Acta Biologica Marisiensis sciendo

COMPARISON OF DIFFERENT ULTRAFILTRATION DEVICES FOR THE STUDY OF PLASMA PROTEIN BINDING OF CARVEDILOL

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Received: 26 July 2023; **Accepted:** 26 August 2023; **Published:** 30 December 2023

Abstract: The aim of the present study was to assess the suitability of different Amicon Ultra and Centrifree ultrafiltration devices for the study of the plasma protein binding process in the case of carvedilol, a highly protein-bound and lipophilic beta-blocking agent. Samples at different levels of concentration were prepared in both proteic and non-proteic matrices (human plasma, 5% human serum albumin solution and saline solution) and subjected to the classical ultrafiltration method using the different devices considered. Furthermore, an attempt to apply a previously described modified ultrafiltration method was also made. The analysis and quantification was achieved using a validated LC-MS/MS method. For the Centrifree devices, the determined unbound fractions of carvedilol and the corresponding binding degree were in accordance to literature data, while for the Amicon Ultra devices a great degree of carvedilol adsorbtion to the sample reservoir was observed, the analyte not being detected in the ultrafiltrate samples. Thus, it was further demonstrated that the type of ultrafiltration device used has a significant influence on the outcome of a plasma protein binding study. In the case of carvedilol, the evaluation of the protein binding interaction could be achieved using the Centrifree ultrafiltration devices, but not the Amicon Ultra devices.

Keywords: ultrafiltration, protein binding, carvedilol, Amicon Ultra, Centrifree

Introduction

The process of plasma protein binding (PPB) of drugs greatly influences both their pharmacokinetic and pharmacodynamic properties, being a key parameter which should be always evaluated for the comprehensive characterization of any compound (Bohnert and Gan, 2013; Yuan et al, 2020; Seyfinejad et al., 2021). Based on the importance of the PPB process, there is an increased interest in developing study methods for the accurate assessment of the binding degree of drugs. Among the different study approaches proposed over time for the assessment of PPB, the classical ultrafiltration (UF) method is still

widely accepted based on its main advantages which include: accuracy, short analysis time and ease of implementation (Howard et al., 2010; Vuignier et al., 2010). However, the method also has some limitations, non-specific binding (NSB) being the most important, but there are a lot of possibilities described in literature as ways to overcome and suppress them (Toma et al., 2021). Several ways to reduce NSB of studied drugs to the UF devices include the following: pre-treatment with different surfactant solutions, determination of NSB using phosphate buffer saline solution and the use of a correction factor, blocking of the NSB sites in the presence of plasma or proteic matrices, using different approaches and modifications of the classical UF technique.

The UF method implies the physical separation of the free and protein-bound fractions of drug through a semipermeable membrane, using the centrifugal force (Howard et al., 2010). The separation is achieved in an UF device which consists of two different compartments delimited by the semipermeable membrane with different molecular weight cut-off. After centrifugation, the ultrafiltrate containing only the free drug fraction can be quantified using an appropriate analytical technique.

It has been demonstrated that the experimental conditions, including the type of the UF device used, can greatly influence the accuracy of the results obtained in a PPB study (Kratzer et al., 2014; Dorn et al., 2018; Toma et al., 2021). Regarding the UF device, the factors that may play a role in their suitability for a particular study, are the type of the semipermeable membrane and also the material from which the other constituent components of the device are made since they can provide NSB sites (Lee et al., 2003; Kratzer et al., 2016).

The UF devices designed and commonly used for PPB studies are represented by the Millipore Centrifree filters, but in some studies, other devices initially designed for concentration of different constituents in biological samples (proteins, RNA, antigens, antibodies, enzymes) have also been successfully used (Du et al., 2014; Downing et al., 2017; Catalani et al., 2018). Furthermore, some authors suggest a validation of these other UF devices to the Centrifree ones, which are considered as reference, since differences in the results obtained based on the UF devices used have been frequently reported (Vogeser et al., 2007; Jensen et al., 2011; Larsen et al., 2011; Arellano et al., 2012; Ciobotaru et al., 2022).

The aim of this study was to assess the suitability of different ultrafiltration devices from the same manufacturer for the study of plasma protein binding of carvedilol (CVD), a highly protein-bound and lipophilic beta-blocking agent, using a validated LC-MS/MS method for quantification.

2. Materials and methods

Chemicals, reagents, and solvents

Pharmaceutical secondary standard of carvedilol was acquired from Sigma-Aldrich (Saint Louis, USA) and metoprolol succinate was purchased from Moehs (Barcelona, Spain). Acetonitrile (Honeywell, Muskegon, USA) and ammonium formate (VWR Chemicals, Radnor, USA) of LC-MS grade were used as solvents or components of the mobile phase. Human Albumin, as proteic matrix, was purched in the form of 200 g/L solution for infusion from Baxalta Innovations GmbH (Wien, Austria), while human plasma was obtained from The Regional Blood Transfusion Center Targu Mures (Romania). Saline solution was purchased from STADA Hemofarm (Timisoara, Romania) and ultrapure water was obtained with the aid of a Millipore Direct-Q 3 (Milford, USA).

LC-MS/MS analysis

A validated LC-MS/MS method previously published was used (Toma et al., 2023). The characteristics of the equipments were: a Perkin Elmer Flexar FX-10 UHPLC (Waltham, USA) and a Sciex QTOF 4600 mass spectrometer (Framingham, USA). The isocratic chromatographic separation was performed on a Phenomenex Luna C18 column 125 × 4 mm, 5 µm (Torrance, USA) with a mobile phase composition of 53% (v/v) 20mM ammonium formate at pH 4.4 and 47% (v/v) acetonitrile. The pump delivered the mobile phase with a flow rate of 0.4 mL/min, the column temperature was set at 25 °C and the injection volume was 4 µL. Metoprolol (MTP) was used as internal standard.

The MS detection was achieved after positive electrospray ionization, in MRM mode and the monitored transitions were the following: for CVD m/z 407.29 → 100.10, 222.15, 224.18, 283.22 and for the internal standard MTP m/z 268.23 → 116.12, 121.08, 133.08, 159.10, 191.14. The ion source parameters were set as follows: electrospray voltage +3300 V, source temperature 500˚C, nebulizing gas 30, drying gas 25, curtain gas 30 and collision energies of 32 for CVD and 24 for MTP (values in arbitrary units).

Preparation of solutions

Stock solutions. The stock solution of 10 µg/mL CVD was obtained by appropriate dilution with ultrapure water of a 1 mg/mL CVD solution prepared in acetonitrile, resulting in a 1% (v/v) acetonitrile concentration in the final stock solution. For the internal standard, the solvent used consisted only of acetonitrile and the final stock solution of 1 µg/mL MTP was obtained by an appropriate dilution of a 500 µg/mL MTP solution.

Standard solutions. Ten standard solutions for the calibration curve over the concentration range of 2.5 – 500 ng/mL CVD were obtained by spiking 150 µL of matrix with 50 µL aliquotes of corresponding intermediate working solutions. Three different matrices were considered: saline solution, human plasma and 5% (w/v) human serum albumin (HSA), prepared by appropriate dilution with saline of the 20% infusion solution.

Sample solutions. The sample solutions with concentrations of 25, 75, 125 and 500 ng/mL CVD were prepared in the three matrices considered following the same protocol applied for the standard solutions.

Experimental ultrafiltration protocol

Classical ultrafiltration method. Different UF devices from the same manufacturer were used: Centrifree® Ultrafiltration Centrifugal Filters (Ultracel® PL Regenerated Cellulose, 30 kDa MWCO, 1 mL), Amicon Ultra-2 and Amicon Ultra-0.5 centrifugal filter units (Ultracel-10K regenerated cellulose membrane, 2 mL and 0.5 mL) from Merck Millipore (Cork, Ireland).

For the determination of the total CVD concentration, a volume of 200 µL of each sample solution was separately added to an Eppendorf microcentrifuge tube, while, for the determination of the free/unbound fraction, 400 µL were added to the UF devices. In order to allow the establishment of the protein-binding equilibrium, all UF devices and microcentrifuge tubes containing CVD samples were incubated at 37°C for 30 minutes. After the incubation period, the 200 µL sample solution aliquotes were immediately processed for analysis, while the UF devices were centrifuged with the aid of an Eppendorf Centrifuge 5430R at room temperature for 15 min. Following the manufacturer's recommendations for each UF device, the following centrifugal forces were applied: 1877 x g for the Centrifree devices, 5214 x g for Amicon Ultra-2 and 4829 x g for Amicon Ultra-0.5. After centrifugation, 200 µL of the

ultrafiltrate obtained were processed for analysis in order to determine the unbound concentration of CVD.

Modified ultrafiltration method. An attempt to apply a modified version of the ultrafiltration method previously described by Taylor and Harker was also made (Taylor and Harker, 2006). For this method, the Amicon Ultra-0.5 centrifugal filter units were used since from all the UF devices considered in the present study, only these were suitable based on their mode of construction. For the modified UF method, for each UF device containing 400 µL CVD sample solution, a partner UF device containing 400 µL control matrix was also prepared. After incubation at 37°C for 30 minutes, all devices were centrifuged at room temperature for 15 minutes, applying a centrifugal force of 4829 x g. Following this first centrifugation, the upper compartments of the UF devices containing the retentate were inverted and placed on the ultrafiltrate collection compartments of their partner UF device. The devices were then centrifuged again for 10 minutes. 200 µL aliquotes of each reconstituted sample obtained were then removed and processed for quantification.

Processing of samples for LC-MS/MS analysis

To all sample and standard solutions, 100 µL aliquotes of 1 µg/mL MTP internal standard solution were added. The solutions were further deproteinized with acetonitrile (1:3 ratio), vortexed for 30 seconds and centrifuged for 10 minutes at 10000 rpm. The supernatants was subjected to the LC-MS/MS analysis.

Statistical analysis

The data sets obtained for determinations made using Centrifree devices were statistically evaluated in terms of normality of distribution using the Kolmogorov-Smirnov test, in terms of homogeneity of variances using the Cochran's C test and in terms of mean difference using the ANOVA single factor test. The statistical tests were applied considering a significance level of 0.05.

3. Results and discussion

Quality parameters of the analytical method

An already validated LC-MS/MS method for quantification was used (Toma et al., 2023). Specificity/selectivity, accuracy, precision and linearity of the method were tested and proved to be suitable. CVD and MTP were separated at retention times of 4.36 (±0.03) min and 2.51 (±0.01) min, respectively, demonstrating selectivity. The method presented good linearity over the concentration range 2.5-500 ng/mL CVD, with correlation coefficients greater than 0.995. Values of accuracy (relative error, Er%) and precision (relative standard deviation, RSD%) were within the acceptance limits according to the EMA Guidelines on bioanalytical method validation (Er% and RSD% < 15%).

Classical ultrafiltration method

The accuracy and relevance of PPB study results using the UF method is greatly influenced by the experimental conditions. Besides pH and temperature, which should be in accordance to the physiological values, a great attention should be paid to the type of UF device used. In the present study, two different types of ultrafiltration devices from the same manufacturer were tested regarding their suitability for the study of CVD binding to proteins. Both types of UF devices present a regenerated cellulose semipermeable membrane, but with different molecular weight cut-off: 30 kDa in the case of Centrifree devices and 10 kDa in the case of Amicon Ultra devices. Other differences between the devices considered, are related to the materials

used for the sample reservoirs and collection tubes. In the case of the Centrifree devices, the sample reservoir is made of styrene/acrylonitrile and the collection tube of polyethylene, whereas in the case of the Amicon Ultra devices, the materials used were styrene/butadiene and polypropylene, respectively.

According to the data sheet of the product, only the Centrifree devices were specifically designed for separating free from bound microsolutes in biological samples, but other PPB studies report good results also obtained using the Amicon Ultra devices, initially designed for concentration of different components of biological samples (antigens, antibodies, enzymes, nucleic acids, microorganisms), protein extraction and purification (Du et al., 2014; Imre et al., 2021).

For the classical ultrafiltration method, samples with concentrations of 25, 75, 125, and 500 ng/mL CVD prepared in human plasma, 5% HSA and saline solution were subjected to ultrafiltration using Centrifree and Amicon Ultra-2 devices. Additionally, Amicon-Ultra 0.5 devices were used for the ultrafiltration of 125 ng/mL CVD samples in the three matrices considered. The results obtained in terms of determined unbound CVD fraction (%) are presented in **Table 1**.

In the case of determinations made in human plasma using Centrifree devices, the chromatographic CVD signal observed for the samples after UF, although present, was bellow the lower limit of quantification of the LC-MS/MS method used (LLOQ – 2.5 ng/mL), thus not allowing further assessments and calculations of the unbound faction. In the case of determinations made using Amicon Ultra devices, no noticeable CVD signal was observed in the chromatograms of samples after UF. Representative chromatograms of samples before and after UF are presented in **Figures 1-3**.

From the results obtained, we could conclude that for the considered analyte, CVD, which is a highly lipophilic compound, Amicon Ultra devices are not suitable for the purpose of plasma protein binding assessments, compared to Centrifree, since no significant presence of the analyte in the ultrafiltrate was detected, not even for the higher concentration samples. In the case of the Centrifree devices, the results obtained for the determinations of CVD in human plasma could indicate a very high degree of binding to plasma proteins, which would be in accordance to literature data sustaining a more than 95% protein bound fraction (Book, 2002).

Table 1. Determined unbound fraction of CVD (%) using different ultrafiltration devices

Type of UF device	c (ng/mL)	Unbound fraction % mean (standard deviation)		
		Human plasma	5% HSA	Saline solution
Centrifree*	25	N/A	7.87 (±0.51)	64.76 (±3.94)
	75	N/A	7.64 (±0.79)	60.09 (±3.26)
	125	N/A	7.57 (±0.75)	66.43 (±2.64)
	500	N/A	8.72 (±0.54)	64.46 (±2.70)
Amicon Ultra-2*	25	N/A	N/A	N/A
	75	N/A	N/A	N/A
	125	N/A	N/A	N/A
	500	N/A	N/A	N/A
Amicon Ultra-0.5**	125	N/A	N/A	N/A

*n=3 ; **n=1 ; N/A – data not available

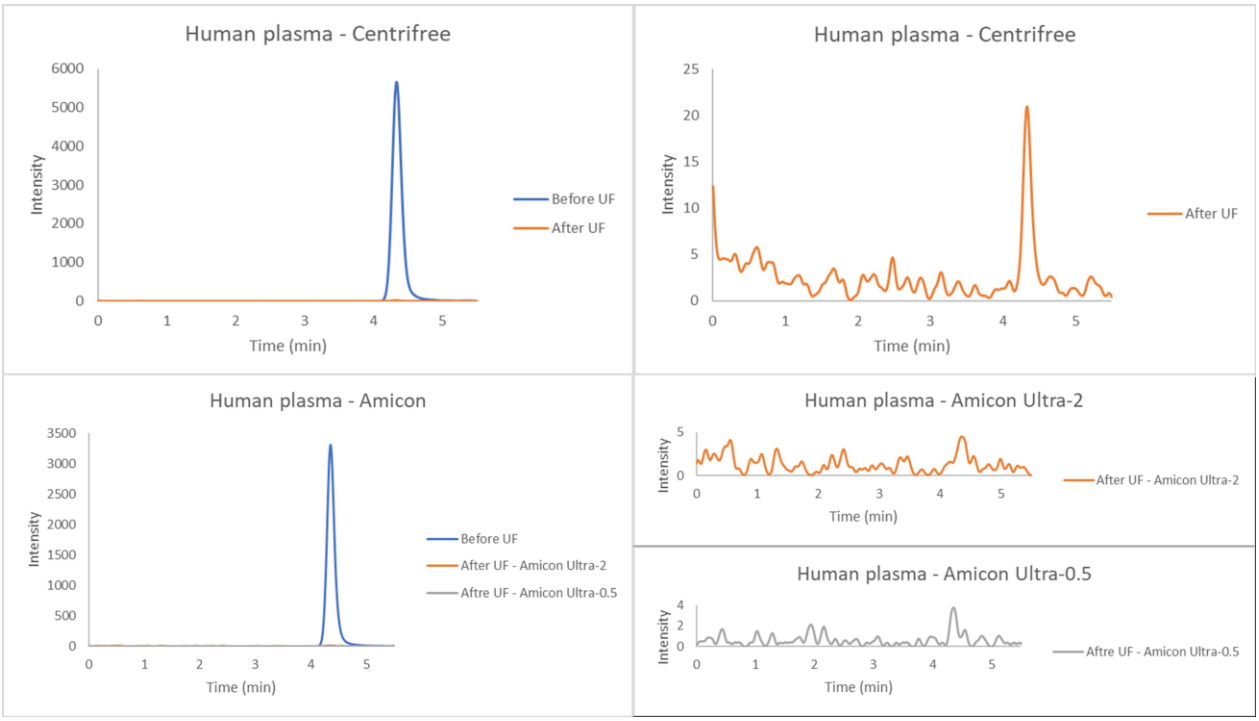

Fig. 1. Representative chromatograms of a 125 ng/mL CVD sample in human plasma before and after ultrafiltration (UF) using Centrifree vs. Amicon Ultra devices

Fig. 2. Representative chromatograms of a 125 ng/mL CVD sample in 5% HSA before and after ultrafiltration (UF) using Centrifree vs. Amicon Ultra devices

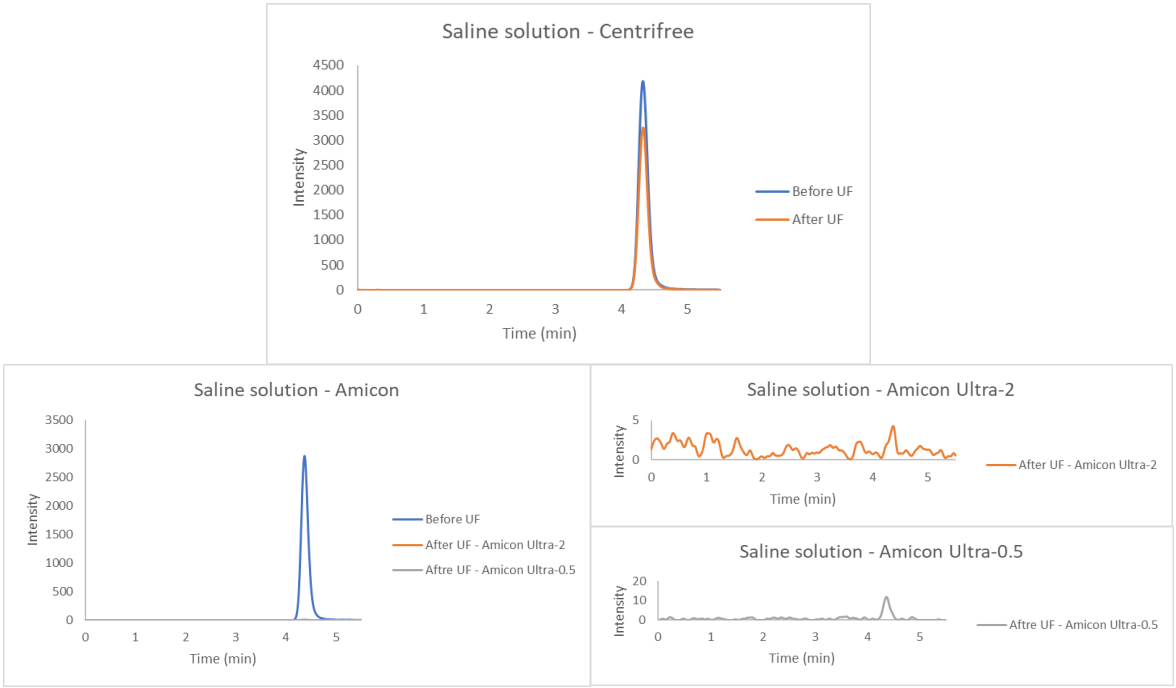

Fig. 3. Representative chromatograms of a 125 ng/mL CVD sample in saline solution before and after ultrafiltration (UF) using Centrifree vs. Amicon Ultra devices

Furthermore, in our experimental conditions, determinations made in HSA solution revealed an overall unbound fraction mean for CVD of 7.95% (±0.053), which implies a protein binding degree of more than 92%, results also in accordance to other literature data (Morgan, 1994). Compared to human plasma, which is a very complex matrix, containing a variety of proteins (albumin, alpha-1-acid glycoprotein, globulins, lipoproteins), the 5% HSA solution was chosen as a simple proteic matrix for the present study in order to also emphasize the difference in the binding behavior of CVD, and the possible influence of the proteic matrix complexity on the results obtained for the different UF devices.

The purpose of the determinations made in saline solution, were to allow an assessment of the possibility of NSB occurrence in the absence of proteins from the matrix. In a previous study, Wang S and Williams NS have shown that NSB is greatly reduced, even in the case of compounds with high lipophilicity, when samples containing protein environments are incubated in the UF device because proteins present a protective effect of blocking the NSB sites (Wang and Williams, 2013). Furthermore, the NSB and adsorption from proteic matrices can also be expected to be significantly lower, as the protein-bound drug fraction cannot be adsorbed.

The results obtained for CVD samples in saline solution indicate that, in the absence of proteins from the matrix, in the case of both types of UF devices considered, a different degree of NSB occurs. While for the Amicon Ultra devices, because of the lack of analyte in the ultrafiltrate, we could conclude that the NSB degree is maximum, in the case of the Centrifree devices an overall mean of less than 35% NSB was observed.

The separate statistical analysis of the data sets obtained for the Centrifree devices, regarding the two matrices considered (5% HSA and saline solution), revealed no significant statistical difference in terms of normality of distribution, variance and mean

free fraction of CVD ($p > 0.05$). These results indicate that, for the considered concentration range (25-500 ng/mL), the binding behavior of CVD to HSA and the adsorbtion which takes place in saline solution, respectively, are not influenced by concentration.

The very poor results obtained for the Amicon Ultra devices could be related to the materials from which the sample reservoir and collection tube are made, leading to a great adsorbtion of CVD, this being the main difference from the Centrifree devices. The difference in the molecular weight cut-off of the semipermeable membrane (10 kDa vs. 30 kDa) should not have an influence on the diffusion of CVD, taking into account its much lower molecular weight (406.5 g/mol). Furthermore, the volume of the sample reservoir in the case of Amicon Ultra devices (2 mL vs. 0.5 mL) seems to not have an influence on the result.

Modified ultrafiltration method

The modified ultrafiltration method described by Taylor and Harker (Taylor and Harker, 2006) was used for assessments regarding samples containing 125 ng/mL CVD in both human plasma and 5% HSA solution. For this method Amicon Ultra-0.5 devices were selected and samples were analyzed in singlicate. In the case of both matrices, a very high recovery of the analyte was observed in the retentate reconstituted samples (111.83% for samples in human plasma and 98.38% for samples in 5% HSA), while no presence of the analyte was detected in the filtrate reconstituted samples.

These results further sustain the very high degree of CVD adsorbtion in the sample reservoir of the Amicon Ultra devices. Even though, in the mentioned study, the research was also focused on highly lipophilic compounds (corticosteroids), the much better results using the modified UF method could be related to the different UF devices used (Microcon).

Conclusions

When studying PPB of drugs using the UF method, a very close attention should be paid to the implied UF protocol and to the UF devices used. The Centrifree filter devices, which were specifically designed for evaluations of PPB, have also proven to be suitable for the study of the protein binding process in the case of the lipophilic compound CVD, in comparison to the Amicon Ultra devices for which very poor results were obtained. The different materials used for the components of the Amicon Ultra devices, compared to Centrifree, seemed to result in a great adsorbtion of the analyte to the sample reservoir, making the devices impractical for use in the desired study approach.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This research was funded by George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Targu Mures, grant number 10127/5/17.12.2020.

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