to obtain the most efficient filtration. Samples were prepared by mixing 200 μL blank human blood, 25 μL 2.0 M phosphate buffer solution (pH 2) and 25 μL of a solution containing cefaclor (1.92 μg mL^{-1}) and internal reference cefradine (1.90 μg mL^{-1}). They were filtered at 6000, 8000, 10,000 and 12,000 rpm for 10 min, respectively. The results show that a weak centrifugal force would result in a small volume of filtrate and a too strong force would damage the hollow fiber. Therefore, we chose 10,000 rpm as the optimal speed for subsequent experiments.

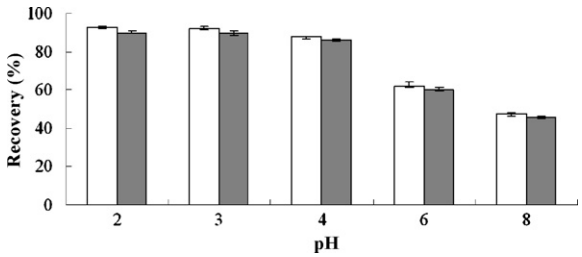

Fig. 2. Influences of buffer pH on recoveries of cefaclor and cefradine. □:cefaclor; ■:cefradine. Samples were prepared by mixing 200 μL blank human blood, 25 μL 2.0 M phosphate buffer solution at pH 2–4, 6 and 8 (adjusted by NaOH solution) and 25 μL of a solution containing cefaclor (1.92 μg mL^{-1}) and internal reference cefradine (1.90 μg mL^{-1}).

3.2.2. Effect of the buffer pH

Since cefaclor is partially bound to plasma proteins under physiological conditions, it has to be released from the drug–protein complex to allow the determination of the total drug concentration. We investigated the effect of pH on drug release and the results are shown in Fig. 2. Samples were prepared as before and treated

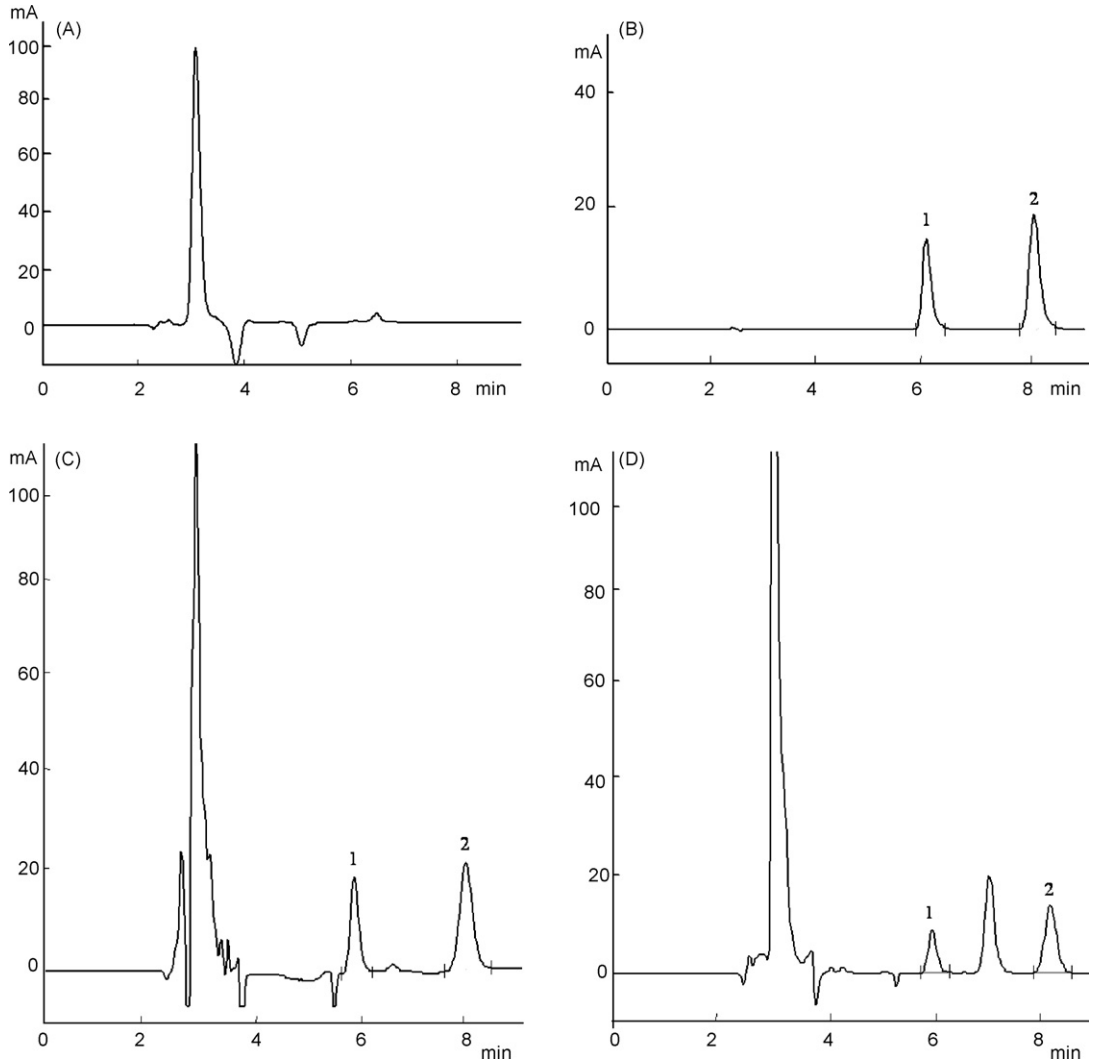

Fig. 3. HPLC-UV chromatograms of (A) human blank plasma, (B) standard solution (cefaclor 1.92 μg mL^{-1} and cefradine I.S., 1.90 μg mL^{-1}), (C) plasma sample prepared by hollow fiber centrifugal ultrafiltration and (D) by protein precipitation with methanol spiked with (1) cefaclor (1.92 μg mL^{-1}) and (2) cefradine (I.S., 1.90 μg mL^{-1}). The separations were performed under isocratic elution conditions using a mobile phase containing acetonitrile (6%), tetrahydrofuran (5%) and 10 mmol L^{-1} sodium dihydrogen phosphate (pH 2.9, 89%). Wavelength for detection was 265 nm.

Table 1
Results of recovery rate and precision of the method which was obtained from plasma samples centrifuged in HF-CF-UF device.

Plasma concentration ($\mu g\,ml^{-1}$)	Relative recovery (%) ($n = 6, \bar{x} \pm s$)	Within-day RSD (%)	Day-to-day RSD (%)	Absolute recovery (%) ($n = 6, \bar{x} \pm s$)
0.120	100.5 ± 1.7	1.7	3.6	86.8 ± 3.1
1.92	99.9 ± 1.2	1.2	2.5	92.5 ± 2.3
30.7	99.2 ± 1.0	1.0	1.9	91.9 ± 1.7

separately with 25 μL of 2.0 M phosphate buffer at pH 2–4, 6 and 8 (adjusted by NaOH solution). As shown, the recovery increased with the decrease of pH over the entire study range and most bound drugs were released in acidic environment. Based on these results, the buffer solution of pH 2 was used to acidify the plasma samples in the following experiments.

3.2.3. Effect of concentration of phosphate buffer

The buffering capability of phosphate solution is important to maintain a stable environment and the drug stability. The capability of buffer was evaluated by monitoring the change of pH of the plasma after adding the phosphate buffer. 25 μL solution containing cefaclor ($1.92\,\mu g\,mL^{-1}$) and internal reference cefradine ($1.90\,\mu g\,mL^{-1}$) was added into 200 μL blank plasma and three different concentrations of pH 2 phosphate buffer (2.0, 1.0 and 0.5 M) were added. The pHs of the acidified samples were 3, 5, and 6, respectively, after treatment with three concentrations of phosphate buffer. Since the lower pH promotes the releases of bound drugs and stable analyte solution was obtained without altering the volume of filtrates when a high concentration of phosphate salt was added, 2.0 M buffer solution was chosen for the pretreatment and sample storage.

In summary, the optimal conditions of the sample preparation for the analysis of cefaclor levels in plasma were to add 2.0 M buffer (pH 2) to plasma and to centrifuge at 10,000 rpm for 20 min.

3.3. Comparison of centrifugal ultrafiltration and protein precipitation

The sensitivity of the method is a key factor for the analysis of drugs in biological samples. When samples were treated by protein precipitation, the concentration of analyte was reduced several times by the organic solvent [21]. In contrast, the concentration of analyte did not change much after treatment with the ultrafiltration method. The chromatograms of samples treated with these two methods are shown in Fig. 3C and D. It shows that the peak-area of the analyte treated with the proposed method was 2–3 times higher than that treated by protein precipitation for the same sample. Moreover, the chromatogram of the sample treated by CF-UF is cleaner compared with the protein precipitation method. Therefore, the CF-UF method is suitable for measuring cefaclor concentration in plasma with higher sensitivity and less interference compared with the traditional protein precipitation procedure. The determined LOD was $0.02\,\mu g\,mL^{-1}$ compared with the reported LOD of $0.2\,\mu g\,mL^{-1}$ by the protein precipitation method [21].

3.4. Method validation

3.4.1. Specificity

The specificity of the assay was evaluated by comparing the chromatogram of plasma sample spiked with analytes with the chromatogram of blank plasma with no drugs or internal standard. The peaks of the drug and IS were well separated and there was no interference from endogenous compounds at the retention times of the analytes (Fig. 3A–C).

3.4.2. Linearity, LOD and LOQ

The calibration curve was constructed by plotting the peak-area ratios (cefaclor/cefradine) with the concentrations of cefaclor. The linear range was 6.00×10^{-2}–$30.72\,\mu g\,mL^{-1}$ with a correlation coefficient of 0.9996 by using a weighted linear regression method. The calibration equation was $A = 0.248C - 3.38 \times 10^{-3}$, where C is the concentration of cefaclor in $\mu g\,mL^{-1}$. The LOD and LOQ were determined separately in five replicates at signal-to-noise ratios (S/N) of 3 and 10, respectively. The determined LOD and LOQ were 0.02 and $0.06\,\mu g\,mL^{-1}$, respectively.

3.4.3. Accuracy, precision, absolute recovery and stability

The accuracy and extraction recovery of the new method are shown in Table 1. Measurements of intra- and inter-day precisions with multiple concentrations were performed to assess the repeatability and reproducibility of the developed method. The relative standard deviations (RSD) of intra-day precision of the three concentrations were 1.7%, 1.2%, and 1.0%, respectively. The inter-day precision of the method was evaluated by assessing sample concentrations at higher, middle and lower linearity ranges on 6 consecutive days. The RSD values of inter-day precision were 3.6%, 2.5% and 1.9%, respectively (Table 1).

To evaluate freeze-thaw stability, samples were subjected to freezing for 24 h at −20 °C and thawing at room temperature for three cycles. The stability at freezing was assessed by keeping samples for 48 h at −20 °C and room temperature stability was assessed by placing samples at room temperature for 6 h. All RSD values for the stability samples were below 3.6%.

Table 2
Plasma concentration–time curves measured by reported method and HF-CF-UF method after single oral dose of 500 mg cefaclor in 6 healthy volunteers ($C\,(\mu g\,ml^{-1})$).

Time (h)	Reported method						HF-CF method					
	1	2	3	4	5	6	1	2	3	4	5	6
0.25	2.43	0.14	0.76	0.06	0.87	2.47	2.54	0.13	0.85	0.10	0.72	2.53
0.5	14.40	0.41	2.48	1.60	6.17	6.27	14.38	0.35	2.57	1.79	6.03	6.39
0.75	22.95	4.37	6.03	8.77	12.83	14.08	22.91	4.07	6.58	9.24	12.24	14.27
1.0	10.78	9.39	10.60	10.09	16.65	19.62	10.62	9.21	10.81	10.78	16.43	19.08
1.5	9.17	8.06	6.28	9.58	11.81	11.11	8.71	8.35	6.16	9.73	11.67	11.96
2.0	4.18	6.44	6.20	6.08	4.35	9.43	4.44	6.28	6.39	6.54	4.52	9.65
2.5	2.47	4.31	5.05	4.82	2.23	4.24	2.42	4.36	5.39	4.99	2.52	4.09
3.0	1.43	2.13	3.91	2.33	1.29	2.40	1.27	2.01	3.51	2.09	1.37	2.29
3.5	0.82	1.10	1.89	1.47	0.98	1.35	0.86	1.01	1.75	1.47	0.82	1.50
4.0	0.41	0.42	0.64	0.45	0.37	0.57	0.29	0.42	0.60	0.44	0.38	0.59
4.5	0.18	0.14	0.37	0.24	0.14	0.31	0.18	0.18	0.34	0.25	0.17	0.21
5.0	0.10	0.08	0.25	0.13	0.12	0.14	0.12	0.08	0.16	0.13	0.14	0.10

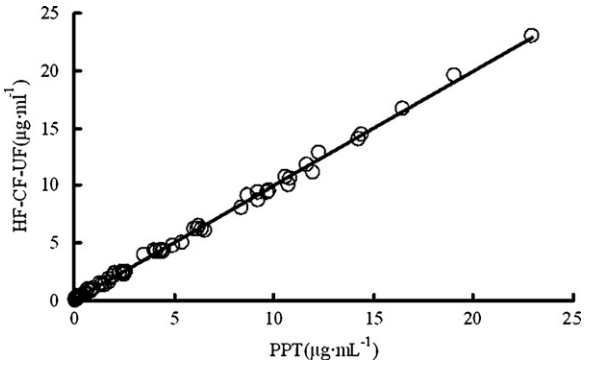

Fig. 4. Correlation of cefaclor levels in plasma between the HF-CF-UF and PPT methods.

3.5. Analysis of human plasma samples

The developed HF-CF-UF technique was finally applied for the analysis of human plasma samples. Six volunteers (all male, aged 18–25 years) were recruited at the Second Hospital of Hebei Medical University and they were administered with a single oral dose of 500 mg cefaclor. A written consent was obtained from each volunteer. Plasma samples prepared from these healthy male adults were analyzed by the reported method with protein precipitation [33] and HF-CF-UF method, respectively. In the protein precipitation method, 0.2 mL plasma and 0.4 mL methanol solution of internal standard were mixed and centrifuged for 10 min at 13,500 rpm. 20 μL supernatant was injected for HPLC analysis. The concentrations of the drug in plasma were determined and the results are listed in Table 2. Statistical analysis of the two sets of results indicated the *P*-value was >0.1 by paired-sample *t*-test. Hence, there is no significant difference between the results by the reported method [33] and the HF-CF-UF method. Concentrations of plasma cefaclor in hamster were compared between HF-CF-UF method and PPT method. The data obtained by HF-CF-UF method nicely correlated with those obtained by PPT method (r^2 = 0.9978, Fig. 4). Thus, HF-CF-UF method used in this study is suitable to measure cefaclor in human plasma.

4. Conclusion

A novel sample preparation method to determine the cefaclor concentration in a relatively small-volume of human plasma was developed. The method is based on hollow fiber filtration. Compared with the reported protein precipitation method, the new method is simpler, which requires only centrifugation for a short time and the filtrate can be injected directly for HPLC analysis without further treatment. The sensitivity of HPLC analysis is higher because the sample is not diluted. Our results also show that sample preparation by HF-CF-UF can be used for the quantitative analysis of cefaclor in human plasma and it may be more reliable than the protein precipitation method.

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