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Short communication

Accuracy assessment on the analysis of unbound drug in plasma by comparing traditional centrifugal ultrafiltration with hollow fiber centrifugal ultrafiltration and application in pharmacokinetic study

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ABSTRACT

In present study, accuracy assessment on the analysis of unbound drug in plasma was made by comparing traditional centrifugal ultrafiltration (CF-UF) with hollow fiber centrifugal ultrafiltration (HFCF-UF). We used metformin (MET) as a model drug and studied the influence of centrifugal time, plasma condition and freeze–thaw circle times on the ultrafiltrate volume and related effect on the measurement of MET. Our results demonstrated that ultrafiltrate volume was a crucial factor which influenced measurement accuracy of unbound drug in plasma. For traditional CF-UF, the ultrafiltrate volume cannot be well-controlled due to a series of factors. Compared with traditional CF-UF, the ultrafiltrate volume by HFCF-UF can be easily controlled by the inner capacity of the U-shaped hollow fiber inserted into the sample under enough centrifugal force and centrifugal time, which contributes to a more accurate measurement. Moreover, the developed HFCF-UF method achieved a successful application in real plasma samples and exhibited several advantages including high precision, extremely low detection limit and perfect recovery. The HFCF-UF method offers the advantage of highly satisfactory performance in addition to being simple and fast in pretreatment, with these characteristics being consistent with the practicability requirements in current scientific research.

1. Introduction

A primary interest in the development of a direct injection technique in HPLC is the need for a simpler and faster analysis of drugs in biofluids. The advantages of direct injection HPLC are easier sample preparation and shorter analysis time. Most importantly, the measurement is closer to the true concentration in biofluids since it escapes from deviations induced by tedious pretreatment procedures [1].

Traditional centrifugal ultrafiltration (CF-UF) is one of the most popular direct injection techniques, which has long been commonly applied in the drug–protein binding studies [2,3]. It entails one step of centrifugation of plasma through ultrafiltration membrane with direct injection of the protein-free ultrafiltrate into HPLC system and thus it is simple and direct. In fact, the ultrafiltration process is exactly a filtration process which is required for direct injection of biological specimen. The only difference is that ultrafiltration membrane replaces ordinary filter membrane. Compared with other direct injection methods including column switching system [4–6], micellar liquid chromatography [7] and some chromatographic columns with specially designed stationary phase [8–10], the traditional CF-UF is free of many problems, such as column plugging, low sensitivity and additional columns, pumps and switching valves. Therefore, the traditional CF-UF provides a more desirable alternative for the direct injection analysis of unbound drug as well as free fraction drug in biological specimen, which is usually measured by "ligand-fishing" technique [11], equilibrium-dialysis [12] and traditional CF-UF [13].

During traditional CF-UF process, small molecules contained in the aqueous component of plasma are forced by a pressure gradient to pass through a selectively permeable membrane and are collected in the ultrafiltrate compartment. Theoretically, the concentration of analyte in ultrafiltrate should be exactly equal to that in plasma. However, the measurement sometimes is not as accurate as predicted. A unidirectional negative deviation which is not negligible has even been observed in a few reports [14], indicating that there are some undiscovered factors affecting the CF-UF process. Some theories including concentration polarization and molecular sieve effect are proposed to explain it, but further investigation has not been carried on and the real reason is still unclear.

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Hollow fiber centrifugal ultrafiltration (HFCF-UF) is becoming an important technique in separating the macromolecules from complicated matrix in recent years. Since the direction of centrifugal force is completely parallel to the membrane [15–17], there is no concentration polarization phenomenon that the small molecules can pass through the UF membrane freely, which would likely contribute to a more accurate result.

In this paper, a comprehensive investigation about accuracy assessment on the analysis of unbound drug in plasma was carried on using metformin (MET) as a model drug. Two kinds of CF-UF method were compared and contrasted on the measurement accuracy under different conditions to explore the factors leading to the measurement bias. The reason of different ultrafiltrate volumes that each CF-UF method actually results in was also explained. Moreover, the HFCF-UF method was validated in detail and successfully applied in pharmacokinetic study. It exhibits its advantages with more accurate results and provides a simpler, faster and more reliable alternative for the measurement of unbound drug in pharmacokinetic study and clinical drug monitor.

2. Experimental

2.1. Materials and apparatus

Metformin hydrochloride reference was obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile (HPLC grade) was purchased from Lab Scan (England). 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 purchased from Taoxin Environment Science and Technology Company (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. The ultrafiltration devices (Centrifree® UFC 501096; 0.5 mL, cut-off 10,000 Da, can sustain up to $1.4 \times 10^4 \times g$) were purchased from Millipore Corp (Billerica, MA).

2.2. HPLC conditions

Analysis was performed on Agilent 1200 series high performance liquid chromatography. Separations were accomplished on a C18 column (250 mm × 4.6 mm, 5 μm, Chromasil, China) at 40 °C. The separations were performed under isocratic elution using a mobile phase containing of acetonitrile (27%) and 0.02 mol L^{-1} potassium dihydrogen phosphate (pH3.0, 73%) containing 0.004 mol L^{-1} sodium dodecyl sulfate and 0.2% triethylamine. The detection wavelength was 232 nm and the flow rate was 1 mL min^{-1}.

2.3. Reference solution preparation

Stock solution of MET was prepared with deionized water and diluted to obtain solution containing 642 μg mL^{-1} MET. A series of working solutions containing MET at appropriate concentrations were prepared by diluting stock solution with deionized water. The concentrations of working solution were 64.2, 32.1, 16.0, 8.00, 4.00, 2.00, 1.00, 0.500, 0.250 μg mL^{-1}, respectively. They were used to spike the blank plasma samples prior to extraction. The stock solution and working solutions were stored at 4 °C.

2.4. Blank plasma

A pool of blank plasma samples which were used for the validation of the method were kindly donated by healthy volunteers who have signed the informed consent.

2.5. Sample preparation

To 900 μL blank plasma in a 2 mL centrifuge tube, 100 μL of the standard solution was added. After vortexing, 500 μL plasma sample containing MET was subjected to traditional CF-UF and HFCF-UF, respectively. After a simple centrifugation at $1.25 \times 10^3 \times g$ for 10 min, 20 μL of obtained ultrafiltrate was directly injected into HPLC for analysis. The traditional CF-UF was performed using Centrifree® tube. The HFCF-UF device, which was consisted of a glass tube and a hollow fiber, was described in detail in the literature by Li et al. [16].

3. Results and discussion

3.1. Estimation and comparison of ultrafiltration volume by two CF-UF methods under different conditions

3.1.1. Estimation of ultrafiltrate volume

The traditional CF-UF device was consisted of two separated parts: sample reservoir and ultrafiltrate compartment. We measured the ultrafiltrate compartment and recorded the weight. The ultrafiltrate volume (V_u) by traditional CF-UF can be calculated by means of Eq. (1):

$$V_u = \frac{W_{1+u} - W_1}{C} \quad (1)$$

where the W_1 and W_{1+u} are the weight of ultrafiltrate compartment before and after ultrafiltration, respectively. Here, the plasma density (C) was regarded as 1 g mL^{-3}.

For HFCF-UF, the ultrafiltrate in the inner cavity of hollow fiber was transferred into an eppendorf tube, of which the weight has been recorded. The obtained ultrafiltrate volume (V'_u) by HFCF-UF can be calculated by means of Eq. (2):

$$V'_u = \frac{W_{2+u} - W_2}{C} \quad (2)$$

where the W_2 and W_{2+u} are the weight of eppendorf tube before and after transfer, respectively.

3.1.2. Comparison of ultrafiltrate volume by two CF-UF methods with different centrifugal conditions

Briefly, 500 μL of plasma containing 0.400 μg mL^{-1} MET was subjected to traditional CF-UF and HFCF-UF at $1.25 \times 10^3 \times g$ for different durations (3, 5, 10, 20, 30 and 40 min), respectively. The estimated ultrafiltrate volume (V_u and V'_u) was calculated according to Eqs. (1) and (2), respectively. The results showed that V_u varied from 50 to 400 μL with different durations while V'_u was constant (about 50 μL). Results were illustrated in Table 1. Obviously, the ultrafiltrate volume by traditional CF-UF was remarkably associated with the centrifugal time, instead of HFCF-UF.

3.1.3. Comparison of ultrafiltrate volume by two CF-UF methods with different plasma conditions

In this section, we examined the ultrafiltrate volume using blank plasma from six volunteers following the above procedure in Section 3.1.2. Ultrafiltrate volume was calculated and listed in Table 1. As far as we can see, V_u was significantly different with different individuals while the impact of plasma on V'_u was ultimately minimal. Therefore, we supposed that the plasma condition, such as blood viscosity, protein level, osmotic pressure and so on, may

Table 1
Data of ultrafiltration volume by two CF-UF methods with different centrifugal conditions, plasma conditions and freeze–thaw circle times.

Centrifugal time (min)	V_u (μL)	V'_u (μL)	Plasma source	V_u (μL)	V'_u (μL)	Freeze–thaw circle times	V_u (μL)	V'_u (μL)
3	52	48	1	182	49	0	190	47
5	115	50	2	208	52	1	197	49
10	190	51	3	137	47	2	205	50
20	245	50	4	159	48	3	217	52
30	302	52	5	92	47	4	225	51
40	394	50	6	177	50	5	238	51

Note: V_u and V'_u stand for ultrafiltrate volume by traditional CF-UF and HFCF-UF, respectively.

exert important influence on ultrafiltrate volume by traditional CF-UF. However, it seemed to be no effect on ultrafiltrate volume by HFCF-UF.

3.1.4. Comparison of ultrafiltrate volume by two CF-UF methods with different freeze–thaw circle times

Since freeze–thaw circle is likely to affect the plasma condition, we examined the effect of freeze–thaw circle times on the ultrafiltrate volume. Plasma samples were frozen at −80 °C for 24 h and thawed at room temperature. The results suggested that with the increasing freeze–thaw circle times, V_u increased gradually. In contrast, V'_u is maintained constant (Table 1). Therefore, it is concluded that freeze–thaw cycle is one of a number of important influences upon ultrafiltrate volume by traditional CF-UF.

3.2. Effect of ultrafiltration volume on the measurement of unbound drug in plasma

Based on the above findings, we studied the effect of ultrafiltrate volume on the measurement of unbound drug in plasma. 20 μL of obtained ultrafiltrate from Section 3.1.2 was directly injected into HPLC for analysis, respectively. There was an observation that the MET concentrations in ultrafiltrate by traditional CF-UF were significantly different with different durations while the measured MET concentrations in ultrafiltrate obtained from HFCF-UF were all around nominal concentrations. Further, the recovery (R%) was calculated by means of Eq. (3):

$$R\% = \frac{C_u}{C_0} \times 100\% \quad (3)$$

where the C_0 and C_u are the nominal concentration of plasma sample and the measured concentration of ultrafiltrate, respectively. Our results showed that the calculated recovery was constant (around 100%) when the volume ratio of ultrafiltrate to sample solution was small. With a higher volume ratio (above 0.4), a significant increase of recovery was observed, even reaching 110%. However, the recovery was invariant for HFCF-UF, around 100%. The results were illustrated in Fig. 1. It was indicated that the ultrafiltrate volume had an important influence on the measurement accuracy. Therefore, it is essential to control the ultrafiltrate volume during traditional CF-UF.

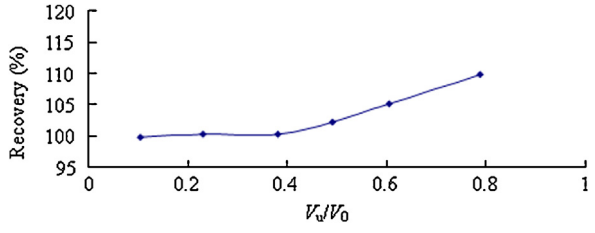

Fig. 1. The change of recovery with different V_u/V_0 on the measurement of MET by traditional CF-UF using 0.400 μg mL^{-1} plasma samples.

3.3. Reason for different ultrafiltrate volume by two CF-UF methods

Both traditional CF-UF and HFCF-UF belong to centrifugal ultrafiltration method. But the centrifugal mechanisms are different. For traditional CF-UF, the direction of centrifugal force is perpendicular to the ultrafiltration membrane [15,16] and the ultrafiltrate is forced to enter the ultrafiltrate compartment. Since the ultrafiltrate and the plasma sample solution are separated, a series of factors including centrifugal time, plasma condition and freeze–thaw circle times may have influence on the ultrafiltrate rate. Thus, the ultrafiltrate volume by traditional CF-UF cannot be well controlled. In contrast, the direction of centrifugal force is completely parallel to the membrane for HFCF-UF. Since hollow fiber is immersed in plasma sample solution, the small molecules in plasma can pass through the hollow fiber membrane freely under centrifugal force. Therefore, ultrafiltrate volume by HFCF-UF can be well controlled by the inner capacity of the U-shaped hollow fiber inserted into the plasma sample under enough centrifugal force and centrifugal time [15], which leads to a more accurate measurement.

3.4. The validation for the developed HFCF-UF method for measurement of MET

We examined selectivity and specificity using blank plasma from six volunteers. The specificity of the assay was evaluated by comparing chromatograms of a blank plasma sample, a blank plasma sample spiked with MET (0.400 μg mL^{-1}) and a volunteer's plasma sample containing MET. The results showed that none of the six blank plasma lots caused significant interference at the retention time of MET. Fig. 2 is one group of represent HPLC-UV chromatograms.

A standard calibration curve was consisted of nine concentration points of 0.0250, 0.050, 0.100, 0.200, 0.400, 0.800, 1.60, 3.21 and 6.42 μg mL^{-1} of MET. A typical equation for the calibration function was $A = 92.15C - 0.022$ ($r^2 = 0.999$). The linear range was 0.0250–6.42 μg mL^{-1}. The LOD and LOQ were 0.005 and 0.0250 μg mL^{-1}, respectively. The detection sensitivity was higher than previously described methods which used PPT [18–20], SPE [21,22] and traditional CF-UF [14] for sample preparation. Higher sensitivity was attributed to no dilution of plasma samples.

For the assessment of accuracy and precision of the method, five replicates of each concentration QC samples were run on 3 consecutive days. Data were presented in Table 2. The figures compared favorably with afore-mentioned data in the literatures [18,22,23]. Higher recovery may be attributed to the following three reasons: (1) Molecular sieve effect and concentration polarization phenomenon is avoided because the direction of centrifugal force is completely parallel to the hollow fiber membrane [15,16]. (2) Sample preparation entails only one step of centrifugation, reducing the deviation introduced by tedious pretreatment procedures. (3) The ultrafiltrate volume can be well-controlled.

Short-term stability, long-term stability, stability after three freeze–thaw cycles, post-processing stability, and stock solution stability were evaluated according to the analytical procedure. For

Table 2
Results of precision and recovery for MET ($n = 5$).

Added ($\mu g\,mL^{-1}$)	Intra-day RSD (%)	Inter-day RSD (%)	Relative recovery (%)	Absolute recovery (%)
0.0250	4.7	3.8	100.7	99.9
0.400	2.0	1.7	99.6	100.8
6.42	1.2	0.9	100.2	100.3

Table 3
Stability analysis of the analytical method for the measurement of MET in plasma.

Stability	Conditions	Accuracy, mean ± SD ($n = 3$)		
		0.0250 $\mu g\,mL^{-1}$	0.400 $\mu g\,mL^{-1}$	6.42 $\mu g\,mL^{-1}$
Short-term stability	At +20 °C for 15 h	0.0250 ± 0.0006	0.387 ± 0.013	6.40 ± 0.07
Long-term stability	At −80 °C for 14 days	0.0250 ± 0.0003	0.397 ± 0.019	6.45 ± 0.03
Freeze–thaw stability	Frozed at −80 °C for 24 h and thawed at +20 °C. Repeated 3 times	0.0247 ± 0.0009	0.403 ± 0.008	6.44 ± 0.07
Post-processing stability	At +20 °C for 15 h	0.0246 ± 0.0005	0.395 ± 0.007	6.40 ± 0.26
Stock solution stability	At 4 °C for 14 days	–	–	6.46 ± 0.06

this purpose, QC samples were freshly prepared at three concentrations: 0.0250, 0.400 and 6.42 $\mu g\,mL^{-1}$. Data were listed in Table 3.

3.5. Application of HFCF-UF method in pharmacokinetic study

The validation of the HFCF-UF method showed satisfactory figures of merit. In order to confirm the practicability, the present method was applied in pharmacokinetic study in 20 healthy volunteers who orally received 500 mg of MET tablet. The HFCF-UF method was clearly adequate for monitoring plasma concentration profiles of MET during the 24 h sampling period. The HFCF-UF method is accurate, stable and reliable. Moreover, the analysis time was significantly reduced owe to simplified pretreatment.

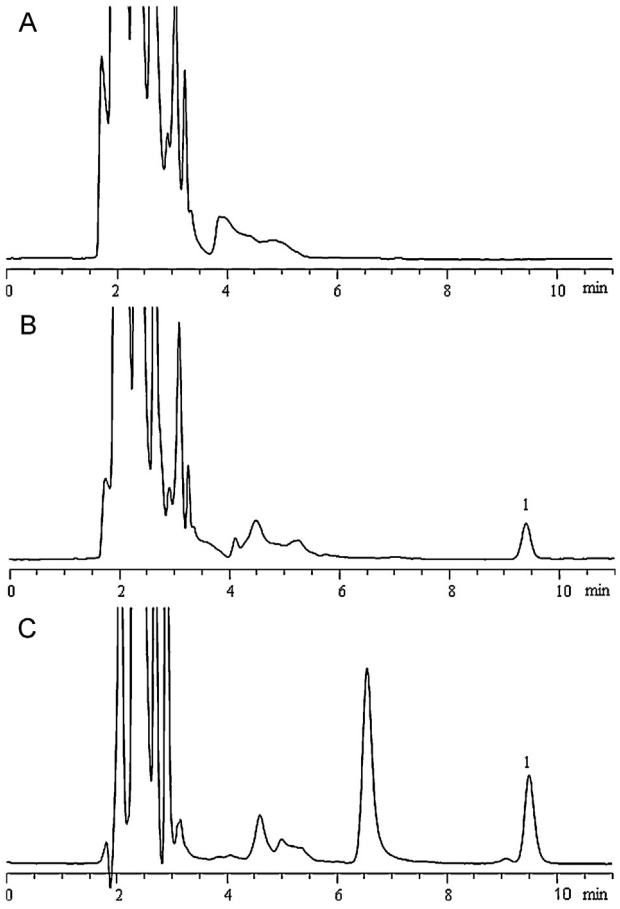

Fig. 2. HPLC chromatograms of MET filtrates of plasma samples by hollow fiber centrifugal ultrafiltration. (A) Filtrate of blank plasma; (B) filtrate of blank plasma spiked with MET (0.400 $\mu g\,mL^{-1}$); (C) filtrate of plasma sample. 1 MET.

4. Conclusions

In present work, accuracy assessment on the analysis of unbound drug in plasma was made by comparing traditional CF-UF with HFCF-UF. The results showed that ultrafiltrate volume was a crucial factor on the accurate measurement of unbound drug in plasma. Traditional CF-UF is a good direct injection method, but the ultrafiltrate volume needs to be well-controlled by well-trained operators. For HFCF-UF, the ultrafiltrate volume can be easily controlled by the inner diameters of hollow fiber without more of the technical operations. As a direct injection method, the developed HFCF-UF achieved a successful application in pharmacokinetic study and dramatic result was received. The analytical methodology proved to have several merits, that is, higher precision and accuracy, lower detection limit and perfect recovery, which are seemed to be better than previous literatures. With these highly practical and desirable features, the developed HFCF-UF method constitutes a simpler, faster and more reliable alternative for the direct injection analysis of unbound drug in biological samples.

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