RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Accuracy on the analysis of free vancomycin concentration by ultrafiltration in various disease states

Jin-feng Zhang [a], Xiu-ling Yang [b], Zhi-qing Zhang [b], Wei-chong Dong [a], Ye Jiang [a*]

a. Department of Pharmaceutical Analysis, School of Pharmacy, Hebei Medical University, Shijiazhuang, Hebei Province 050017, China

b. Department of Pharmacy, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei Province 050000,China

*Corresponding author. Tel (Fax):+86 311 86266025;

E-mail addresses: jiangye@hebmu.edu.cn, jiangye1@126.com (Y. Jiang).

ABSTRACT

In the present study, accuracy on the analysis of free vancomycin (VCM) by ultrafiltration in various disease conditions was assessed. With VCM as a representative drug, we used clinical plasma samples to investigate the effect of plasma conditions on volume ratio of ultrafiltrate to sample solution (V_u/V_s) and the consequential effect on measured free drug concentration (f_c). Our results demonstrated that plasma conditions had a significant impact on V_u/V_s by centrifugal ultrafiltration (CF-UF). The V_u by CF-UF ranged from 97μL to 279μL among different individuals under the same centrifugation conditions. Total protein levels and the osmotic pressure of plasma were the main influence factors of V_u/V_s in disease states. In contrast, the V_u/V_s by hollow fiber centrifugal ultrafiltration (HFCF-UF) were less influenced by plasma conditions. As a consequence, the results of f_c determined by HFCF-UF were more accurate than that by CF-UF for patients with different disease states. HFCF-UF displayed great advantages in clinical samples for accurate analysis of f_c. It has been successfully applied to monitor free VCM in clinical plasma samples in routine therapeutic drug monitoring (TDM).

Keywords: Free vancomycin; Therapeutic Drug Monitoring; Ultrafiltrate volume; Plasma conditions

1. Introduction

It is well known that only the free fraction of a drug is able to penetrate to the target site and is pharmacologically active.[1,2] Especially in severe or confounding disease states (e.g., uremia, renal failure or hypoalbuminemia), the suboptimal treatment or toxic effect of drugs may occur at the total concentration remaining within the therapeutic range.[1-3] Therefore, the analysis of free drug concentration (f_c) in plasma is more accurate for TDM.[1-4]

In recent years, centrifugal ultrafiltration (CF-UF) is frequently used for the analysis of f_c in clinical laboratories.[5-9] However, the V_u/V_s by CF-UF was not well controlled and affected the accurate monitoring of f_c.[9,10] Constant and tiny V_u/V_s ratios are critical for real representation of f_c in patients.[10] Therefore, the control of V_u/V_s is a challenge in the clinical laboratories when large batches of samples need to be monitored simultaneously. The dependency of V_u/V_s on both centrifugation force and centrifugation time has been demonstrated in pooled normal plasma.[7-10] Therefore, some reports attempted to control centrifugation time and centrifugation force to obtain uniformity and appropriate V_u/V_s when CF-UF was applied.[7,8] Sometimes the results of precision for the validation of CF-UF were unsatisfactory.[5-8,11] Some authors demonstrated that the V_u/V_s by HFCF-UF could be controlled by the inner diameters of both the glass tube and hollow fiber.[10] However, those studies were all carried out in pooled plasma from healthy volunteers, and were rarely assessed in clinical plasma samples.

It should be noted that plasma conditions (e.g., protein levels, viscosity, cholesterol levels, osmotic pressure, etc.) of the patients vary with individuals[12,13] and are significantly different from that of healthy subjects,[1,14,15] whose pooled plasma is usually used for the development and validation of assay method. The pooled plasma of normal subjects has consistent plasma conditions, and therefore acceptable V_u/V_s can be realized through strictly controlled centrifugation time and centrifugation force under the same centrifugation conditions. Using pooled plasma samples with same plasma conditions could give compromised results for the validation of the assay

method. Nevertheless, the adequacy of the method for analysis of clinical samples from patients in various disease states may be suspected. The main populations of TDM are critically ill patients, whose plasma conditions differ greatly. It is unclear whether the uniformity and appropriate V_u/V_s can be controlled under the same centrifugation conditions for those samples. However, no studies have investigated the effect of plasma conditions on V_u/V_s and the related effect on monitoring of f_c in clinical samples.

Vancomycin (VCM) is a drug which has been the cornerstone of treatment in critically ill patients with serious bacterial infections (Coagulase-negative staphylococci, *Staphylococcus aureus*, enterococci and meticillin-resistant *S.aureus*).[16,17] The physiological and pathological conditions (live cirrhosis, chonic renal disease, hypoproteinemia) of those patients was very complex which made the plasma conditions such as protein levels, bilirubin levels, cholesterol levels, plasma osmotic pressure (etc.) appear significantly different. To date, published data on the unbound fraction of VCM (f_u) in patient samples exhibits high variability, with ranges from 3.7% to 82%.[11,18-25] Numerous studies have been put forward in an attempt to investigate the wide range of f_u, and the large variations were related to the large inter-individual variability.[20,21] CF-UF was commonly used for analysis of free VCM in plasma.[11,18,23] None of them considered the associated factors of methodology due to the effect of V_u/V_s resulting from plasma conditions of the patients on the monitoring of free VCM, which may likewise have an impact on this high variability.

In our present study, we used plasma samples of critically ill patients treated with VCM to explore the effect of plasma conditions on V_u/V_s and the related effect on measured free VCM. Furthermore, we validated an accurate method that was less affected by plasma conditions and it was successfully applied to monitor free VCM in clinical plasma samples in routine TDM.

2. Experimental and methods

2.1. Chemicals and Materials

Vancomycin standard was purchased from the National Institute of Control of Pharmaceutical and Biological Products (Beijing, China). Methanol of HPLC grade

was obtained from DIKMA (Lake Forest, CA). Deionized water was prepared using the Milli-Q50 water purification System (Millipore, Bedford, MA). Blank plasma was collected from The Second Hospital of Hebei Medical University (Hebei, China). All other chemicals and reagents were of analytical grade. The hollow fiber (molecular cut-off was 10kDa, wall thickness was 150μm, inner diameter was 1000μm) was obtained from Kaijie Membrane Separation Technology (Hangzhou, China). The slim glass tubes (7cm of height and 3cm of inner diameter) were purchased from Yongda Instrument and Chemical Company (Tianjin, China). The ultrafiltration devices (UFC 501096; 0.5mL, cut-off 10kDa) were purchased from Millipore Corp (Billerica, MA)

2.2. Apparatus and instruments

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 data were collected by a HW-2000 chromatograph data workstation (Qianpu. Corp, Nanjing, China). A temperature controllable centrifuge from Baiyang (Shanghai, China) was used, XW-80 Vortex mixer (Shanghai medical university Instrument Co.,Shanghai, China) was applied.

2.3. Chromatographic conditions

Separations of VCM were accomplished on a Diamonsil C_{18} column (150 mm×4.6mm, 5μm, Dikma, China) under an elution with methanol/0.05mol·L^{-1} potassium dihydrogen phosphate buffer solution (pH=3.2) at 20/80 (v/v) at a programmed flow-rate of 1mL/min at room temperature. The injection volume was 20μL and the detection of VCM was carried out at the wavelength of 236nm.

2.4. Preparation of solutions, quality controls (QC_S) and calibration standards (C_S)

The stock solution of VCM was prepared in phosphate buffered saline (PBS) (KH_2PO_4 (67mmol·L^{-1}) and NaCl (9g·L^{-1}) adjusted to pH 7.4 with NaOH) at a concentration of 2000μg·mL^{-1}. A series of VCM working standards at appropriate concentrations were prepared by diluting stock solution with PBS. All the stock solution and working standards were kept at 4°C.

Total VCM QC_S were prepared by spiking appropriate aliquots of the above-mentioned solutions of VCM into human blank plasma at three concentration

levels of 1.00, 10.0, 50.0μg·mL^{-1}. Free VCM QC_S were prepared at concentrations of 0.25, 2.00, 20.0μg·mL^{-1} in PBS to ensure a minimum percentage of non-specific binding (NSB) to filter materials. The C_S for the determination of free (0.25, 0.5, 1, 2, 5, 10, 20, 50μg·mL^{-1}) and total VCM (0.5, 1, 2, 5, 10, 20, 50, 100μg·mL^{-1}) were prepared in the same way using PBS and human blank plasma, respectively. Those concentrations were selected based on the concentration profiles from patients administered intravenously with VCM of test article.

2.5. Sample preparation

2.5.1. Total VCM sample preparation

Plasma was thawed at room temperature, added with 20μL of 10% Zinc Sulfate to 200μL of plasma sample in a 2mL Eppendorf tube, the mixture was vortexed for 5 min and then centrifuged at 4.0 × 10^{3}g for 10min at 37°C. 20μL of the supernatant was injected into HPLC for analysis.

2.5.2. CF-UF for Free VCM

500μL plasma was incubated in a water bath (37°C) for 10min and was subjected to CF-UF using a Centrifree tube at 2.0 × 10^{3}g for 10min at 37°C. 20μL ultrafiltrate was injected into HPLC for analysis.

2.5.3. HFCF-UF for Free VCM

For the first step, hollow fiber was sonicated in 50% methanol for 10min to remove any contaminants and dried then in air. For the second step, it was manually cut into 15cm segments and placed into the tube, then 500μL plasma was transferred into the glass tube and the tube was incubated in a water bath (37°C) for 10min, After centrifugation at 1.25 × 10^{3}g for 10min at 37°C, the ultrafiltrate (about 50μL) was pushed out from the lumen of the hollow fiber using a syringe. Finally, 20μL of the ultrafiltrate was injected into HPLC for analysis.

2.6. Study design and Clinical samples

This study is a prospective non-interventional cohort study. All patients were admitted to The Second Hospital of Hebei Medical University (Hebei, China). The patients who were receiving VCM by continuous infusion for documented Gram-positive infection and required therapeutic drug monitoring were considered for

inclusion. The protocol was approved by the Ethics Committee of the Second Hospital of Hebei Medical University, each subject or their guardians gave written informed consent to participation. Patient data were collected by a review of the medical records and data collection was conducted by trained staff at The Second Hospital of Hebei Medical University. The medical history (diabetes mellitus, hypoproteinemia, hypertension and chonic renal disease), total protein, albumin, globulin, prealbumin, total bilirubin, direct and indirect bilirubin, osmotic pressure of plasmas, total cholesterol and triglycerides were available for each patient.

Approximately 3mL blood from each patient was collected in a centrifuge tube containing heparin before VCM infusion, and immediately centrifuged. The obtained plasmas were then stored at -80°C immediately until analysis. Free and total VCM concentrations were measured according to validated HFCF-UF method. These results were evaluated and reported in medical record of the patients. For the purposes of present study, for the plasma samples with sufficient volume, free VCM was monitored both by CF-UF and HFCF-UF, respectively. The ultrafiltrate volume (V_u) was also calculated at the same time. Any replicate measurements from patients with multiple samples were treated as single events. No study interventions were undertaken.

2.7. The evaluation of ultrafitrate volume with CF-UF and HFCF-UF

The CF-UF device consists of sample reservoir and filtrate collection cup. Firstly, the weights of sample reservoir and filtrate collection cup were recorded as W_1 and W_2, respectively. A volume of 500μL (V) plasma was added to the sample reservoir, and the weight was recorded as W_{1+v}. So the plasma density (ρ) was calculated by means of Eqs. (1):

$$\rho = \frac{W_{1+v} - W_1}{V} \quad (1)$$

Then the CF-UF device was centrifuged at 2.0×10^3g for10min at 37°C. The filtrate collection cup was weighted again as W_{2+u}, and the ultrafiltrate volume (V_u) was calculated by means of Eqs. (2):

$$V_u = \frac{W_{2+u} - W_2}{\rho} \quad (2)$$

HFCF-UF device consists of a slim glass tube and a U-shaped hollow fiber. Firstly, a volume of 500μL plasma was added to the slim glass tube and the device was centrifuged at 2.0×10^3g for10min at 37°C. The ultrafiltrate in the lumen of the hollow fiber was transferred into an eppendorf tube. The weight of eppendorf tube was recorded as W_3 and W_{3+u} before and after transfer, so the ultrafiltrate volume (V_u') was calculated by means of Eqs. (3):

$$V_u' = \frac{W_{3+u} - W_3}{\rho} \quad (3)$$

2.8. Method validation

As part of standard clinical practice, the results of TDM for free VCM determined by HFCF-UF were reported in our hospital. Therefore, in order to meet the requirement for biological specimen and ensure accurate monitoring of free VCM in plasma with HFCF-UF, the method was validated according to FDA guidelines for bioanalytical method validation.[26] Specificity, linearity, accuracy, precision and stability of the analyte at various test conditions and recovery were all evaluated.

The specificity of the method was evaluated by comparing chromatograms of blank plasma sample, VCM standard solution, blank plasma spiked with VCM and plasma sample from patients for the test of endogenous interferences. Potential chromatographic interference by combined drugs and other commonly administered drugs (meropenem, biapenem, ambroxol, tienam, ceftazidime, etc.) was also studied.

The linearity was evaluated on five consecutive days by constructing freshly prepared calibration samples over the concentration range of 0.25-50μg·mL^{-1} for free VCM and 0.5-100μg·mL^{-1} for total VCM, respectively. The linearity of the relationship between peak area and concentration was determined by the correlation coefficient (R) using a $1/c^2$ weighted linear least-squares regression model. The relative standard deviation was calculated for all calibration curves (n=5). The limit of detection (LOD) and limit of quantification (LOQ) were defined as the concentration

of the compound at which the signal versus noise ratio (S/N) was equal to 3 and 10, respectively.

The intra-day, inter-day accuracy and precision of the method for free VCM were evaluated from quintuplicate analysis of each QC_S sample concentration in one day and repeated for five days. The accuracy was obtained by calculating the bias (%) and the precision by calculating CV (%).

Recovery of the method for free VCM (ratio percentage) was investigated using QC_S at three concentration levels. The Recovery was calculated by comparing the peak area obtained from the QC_S after preparation with HFCF-UF to the peak area obtained from those of corresponding standard solutions at the same concentration.

Six aliquots of QC_S samples at each level of three concentrations were prepared to investigate the stability of samples. Stability of total VCM in plasma and free VCM in PBS (short-term and long-term storage, freeze/thaw cycles, post-processing) were established. Short-term stability was evaluated by maintaining the samples at room temperature for 24h before analysis and the concentrations were compared to those obtained for freshly prepared samples. Long-term stability was assessed by comparing the concentration of QC_S samples kept at the storage temperature (-70°C) for 3 months with that of QC_S newly prepared. For the analysis of Freeze-thaw stability the samples were subjected to freezing for 24 h at -20°C and thawing at room temperature for three cycles, then the concentrations were compared with that of newly prepared QC_S. The post-processing stability of VCM at room temperature was also studied by analyzing the QC_S samples over a period of 12h and the results were compared to those obtained for freshly prepared samples. The stability was evaluated by the calculated accuracy: accuracy (%) = $(C_{found} - C_{initial})/ C_{initial}) \times 100$. C_{found} is the concentration of found in the QC_S spiked VCM and $C_{initial}$ is the theoretical concentration in the QC_S spiked VCM. The value of accuracy should be within ±15%.

2.9. Statistical analysis

Statistical analysis was performed using SPSS Statistics 17.0. Clinical characteristics were reported by their median and range. Unbound VCM fraction was calculated as the ratio of unbound to total drug concentrations as reported in the

literature. A comparison between unbound VCM fraction as determined by CF-UF and HFCF-UF method was done by using a paired Student *t* test. Linear regression analysis was used to evaluate the impact of different plasma conditions on the ultrafiltrate volume as determined by CF-UF. Scatter plots were used to elucidate the relationships between the dependent variability and ultrafiltrate volume. Two-sided α error < 0.05 were considered to indicate a statistically significant difference and reported as *P*-values.

3. Results and Discussions

3.1. Method validation

3.1.1. Specificity

No interference was detected in the plasma sample at retention times of the VCM. Representative chromatograms are presented in Fig. 1A-D.

3.1.2. Linearity, LOD and LOQ

The linear relationship between peak area and free concentration of VCM were described by the calibration equation: A=50865C+1288.1 (R^2=0.9999) in the range of 0.25-50μg·mL^{-1}. The linear relationship between peak area and total concentration of VCM in the range of 0.5-100μg·mL^{-1} were described by the calibration equation: A=28296C+4660.7 (R^2=0.9995). The LOD and LOQ were 0.1 μg·mL^{-1} and 0.25μg·mL^{-1} at the signal-to-noise ratios (S/N) of 3 and 10, respectively (n=5).

3.1.3. Accuracy, precision and recovery

The accuracy of the method ranged from 96.7% to 100.7% at three concentration levels. All RSD of intra-day, inter-day precision were less than1.62%.

Recovery was all about 100% for free VCM at three levels of QC_S (n=5) with RSD less than 4.8%, showing good consistency.

3.1.4. Stability

The stability of total VCM in plasma and free VCM in PBS was investigated under a variety of storage and processing conditions. VCM was stable at room temperature for 24h, at room temperature for 12h post-processing and -70°C for 3 months. VCM was also stable after three freeze-thaw cycle. All of the values for the

stability samples were within ±15% indicating that there is no need to worry the stability of the analyte during the whole analysis (the analyte were stable during the whole analysis).

3.1.5. Application to clinical study

A total of 102 samples from 43 patients were collected for TDM as part of patients' routine care. The mean (range) of total and free VCM plasma concentrations were 8.33μg·mL^{-1}(1.68-30.6μg·mL^{-1}) and 4.86μg·mL^{-1}(1.17-12.9μg·mL^{-1}). The mean unbound VCM fraction (f_u) was 46.6% (39.8-69.7%). The CV for inter-individual variability of f_u was 28.4%. The effective trough concentrations of VCM range form 15μg·mL^{-1} to 20μg·mL^{-1} for total concentration and 7.5μg·mL^{-1} to10μg·mL^{-1} for free concentration based on the protein binding ratio (approximately 50%). Out of 102 observations, 72 total VCM concentrations were within the therapeutic range of 15-20μg·mL^{-1}. However, there are approximately 21 which free VCM concentrations were out of the therapeutic range (7.5-10μg·mL^{-1}) among those 72 "therapeutic" total VCM concentrations. Consequently, in clinical practice the free VCM concentration should be used as the surrogate marker of VCM efficacy.

3.2. The evaluation of non-specific adsorption

Non-specific adsorption is a universal phenomenon in membrane isolation technique due to non-specific binding (NSB) to filter materials of the tested compounds. Therefore, the HFCF-UF procedure was examined for NSB of VCM. Four types of hollow fiber materials including polysulfone, polyvinyl chloride, polyvinylidene difluoride and polypropylene were used to evaluate NSB. Five replicates of VCM standard solutions were prepared in phosphate buffered saline at three concentrations (0.5, 5, 50μg·mL^{-1}), then the next operation was according to the HFCF-UF for free VCM. NBS was evaluated by analyzing and comparing the VCM levels before and after passage of the filter units. The ratio of obtained concentrations after and before HFCF-UF was about 98%±5% and RSD was less than 3.1%. So it can be considered there is no significant NBS with HFCF-UF. In present study, hollow fiber of polyvinylidene difluoride was used to separate unbound VCM. The CF-UF has been demonstrated no significant NBS existed.[18,19]

311 *3.3. The results of clinical sample by HFCF-UF and CF-UF*

312 A total of 31 plasma samples (from 25 patients) with sufficient volume were
313 analyzed by both CF-UF and HFCF-UF. Of those patients, 2 had two samples
314 collected and 2 had three samples collected. Chonic renal disease was diagnosed in 11,
315 hypoproteinemia in 9, diabetes mellitus in 4, and hypertension in 5 patients. The
316 clinical characteristics and relevant medical history of the studied patients are shown
317 in Table 1A. As expected, the plasma conditions were different between patients for
318 various disease states.

319 As shown in Fig. 2A, the V_u by CF-UF was significantly different among
320 different individuals under the same centrifugation conditions, ranging from 97μL to
321 279μL. There was a larger V_u in patients with hyporproteinemia or chonic renal
322 disease than in patients with diabetes or hypertension (shown in Fig. 2B). In contrast,
323 the V_u by HFCF-UF was less affected by plasma conditions (about 50μL). Importantly,
324 a comparison between unbound fraction of VCM (f_u) as determined by CF-UF and
325 HFCF-UF was statistically significantly different ($p < 0.0001$, Fig. 3A). The f_u ranged
326 from 41% to 78% as determined by CF-UF and the f_u ranged from 42% to 58% as
327 determined by HFCF-UF, respectively. Comparing with HFCF-UF, there was a
328 general bias toward an overprediction of f_u when determined by CF-UF, especially for
329 patients with hyporproteinemia or chonic renal disease (Broken circle in Fig. 3B).
330 However, the results of f_u as determined by CF-UF and HFCF-UF were about the
331 same in patients with diabetes or hypertension (the circle of solid line in Fig. 3B). As
332 a whole, there was a higher variability of f_u when determined by CF-UF compared
333 with HFCF-UF.

334 Table 1B shows the results of the bivariate regression analysis for patient
335 characteristics or plasma conditions and V_u with CF-UF. The variables included in
336 regression analysis were total protein, albumin, globulin, osmotic pressure of plasmas,
337 total bilirubin, direct and indirect bilirubin, total cholesterol and triglycerides. Total
338 protein and the osmotic pressure of plasma may be the main influence factors of V_u.
339 Scatter plots of the two individual covariates retained are shown in Fig. 4A-B. As
340 observed, the V_u decreased with the increase of total protein and the osmotic pressure

of plasma.

3.4. The effect of ultrafiltrate volume on the free VCM concentration with CF-UF

For CF-UF, the centrifugation device was strictly controlled centrifugation time and centrifugation force to obtain uniform and satisfactory V_u (about 70μL) in present study. Despite the results for the validation of HFCF-UF and CF-UF using pooled plasma samples with same plasma conditions were comparable. However, for clinical samples, we observed the V_u by CF-UF was significantly different between patients under the same centrifugation conditions in spite of the centrifugation device was well controlled. Moreover, there was a significantly different of unbound VCM fraction between that determined by CF-UF and HFCF-UF. Therefore, we further evaluated the effect of V_u/V_s on the free VCM concentration using CF-UF method. Briefly, total QC_S at three concentration levels of 1.00, 10.0, 50.0μg·mL^{-1} were prepared with human blank plasma. 500μL plasma was subjected to CF-UF for different durations (2, 5, 10, 20, 30min). V_u was calculated at the same time.

Table 2 clearly shows free VCM concentrations significantly increased with the V_u increasing by CF-UF, a clinical significant 25.5% increase in free VCM when the V_u increased from 53μL to 329μL. Therefore, it can be concluded that free VCM concentration is influenced by the V_u.

The reasons for different ultrafiltrate volume by CF-UF and HFCF-UF are the different shape of the filters and different centrifugal mechanisms of the two ultrafiltration methods. As shown in Fig. 5A, in CF-UF device, the ultrafiltration membrane is a flat membrane. The plasma sample solution and the ultrafiltrate are separated in CF-UF, and the ultrafiltrate is forced to enter the filtrate collection cup. The amount of ultrafiltrate increases with the centrifugation force and centrifugation time, so the CF-UF is a non-equilibrium separation.[27] In addition, the plasma proteins during ultrafiltration are forced to deposit on the membrane surface and may influence the ultrafiltration rate. Therefore, the V_u with CF-UF could be influenced by many factors based on the characteristics of its device and can not be well controlled. However, in HFCF-UF device, as shown in Fig. 5B, the ultrafiltration membrane is a hollow fiber membrane, and the direction of centrifugation force is completely

parallel to the hollow fiber membrane. The hollow fiber is immersed in sample solution and the small drug molecules in plasma can pass though the membrane freely under centrifugal force. The V_u by HFCF-UF can be controlled by the inner of both glass tube and hollow fiber,[10] and is not affected by the centrifugation force and centrifugation time. So the HFCF-UF is an equilibrium separation. Moreover, the plasma proteins during ultrafiltration will deposit in the bottom of the glass tube and do not influence the ultrafiltration rate.

3.5. Discussions of results

For CF-UF, the dependency of the V_u on both centrifugation force and centrifugation time has been demonstrated in pooled normal plasma.[7-10] Therefore, the centrifugation setting should be controlled with respect to time and force to obtain uniform and a satisfactory V_u.[7,8] Sometimes the results of precision for the validation of CF-UF were unsatisfactory (e.g., Intra-day and inter-day precision were 14.8% and 15.4% for free VCM).[5-8,11] Using pooled plasma samples with the same plasma conditions could give compromised results for the validation of the CF-UF method by strictly controlled process parameters in this study, but for clinical samples with different diseases, we observe that the V_u by CF-UF is also affected by plasma conditions. The osmotic pressure of plasma and total protein levels may be the main influence factors of V_u in disease states. It has been reported that the protein levels varied widely in patients (albumin 11.1-32.2g/L) and can change strikingly during acute and convalescent periods.[24] In this study, the total protein levels ranged from 41g/L to 83g/L and the osmotic pressure of plasmas varied from 262.4 to 329.8mmol/L, resulting in a change of V_u ranging from 97μL to 279μL. It is generally accepted that plasma conditions depend on the physiological and pathological conditions of the patients and can change quickly for various disease states, such as uremia, diabetes, renal failure or hypoalbuminemia.[1,12-15] Protein levels of patients with uremia, trauma and hypoalbuminemia were significantly lower than that of normal subjects.[12] Plasma viscosity of patients with diabetes, hypertension or mixed hyperlipidemia was obviously higher than normal subjects and patients with other diseases.[13] Colloid osmotic pressure was significantly lower in patients with

pulmonary edema and those who were critically ill.[28] The difference of plasma conditions can cause alteration of the protein binding ratio, thereby changing the unbound drug concentration.[29] Impact of creatinine clearance and total bilirubin on unbound mycophenolic acid was reported in pediatric and young adult patients.[30] Plasma albumin and plasma urea concentrations were also identified as significant covariates influencing the unbound phenytoin fraction.[31] A Smits *et al* demonstrated that the effect of albuminaemia, indirect bilirubinaemia and postmenstrual age on the unbound cefazolin fraction.[32] However, our study demonstrates that plasma conditions also affect the V_u/V_s by CF-UF and that affects the monitoring of free drug concentration. Our results indicated that there was a larger V_u in patients with hyporproteinemia or chonic renal disease than in patients with diabetes or hypertension. This may be due to the plasma from patients with hyporproteinemia and chonic renal disease has lower protein level and higher osmotic pressure of plasmas than that from patients with diabetes and hypertension. Therefore, for clinical samples, the V_u by CF-UF cannot be well controlled under the same centrifugation conditions, and only one sample with CF-UF at a time was performed under given plasma conditions. However, it is inefficient and time consuming which is not suitable for clinical routine TDM of large batches of samples. In contrast, the V_u by HFCF-UF can be controlled by the inner of both glass tube and hollow fiber and was less influenced by plasma conditions. Therefore, it could be a rapid and reliable method for TDM of free concentration in the future.

To date, the clinical relevance of monitoring free concentrations for VCM in the clinical routine has not yet been addressed, although the clinical significance of monitoring free VCM has been realized. It is attributed to high variability of the VCM unbound fraction (f_u) reported in the literature.[11,18-23] In an attempt to account for the wide range of reported f_u, considerable interest has been concentrated largely on the factors of patient or plasma conditions on free VCM. Zokufa HZ *et al* demonstrated that there is a significant correlation between f_u and albumin concentration in 10 patients with burn injuries.[21] The f_u was also reported to be correlated with α_1 glycoprotein (AAG) in 10 MRSA-infected patients.[20] Moreover, in vitro study, f_u is

depended on both albumin and immunoglobulin A.[22] None of them took the associated factors of methodology for the monitoring of free VCM in patients into consideration. Our observations indicate that the V_u/V_s by CF-UF exert a significant effect on free VCM concentration. Unfortunately, it can be found that different centrifugation force and centrifugation time for separation of the bound and unbound VCM was applied when tracing previous studies.[11,18-23] However, the V_u and V_u/V_s were only mentioned in two studies (70μL and 200μL).[24,25] It has been recommended that the V_u should occupy less than one-fifth of the plasma volume during CF-UF,[7] so the obtained V_u that is more than 100μL is unreasonable in above studies (plasma volume was 500μL). In conclusion, lack of standardized methodology for monitoring free VCM concentrations with CF-UF partly explained the wide percent range free VCM values reported in the literature. The present study validated an accurate and reliable HFCF-UF method that can be the reference methodology for TDM of free VCM. Future studies should be performed to explore the true extent of f_u in patients and address the clinical relevance of monitoring free VCM concentration in clinical routine.

4. Conclusions

In this work, accuracy on the analysis of free VCM by ultrafiltration in various disease conditions was investigated. The results showed that plasma conditions significantly affected V_u/V_s by CF-UF, Therefore, in order to avoid the overestimation or underestimation of real free concentration of the patients, future studies should take the factors into consideration when CF-UF is being applied. For HFCF-UF, the V_u/V_s can be well controlled by the inner of both glass tube and hollow fiber and were less affected by plasma conditions. The reported large variability of unbound VCM fraction should be due in part of analytical issue with CF-UF rather than a real interpatient variability of unbound fraction. The developed HFCF-UF achieved a successful application in TDM of free VCM and can be a reliable alternative for accurate monitoring of free VCM. As a whole, the HFCF-UF used for clinical purposes can benefit from the development and validation of a standardized method escaping from the controlling of centrifugation time and centrifugation force as well

as the influence of plasma conditions of patients, which provides more advantages than CF-UF. It can be a very powerful and reliable future sample preparation technique for monitoring of free drug level in routine TDM.

Acknowledgments

The authors gratefully acknowledge financial support from the Natural Science Foundation of Hebei Province in China (Project No. H2012206043).

References

1. A. Dasgupta, Clinica chimica acta. 377 (2007) 1-13.

2. C.K. Svensson, M.N. Woodruff, J.G. Baxter, D. Lalka, Clinical pharmacokinetics. 11 (1986) 450-469.

3. K. Chan, R.G. Beran, Seizure, 17 (2008) 572-575.

4. B.A. Atcheson, P.J. Taylor, C.M.. Kirkpatrick, S.B. Duffull, D.W. Mudge, P.I. Pillans, S.E. Tett, Ther Drug Monit. 26 (2004) 284-286.

5. N.V. De Moraes, G.R. Lauretti, M.N. Napolitano, N.R. Santos, A.L.P.C. Godoy, V.L. Lanchote, J. Chromatogr., B 880 (2012) 140-147.

6. Y. Chen, M. Yazdanpanah, X.Y. Wang, B.R. Hoffman, E.P. Diamandis, P.Y. Wong. Clin. Biochem. 43 (2010) 490-496.

7. J. Zhang, D.G. Musson, J. Chromatogr. B 843 (2006) 47-56.

8. W. Li, H. Lin, H.T. Smith, F.L.Tse. J. Chromatogr. B 879 (2011) 1927-1933.

9. G.A. McMillin, J.E. Juenke, A. Dasgupta, Ther. Drug Monit. 27 (2005) 630-633.

10. W.C. Dong, Z.Q. Zhang, X.H. Jiang, Y.G. Sun, Y Jiang. Eur. J. Pharm. Sci. 48 (2013) 332-338.

11. K. Berthoin, E. Ampe, P.M. Tulkens. Int. J. Antim. Agents 34 (2009) 555-560.

12. H.R. Butt, A.M. Snell, A. Keys. Arc. Int. Med. 63 (1939) 143.

13. W. Koenig, M. Sund, E. Ernst, U. Keil, J. Rosenthal, V. Hombach. *Ame.* J. Hyper. 4 (1991) 529-536.

14. N.E. Stathakis, A. Fountas, E. Tsianos. J. Clin. Path. 34 (1981) 504-508.

15. S. Takano, S. Kimura, S. Ohdama, N.Aoki, Blood 76 (1990) 2024-2029.

16. R.C. Moellering. Clin Infect Dis.42 (2006) S35-9.

17. R.G. Finch, G. M. Eliopoulos. J. Anti. Chem. 55 (2005) ii5-13.

18. B.H. Ackerman, E.H. Taylor, K.M. Olsen, W.E.D.A.D. Abdel-Malak, A.A. Pappas. *Drug Intell. Clin. Pharm*., 22 (1988) 300-303.

19. L.M. Albrecht, M.J. Rybak, L.H. Warbasse, D.J. Edwards. Drug Intell. Clin. Pharm. 25 (1991) 713-715.

20. K. Morita, A. Yamaji. Ther. Drug Monit. 17 (1995) 107-112.

21 H.Z. Zokufa, L.D. Solem, K.A. Rodvold, K.B.Crossley, J.H.Fischer, J.C. Rotschafer, J. Burn Care Rehabil. 10 (1989) 425-428.

22. L Li, M. V Miles, H Lakkis, A.L. Zaritsky. Pharmacotherapy 16 (1996) 1024-1029.

23. J.M. Butterfield, N. Patel, M.P. Pai, T.G. Rosano, G.L. Drusano, T.P. Lodise. Anti. Agents and Chem. 55 (2011) 4277-4282.

24. M.G. Kees, S.G. Wicha and A Seefeld, F. Kees, C. Kloft, J. Clin. Pharm. 54 (2013) 318.

25. K. Berthoin, E. Ampe, P.M. Tulkens, S.Carry. Int. J. Anti. Agents 34 (2009) 555-560.

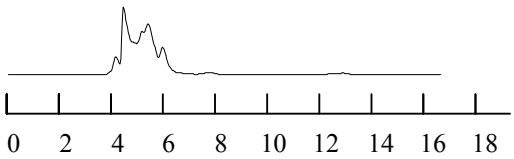
26. Food and Drug Administration (FDA), Guidance for Industry in Bioanalytical Method Validation, Rockvile, MD,USA, 2001.

27. S.S. Holm, S.H. Hansen, J Faber, P. Staun-Olsen. Clin. Biochem. 37 (2004) 85-93.

28. M.P. Morissette. Can. Med. Ass. J. 116 (1977) 897.

29. K. M. Piafsky. Clin. Pharm. 5 (1980) 246-262.

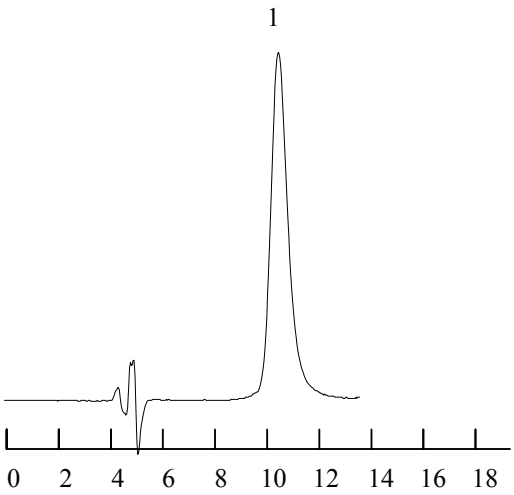
510 30. H. Kim, J. Long-Boyle, N. Rydholm, P. J. Orchard, J. Tolar, A. R. Smith, R.
511 Brundage. J. Clin. Pharm. 52 (2012) 1665-1675.
512 31. ter. R. Heine, van E. M. Maarseveen, van der M. M. L Westerlaken, K. P. Braun, S.
513 M. Koudijs, J. S. M. Maarten, M. M. Malingre. J. Chi. Neur. (2013).
514 .32. A. Smits, A. Kulo, R. Verbesselt, G. Naulaers, de J. HOOH, P. Vermeersch, K.
515 Allegaert. Euro. J. Clin. Micro. Infec. Dis. 31 (2012) 3359-3365.



A

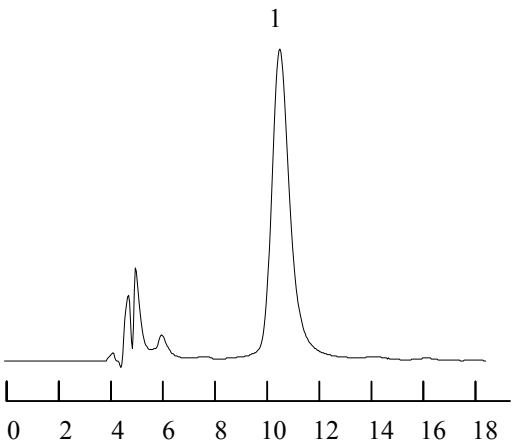
516

517



B

518



C

519

520

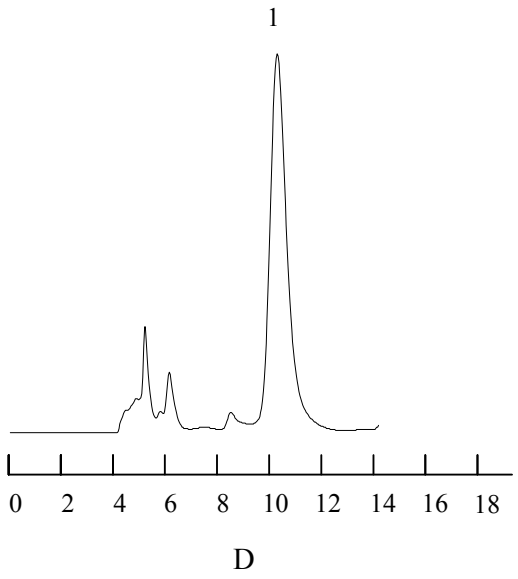


Fig. 1. Typical HPLC chromatograms of drug-free plasma (A), vancomycin standard solution (B), Human blank plasma spiked vancomycin (C), Plasma sample collected from a patient (D) (1 vancomycin).

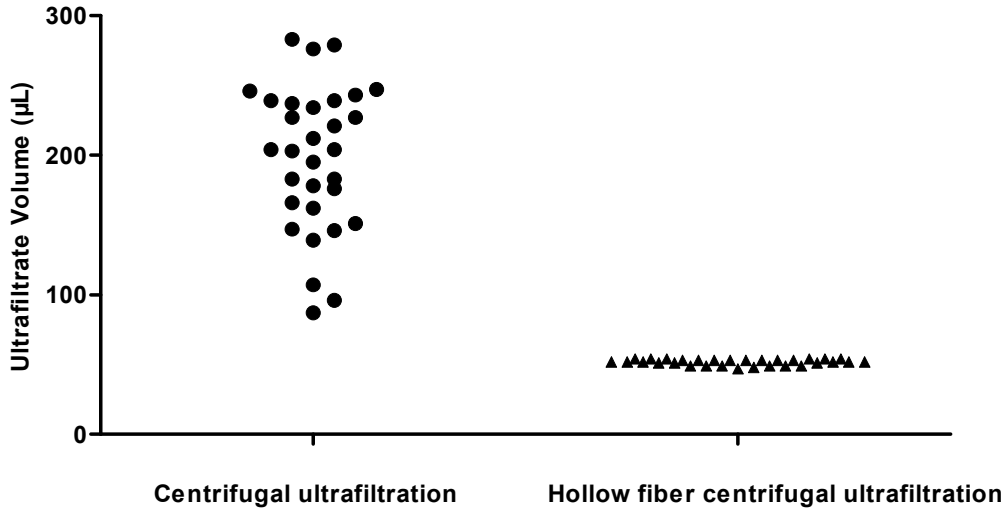
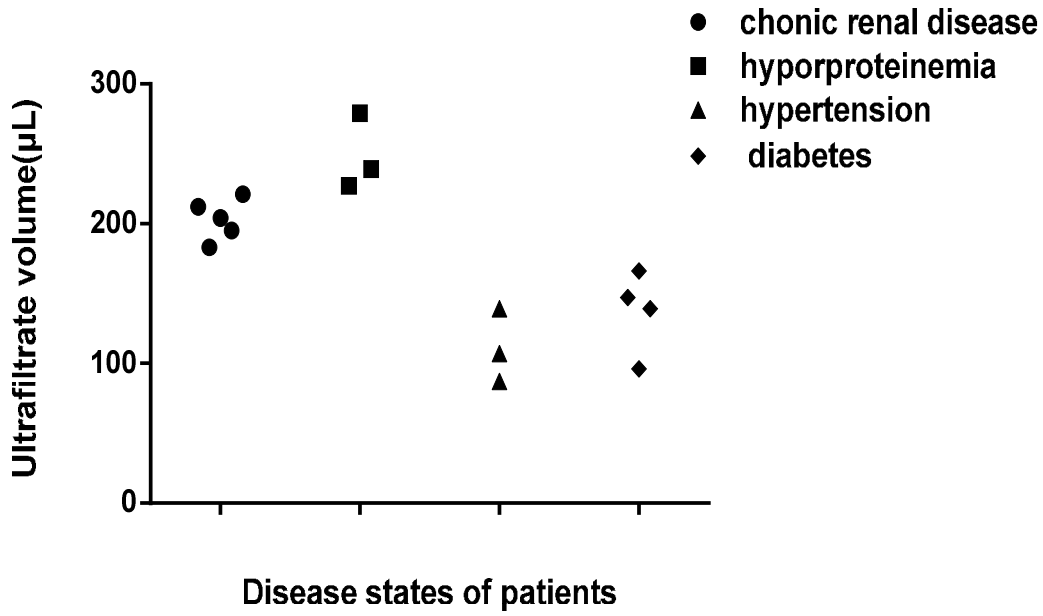
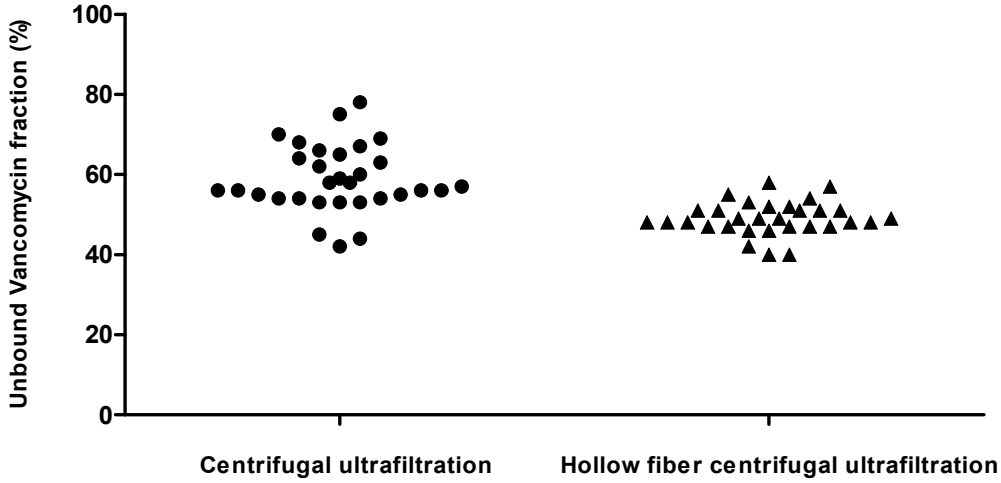


Fig. 2A. Comparative ultrafiltrate volume as determined by CF-UF and HFCF-UF for 31 samples with sufficient volume to be analyzed by both methods (the V_u by CF-UF ranged from 97µL to 279µL and the V_u by HFCF-UF remained constant for 31 samples under the same centrifugation conditions).



532

533 **Fig. 2B.** Variation of ultrafiltrate volume among patients with different main
534 pathology (There was a larger V_u in patients with hyporproteinemia or chonic renal
535 disease than in patients with diabetes or
536 hypertensio



537
538

539 **Fig. 3A.** Comparative unbound VCM fraction as determined using CF-UF and
540 HFCF-UF for 31 samples with sufficient volume to be analyzed by both methods (The
541 f_u ranged from 41% to 78% as determined by CF-UF and the f_u ranged from 42% to
542 58% as determined by HFCF-UF, respectively. A comparison between unbound
543 fraction of VCM (f_u) as determined by CF-UF and HFCF-UF was statistically
544 significantly different ($p < 0.0001$)).

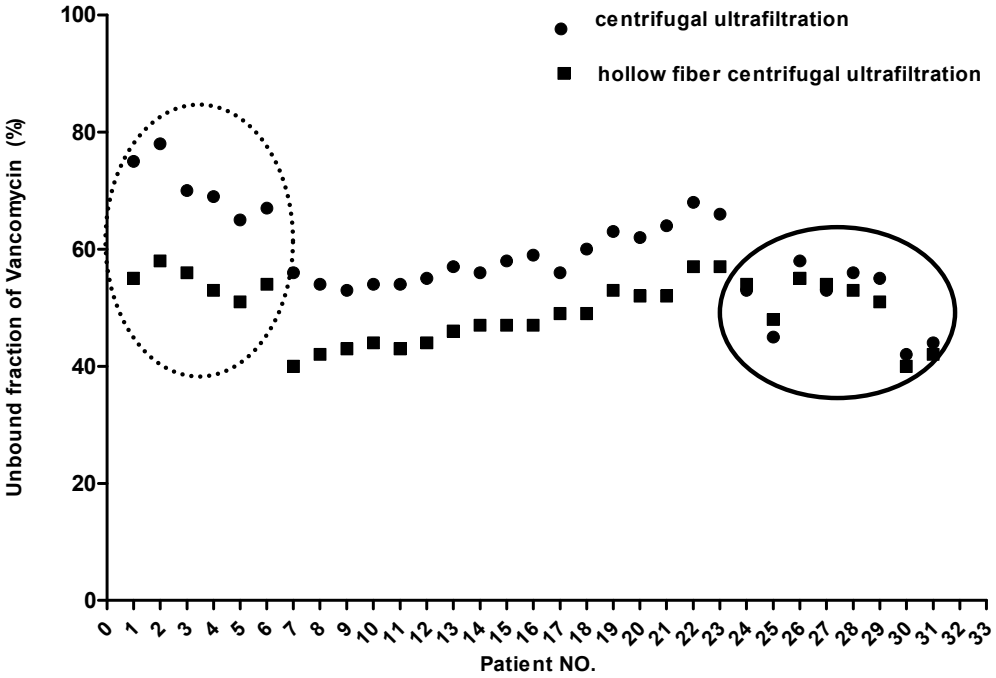
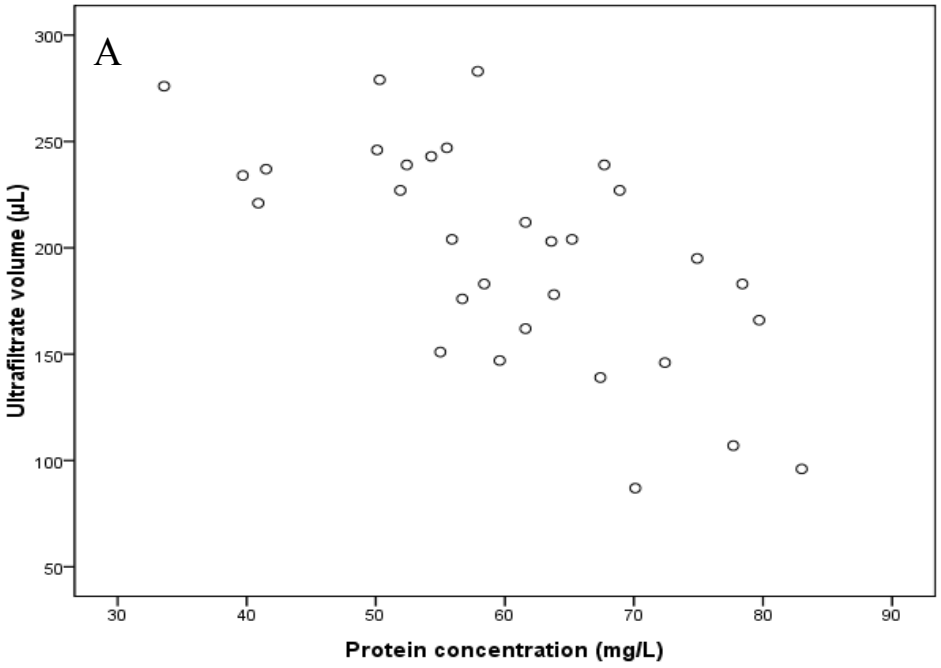


Fig. 3B. Comparative unbound VCM fraction of 31 samples as determined using CF-UF and HFCF-UF (Broken circle represents the f_u of patients with hyporproteinemia or chonic renal disease as determined by CF-UF and HFCF-UF had greater differences; the circle of solid line represents the f_u of patients with diabetes or hypertension as determined by CF-UF and HFCF-UF were about the same).



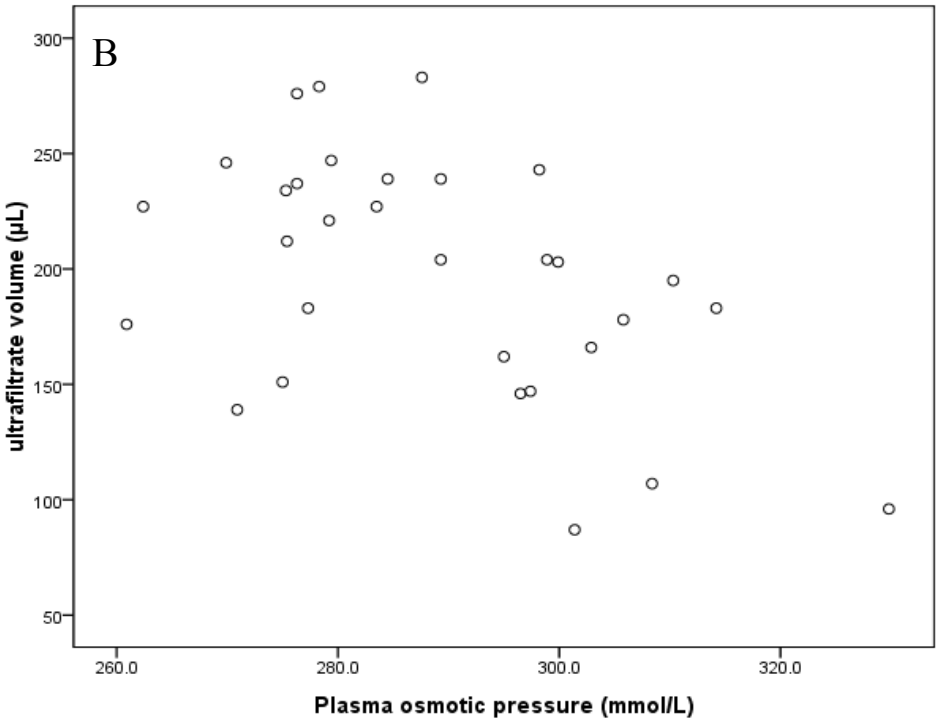


Fig. 4. Linear regression of ultrafiltrate volume as a function of: (A) total protein levels; (B) plasma osmotic pressure (the V_u decreased with the increase of total protein and the osmotic pressure of plasma).

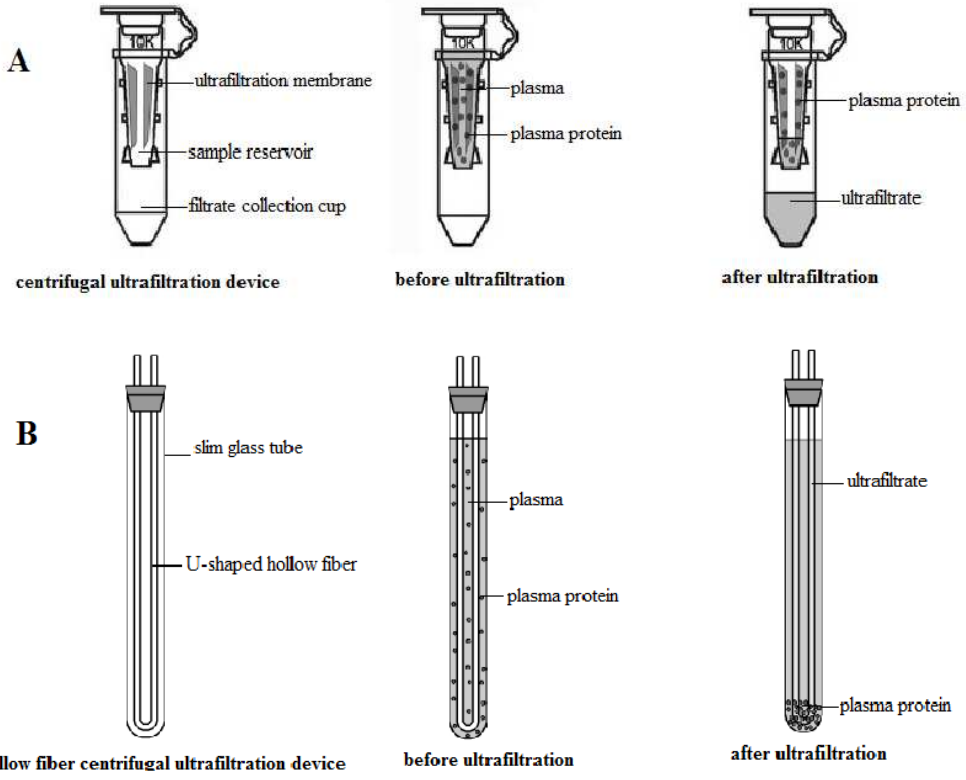


Fig. 5. The schematic representation of centrifugal ultrafiltration (A) and hollow fiber centrifugal ultrafiltration (B).

Table 1A

Clinical characteristics of the included study patients (n=25), reported as the median and range or number of cases.

Age (years)	44 (19-78)
Sex (male/female)	12/10
Total protein (g/L)	52.3 (41-83)
Albumin (g/L)	29.6 (19.8-46.2)
Globulin(g/L)	25.4(18.8-39.5)
Total bilirubin (mg/dL)	5.6 (3.6-231.3)
Direct bilirubin (mg/dL)	3.4 (1-191.3)
Indirect bilirubin(mg/dL)	2.8 (0.5-46.9)
Plasma osmotic pressure (mOsm/kg)	293.2 (262.4-329.8)
Total cholesterol (mmol/L)	4.35 (1.54-6.78)
Triglycerides (mmol/L)	1.83 (0.61-5.8)
Diabetes mellitus (No. of patients)	4
Hypoproteinemia (No. of patients)	3
Hypertension (No. of patients)	3
Chonic renal disease (No. of patients)	5

Table 1B

Results of the Bivariate Regression Analysis for Patient Characteristics and ultrafiltrate volume.

Variable	β Coefficient	P Value
Total protein (g/L)	-0.685	0.004
Albumin (g/L)	-0.483	0.332
Globulin(g/L)	-0.284	0.386
Total bilirubin (mg/dL)	0.090	0.390
Direct bilirubin (mg/dL)	0.030	0.446
Indirect bilirubin(mg/dL)	0.063	0.485
Plasma osmotic pressure (mmol/L)	0.503	0.013
Total cholesterol (mmol/L)	-0.19	0.137
Triglycerides (mmol/L)	-0.11	0.181

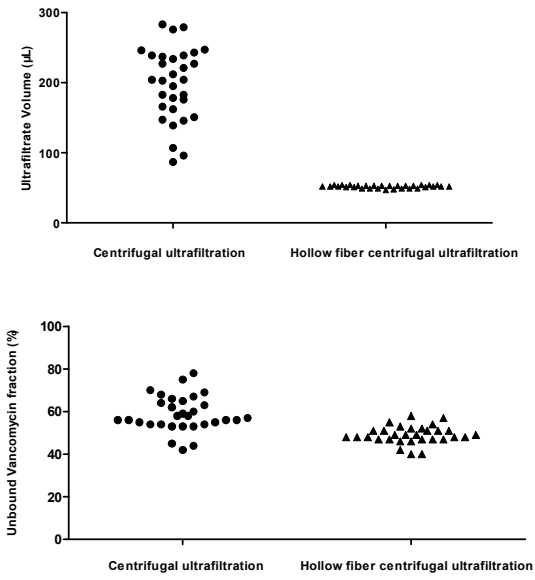
Added ($\mu g \cdot mL^{-1}$)	Time (min)	Ultrafiltrate volume(μL) (Mean±SD n=5)	Free concentration[a] ($\mu g \cdot mL^{-1}$)

Table 2

The results of effect of Ultrafiltrate volume on free VCM concentration with CF-UF.

	5	53(14.2)	4.45
	10	147(12.8)	5.43
10.0	15	208(13.4)	5.92
	20	256(9.7)	6.53
	30	329(8.9)	6.97
	5	56(13.2)	9.08
	10	153(11.9)	10.13
20.0	15	216(10.4)	10.68
	20	276(8.3)	11.05
	30	336(8.1)	11.56
	5	62(14.7)	22.68
	10	151(13.6)	23.59
50.0	15	219(12.9)	24.19
	20	268(9.3)	24.77
	30	329(9.0)	25.06

a: Free VCM concentrations significantly increased with the V_u increasing by CF-UF, a clinical significant 25.5% increase in free VCM when the V_u increased from 53μL to 329μL.



The different ultrafiltrate volume results in different unbound vancomycin fraction as determined by centrifugal ultrafiltration and hollow fiber centrifugal ultrafiltration.