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Pretreatment of plasma samples by a novel hollow fiber centrifugal ultrafiltration technique for the determination of plasma protein binding of three coumarins using acetone as protein binding releasing agent

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ABSTRACT

A novel and practical sample pretreatment method based on hollow fiber centrifugal ultrafiltration (HFCF-UF) was developed to determine plasma protein binding by using HPLC. The samples for analyzing unbound and total concentrations could be prepared in parallel simultaneously by the same device. It only required centrifugation for a short time and the filtrate could be injected directly for HPLC analysis without further treatment. Coumarins were selected as the model drugs. Acetone was chosen as the releasing agent to free the binding drug from the drug–protein complex for the total drug concentration determination. Non-specific bindings (NSBs) between the analytes and hollow fiber membrane materials were investigated. The type and volume of protein binding releaser were optimized. Additionally, centrifugal speed and centrifugal time were considered. Under the optimized conditions, the absolute recovery rates of the unbound and total concentrations were in the range of 97.5–100.9% for the three analytes. The limits of detection were in the range of 0.0135–0.0667 μg mL^{-1}. *In vitro* plasma protein binding of the three coumarins was determined at three concentrations using the validated method and the relative standard deviations (RSDs) were less than 3.4%. Compared with traditional method, the HFCF-UF method is simple to run, no specialized equipment requirement and is a more accurate plasma pretreatment procedure with almost excellent drug–protein binding equilibrium. Therefore, this method can be applied to determine the plasma protein binding in clinical practice. It also provides a reliable alternative for accurate monitoring of unbound or total drug concentration in therapeutic drug monitoring (TDM).

1. Introduction

Drug–protein binding has an important effect on the pharmacology and toxicology of drugs. The extent of protein binding may determine the relative concentration of a drug which is available for the drug–receptor interaction at the site of action [1,2]. It is well known that only the unbound drug can reach the active site by diffusing across the membranes and exert pharmacological effect by interacting with its receptors [3–5]. This is especially important for medium or high protein binding drug where fluctuation in the free fraction can bring a significant impact on drug efficacy and safety [6,7]. Therefore, it is necessary to determine the extent of plasma protein binding to adjust the therapeutic dose and understand the pharmacologic behavior of a drug.

Until now, a variety of methods have been used for drug–protein binding measurement, such as equilibrium dialysis (ED), microdialysis (MD), ultrafiltration (UF) and high-performance frontal analysis (HPFA). However, some disadvantages are exhibited among them. ED is time-consuming, and dialysis temperature and time usually need to be strictly controlled. Another downside of this method is that the plasma sample is greatly diluted while adding large amount of dialysate. This may lead to disruption of the drug–protein binding equilibrium. Consequently, poor sensitivity and deviation from true plasma condition are inevitable [7,8]. MD sampling has been demonstrated as an alternative technique to study protein binding both *in vitro* and *in vivo*. However, accurate determination of free concentration of a drug by MD sampling requires careful calibration of the MD probe with respect to recovery [9,10]. UF usually presents a large volume ratio of ultrafiltrate to sample solution, and this ratio can not be controlled

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effectively [11–13]. In addition, because the flow direction of the sample solution is perpendicular to the ultrafiltration membrane, it often suffers from concentration polarization during the separation process, which will break the protein-binding equilibrium and exert an influence on the determination of free drug concentration [14–16]. Even more unfortunately, all of the above methods can only be used to analyze the unbound drug without the capability to achieve simultaneous determination of both unbound and total drug concentrations. Although some researchers tried to solve this problem by HPFA [17–20], since the plasma sample needs to be introduced into the column and eluted by the mobile phase, the equilibrium between the protein-unbound and -bound fractions is broken which gives the inaccurate results. Moreover, nonideal chromatographic behaviors occur frequently because of severe chromatographic column deterioration caused by the protein from plasma samples.

Hollow fiber centrifugal ultrafiltration (HFCF-UF), a simple device composed of a slim glass tube and a U-shaped hollow fiber, has been introduced for the separation of unbound drug fraction from plasma sample [21–23]. Since the direction of centrifugal force is completely parallel to the hollow fiber membrane, concentration polarization is avoided and the small molecules can pass through the membrane freely [24,25]. Furthermore, ignorable amount of ultrafiltrate is withdrawn from the hollow fiber compared with plasma sample in the slim glass tube, therefore, drug–protein binding equilibrium in plasma sample is stable, which would likely contribute to a more accurate result [21–23].

Coumarins, such as bergenin, daphnetin and scopoletin (Fig. 1), are the major bioactive components in the roots of *Stellera chamaejasme* L. which is usually used in traditional Chinese medicine for tuberculosis, tumor and tinea therapy [26–28]. They are also the main components in a wide variety of Chinese medicinal herbs, such as *Daphne tangutica*, *Lycium chinense*, *Angelica dahurica* and *Saxifraga stolonifera* [29,30]. Pharmacology research illuminates that coumarins have the effects of anti-tumor, anti-oxidant, anti-inflammatory, anti-coagulant and anti HIV virus [31,32]. Therefore, these Chinese herbals are extensively used in Chinese traditional medicine as either single or compatibility with other drugs. However, coumarins are toxic and there is a narrow margin of safety between a therapeutic and a toxic dose. Taking higher doses of Chinese herbal medicine containing coumarins may lead to symptoms such as dizziness, diarrhea, vomiting, and even hepatic and pulmonary toxicity [32]. Especially when they are administrated with other drugs, the activity changes or even toxicity may exhibit [33,34]. The reasons lending to this phenomenon are complex while one of which may be the effect of protein binding rate. It is well known that when two or more drugs are administrated in combination, the competition for the protein among them may occur [35,36]. While, the change of protein binding can alter the unbound drug concentrations which is responsible for drug efficacy and potential drug toxicity. The influence is especially obvious for critically ill patients [37]. Until now, some studies have only been reported about the determination of total concentrations of coumarins in biological matrix with protein precipitation (PPT) method [38–40]. However, as a kind of protein bound drugs with many excellent pharmacological effects but potential adverse reactions, none of them took protein binding into consideration.

In this study, we developed a relatively straight forward procedure using HFCF-UF to determine plasma protein binding of three coumarins. The samples for analyzing unbound and total concentrations could be prepared in parallel simultaneously by the same device. Acetone was chosen as the releasing agent to free the binding drug from the drug–protein complex for analyzing the total drug concentration. It is simple, sensitive, accurate and can directly determine drug concentrations under near physiological conditions without destroying the balance of drug–protein binding. The analytes can be filtered into inner of the hollow fiber by only simple centrifugation and almost no interference produces due to strong interception ability of the hollow fiber. The proposed method was also compared with traditional pretreatment. The results indicated that it is a reliable alternative for quantifying drug–protein binding as well as unbound or total drug concentration in therapeutic drug monitoring (TDM).

2. Experimental

2.1. Materials

The standards of bergenin, daphnetin and scopoletin were obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). The hollow fibers (1000 μm outside diameter, 150 μm wall thickness, 10 kDa molecular weight cut-off) were purchased from Kaijie Membrane Separation Technology (Hangzhou, China). Methanol (HPLC grade) was from Grace & Company Inc. (USA). Acetone was supplied by Lan Xiang Chemical Co. Ltd. (Nanchang, China). Ultrapure water was obtained from a Millipore (Bedford, MA, USA) Milli-Q water-purification system. All other reagents were of analytical grade. The slim glass tubes (4.5 mm outside diameter, 0.5 mm wall thickness, 7 cm height) were provided by Yongda Instrument and Chemical Company (Tianjin, China). Blank plasma was kindly donated by healthy volunteers from the Second Hospital of Hebei Medical University (Hebei, China) who had signed the informed consent. Semi-permeable membranes with a molecular cut-off of 10 kDa for ED were obtained from Huameizhuandao Technology Co., Ltd. (Beijing, China). The membranes were washed with distilled water, and then soaked in phosphate buffered saline (PBS) (KH_2PO_4 (67 mmol L^{-1}) and NaCl (9 g L^{-1}) adjusted to pH 7.4 with NaOH solution) before being placed into the plasma for analysis.

2.2. Apparatus and HPLC conditions

The HPLC system was consisted of an L-6200A ternary pump (Hitachi, Japan) and a 785A UV detector (Applied Biosystems, USA). Chromatographic separation was performed on a Diamonsil C_{18} column (150 mm × 4.6 mm, 5 μm, Dikma, China) at room temperature. The mobile phase was composed of methanol: 1% HAc buffer (pH 3.0) (35:65, v/v), and eluted at a flow rate of 1.0 mL min^{-1}. The injection volume for all experiments was 20 μL. The wavelength was set at 327 nm. A Z-52 temperature controllable centrifuge (Baiyang Electronic Measurement Technology Ltd. Shanghai, China) and a XW-80 vortex mixer (Shanghai Medical University Instrument Co., Shanghai, China) were employed as well.

2.3. Preparation of calibration standards and quality control (QC) solutions

Appropriate amounts of bergenin, daphnetin and scopoletin were separately weighed and dissolved in methanol to prepare three individual stock solutions. A series of standard mixture working solutions were then prepared by mixing certain amounts of the three stock solutions and diluted with PBS. All the stock solutions and mixture working standards were stored at 4 °C.

Calibration standards to determine the unbound and total concentrations of the three analytes were prepared by adding appropriate amounts of mixture working solutions into PBS and blank plasma, respectively. Their concentration ranges were given in Table 1.

QC solutions at three different levels (0.445, 1.78 and 14.2 μg mL^{-1} for bergenin; 0.108, 0.430 and 3.44 μg mL^{-1} for daphnetin and 0.109, 0.435 and 3.48 μg mL^{-1} for scopoletin) for the