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Determination of the extent of protein binding of antibiotics by means of an automated continuous ultrafiltration method

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Abstract

The extent of the protein binding of a drug is an important pharmacokinetic parameter. In this study, the determination of the protein binding of the antibiotics gatifloxacin, moxifloxacin, linezolid and telithromycin to bovine serum albumin (BSA) and human serum albumin (HSA) was performed by means of an automated continuous ultrafiltration method. The continuous ultrafiltration has several advantages compared to classical methods like equilibrium dialysis or discontinuous ultrafiltration. The method is appropriate for a fast determination of the extent of the protein binding of a drug. In one single experiment the calculation of the protein binding over a wide range of different drug/protein ratios is possible. Comparing the results obtained with the continuous ultrafiltration with binding data reported in literature shows good correlation and proves the reliability of the method.
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Keywords: Protein binding; Automated continuous ultrafiltration; Antibiotics; HSA; BSA

1. Introduction

The extent of protein binding is important to know because it is only the unbound fraction of a drug, which is able to bind to a receptor or an enzyme and along with this to induce a pharmacological effect. In addition, the drug fraction bound to plasma protein cannot be enzymatically metabolized and further down the route excreted. To be more precise, the extent of protein binding largely influences adsorption, distribution, metabolism and excretion as well as toxicology, taken together the ADMET parameter (Benet and Hoener, 2002). Thus, the precise in vitro determination of the extent of protein binding which mimics the in vivo conditions is not only decisive for drugs already in clinical use but also in drug development. Having identified a lead compound for a certain target the optimization of the lead has to consider both high affinity and selectivity for one target, e.g. a receptor or enzyme, and ADMET parameters. In the latter case, a fast and cheap method is needed in order to be able to screen large numbers of newly synthesized potential active compounds. Whereas the classical methods, such as dialysis, electrophoresis, gel chromatography or ultracentrifugation (Oravcova et al., 1996), suffer from several drawbacks like being time-consuming, using expensive materials and the determination of the extent of protein binding at a certain concentration of the drug and the protein, the automated continuous ultrafiltration method is fast, cheap and close to in vivo conditions. Since the protein binding corresponds to the simple mass-action equation, the formation of the drug/protein complex depends on the concentration of both components. The method applied should be able to characterize the extent of protein binding over a broad range of concentration, which is perfectly fulfilled by the continuous ultrafiltration.

The method originally developed by Kinawi and Teller (1979), Nickel and Schlerf (1983) and Oehlmann (1996) was used by Zlotos et al. (1998) for the determination of fluoroquinolone antibiotics. It is based on a titration method of a continuous ultrafiltration using an especially constructed measuring cell containing an ultrafiltration membrane of defined pore size and an UV detector. The cell is loaded with a fixed concentration of human or bovine albumin or plasma and the drug to be examined is pumped through the cell consisting of an ultrafiltration membrane of appropriate molecular weight cut-off (see Fig. 1). In detail, the inlet of the ultrafiltration cell is connected

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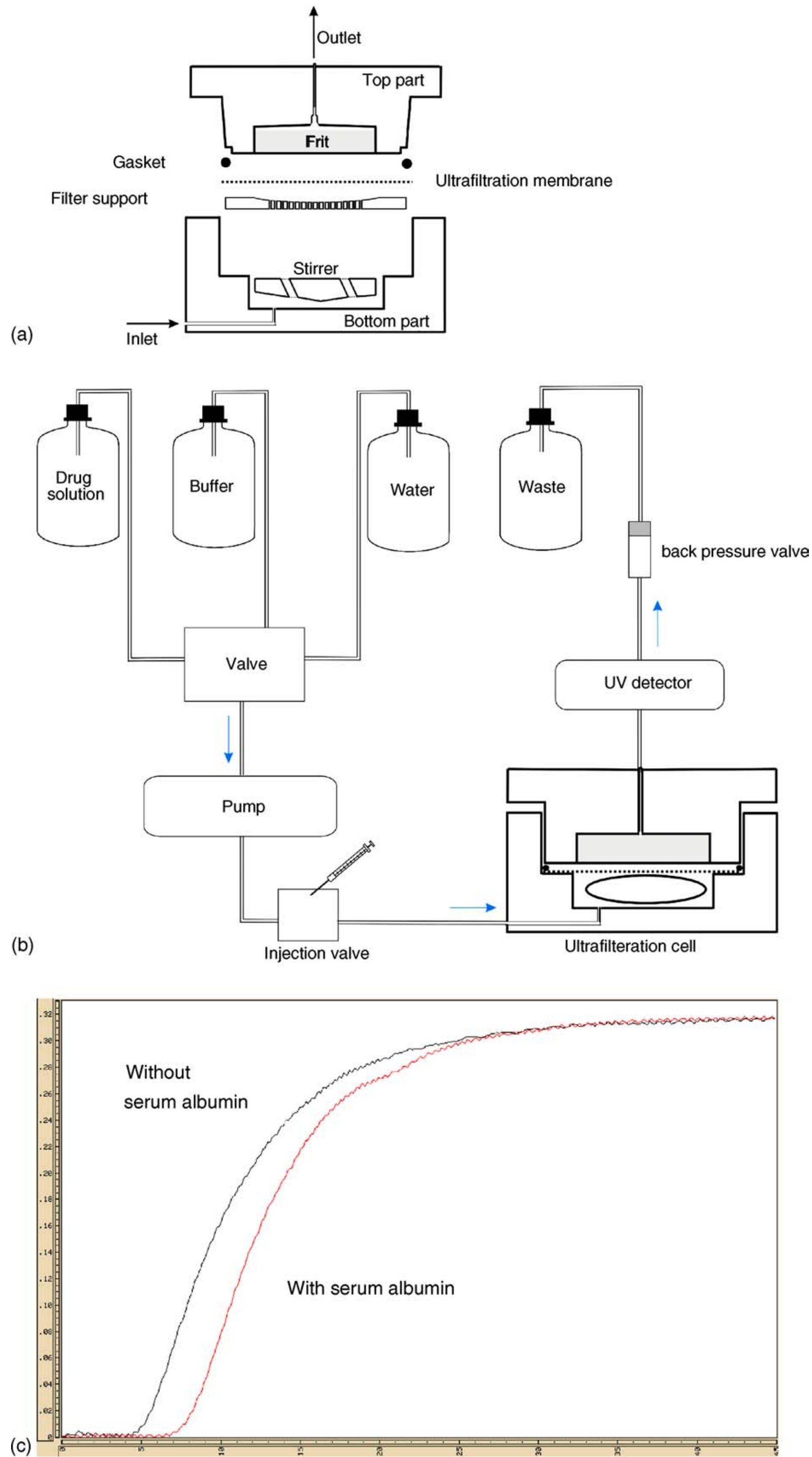

Fig. 1. Schematic representation of the cell (a), the entire instrument (b) and the elution curves (c) obtained in absence and presence of the serum albumin.

to three reservoirs for drug and buffer solution and for water, a valve to switch between the solutions, a low-pressure pump and an injection valve for injection of the protein and the outlet is equipped with an UV detector and a back-pressure valve.

The steps of the experiment are: firstly, the buffered drug solution is pumped continuously through the self-constructed ultrafiltration cell. The absorbance of the ultrafiltrate is measured (left curve, Fig. 1c). Secondly, an ultrafiltration membrane is put into the cell. Thirdly, after injection of the protein solution, the buffered drug solution is again pumped through the cell under constant flux and the absorbance of the filtrate is measured (right curve, Fig. 1c). Fourth, plotting the absorption values versus the time gives the elution curves as shown in Fig. 1. The area between these curves is proportional to the amount of drug bound to the protein in the ultrafiltration cell. Due to the interaction of the drug and protein molecules, the curve obtained from the drug/protein mixture is shifted to the right compared to the curve obtained from the drug solution. The stronger the protein binding is the greater the shift of this curve. For evaluation of the data, the area between these two titration curves is calculated using software originally developed by Nickel and coworkers (1996).

Since the original cell and computer software were especially designed for the determination of high extents of protein binding (Oehlmann, 1996) there was a need of optimization with regard to the precise determination of the protein binding of low and medium extent. Thus, the aim was to further optimize the cell and the computer program, which is automatically operating the measurement and computing the extent of the protein binding from the titration curves. In order to check the reliability of the new apparatus, drugs of a low and medium extent of protein binding were studied, i.e. the newly launched antibiotics gatifloxacin, moxifloxacin, linezolid and telithromycin (see Fig. 2) whose protein binding covers the range of 20–70% (Blaschek et al., 2004).

2. Material and methods

2.1. Materials

Gatifloxacin was supplied from Grünenthal AG (Aachen, Germany), moxifloxacin from Bayer AG (Leverkusen, Germany), linezolid from Pfizer (Kalamazoo, USA) and telithromycin from Aventis Pharma Deutschland (Frankfurt, Germany). The phosphate buffer 0.03 M (pH 7.4) containing 0.1 M NaCl is mixed from appropriate amounts of sodium hydrogen phosphate, sodium dihydrogen phosphate and sodium chloride each supplied by Merck AG (Darmstadt, Germany). Proteins applied were bovine (BSA, 65 kDa) and human (HSA, 66 kDa) serum albumin, respectively. BSA was supplied from Fluka (Taufkirchen, Germany) and HSA from VWR International (Nürnberg, Germany). The injected protein solution of a concentration of 40 mg/ml is made by dissolving the protein in the aforementioned phosphate buffer (pH 7.4). The drug solutions are made by dissolving different amounts of the drugs in the phosphate buffer resulting in concentrations between 10 and 30 mg/ml.

2.2. Continuous ultrafiltration

During the course of the experiments, phosphate buffer 0.03 M, pH 7.4 and the drug solution are pumped alternately. The solutions are pumped through the ultrafiltration cell (cell dimension 2.8 ml) containing an ultrafiltration membrane with a molecular weight cut-off of 30 kDa (type PLTK030 by Millipore, Bedford, USA), which holds the protein molecules in the cell. Only free and unbound drug molecules can pass the membrane and will be detected. The absorption is plotted versus time and the resulting adsorption time curves are transformed into titration curves. Two curves are needed, one with and one

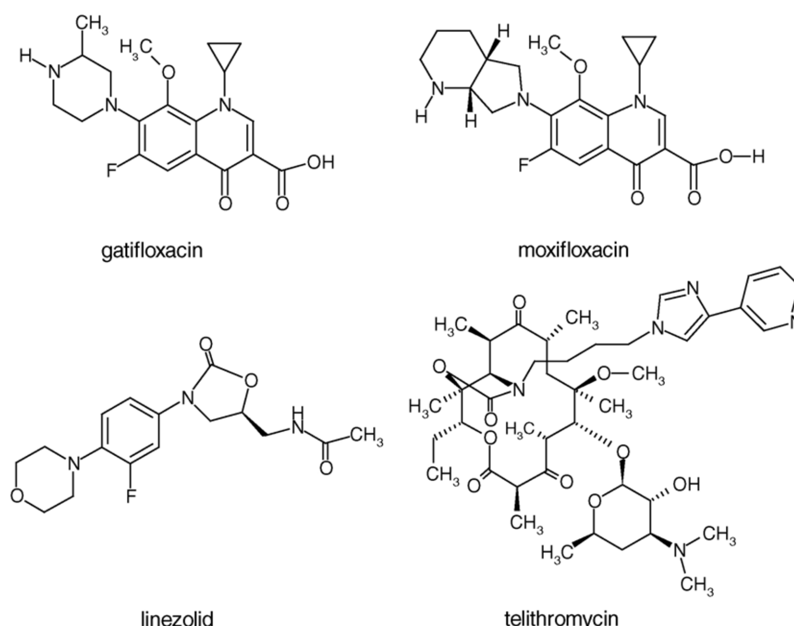

Fig. 2. Structural formulae of the antibiotics studied.

Table 1
Extent of protein binding and standard deviation found by means of the continuous ultrafiltration using HSA and BSA, and protein binding data reported in the literature

Drug	Protein binding towards BSA (%)	Protein binding towards HAS (%)	Literature
Gatifloxacin	13.9 ± 5.4	21.2 ± 5.6	~20% (Blaschek, 2003), 20 ± 5% (Nakashima et al., 1995), (plasma each)
Moxifloxacin	55.5 ± 4.0	53.5 ± 6.6	52 ± 8% (Müller et al., 1999), 39.4 ± 2.4% (Hardman et al., 2001), (plasma each)
Linezolid	52.7 ± 6.2	27.0 ± 1.5	31% (Stalker and Jungbluth, 2003), (plasma)
Telithromycin	59.4 ± 9.6	64.4 ± 4.4	60–70% (Blaschek, 2003), (plasma)

without protein molecules in the ultrafiltration cell. The evaluation of the data is performed by the computer program *PBCalc* written by Nickel and Reyer. The protein binding of a drug can be calculated over a wide range of drug/protein ratios.

3. Results and discussions

The constructed measuring system was further developed and optimized for the determination of drugs with a protein binding between 20 and 100%. The determination of low protein binding is more difficult than in the case higher protein binding extents because the adsorption time curves differ only slightly. Thus, the method was optimized with regard to the filter and the corresponding support, the computer software and the air often dissolved in the aqueous solution.

Great problems during such experiments raised due to air bubbles separated from the solution by the pumping which creates spikes that interfere with the measuring signal and, thus, make the analysis and evaluation of the data impossible. A back-pressure valve now integrated in the new system solves this problem. It prevents the appearance of air bubbles in the detector by keeping a certain pressure of 2–3 bar at a flow rate of 0.5 ml/min in the system.

The ultrafiltration cell was optimized concerning handling and tightness. The newly developed filter support made from acrylic glass allows the use of different ultrafiltration membranes, which would otherwise burst due to the pressure. In addition, membranes can be re-used several times which substantially reduces the expenses of the determinations. Therefore, the membranes have to be re-conditioned with 0.1 M NaOH solution in order to wash out the adsorbed protein and subsequently with ultrapure water for 1 h. Possible protein adsorption to the membrane can be detected by inspection of the first curves of subsequent experiments, which are obtained with the drug solution. Membranes can be stored away over night in 10% aqueous ethanol.

In order to avoid or minimize the adsorption of the protein to the membrane several membranes from different suppliers being composed of cellulose acetate, polyethersulfone, regenerated cellulose and cellulose esters, respectively, were checked. The regenerated cellulose membrane provided by Millipore showed the lowest adsorption of the protein to the membrane and was, thus, used throughout. In addition, this membrane could be re-used several times without any problems.

The software was adapted for the new system and further optimized. Different data masks, which guide automatically through the experiment step-by-step (Heinze, 2004), allow the fast input of all parameters necessary for the experiments. Furthermore, more data are recorded during the experiments to improve the calculations and the software is able to control more than one experiment at a time.

Using the new system, the extent of the protein binding was evaluated for the antibiotics linezolid, teilthromycin, gatifloxacin and moxifloxacin from three determinations with BSA and HSA. The corresponding standard deviations are displayed in Table 1. For sake of comparison the values found in the literature with various methods are also given. The standard deviations of the reference data are given if available.

The standard deviations found in all cases are rather low and often in the same range as described in the literature with the classical methods, indicating a good reproducibility of the continuous ultrafiltration. Especially in the case of moxifloxacin the standard deviations are lower than the ones reported in literature.

Since HSA and BSA used are not identical, differences in the amount of the protein binding may occur. A comparison of data obtained with BSA and HSA is therefore sometimes hardly possible. The results show that the percentage of the protein binding of the oxazolidinone linezolid is strongly dependent on the protein used. The binding obtained with BSA is twice as high as with HSA. The fluoroquinolone gatifloxacin shows a higher binding to HSA compared to BSA. For moxifloxacin and telithromycin, the extents of the protein binding to BSA and HSA are in the same range. However, these results point to the fact that BSA being much cheaper than HSA cannot be used a representative of HSA or plasma. In contrast, all results of the experiments performed with HSA are in good accordance with data reported in the literature where the binding to plasma was determined in all cases.

4. Conclusion

The optimized continuous ultrafiltration method is appropriate for fast, precise and cheap determination of the extent of protein binding. In order to determine the protein binding of several potential drugs at a time or to determine the protein binding of one drug to various ingredients of plasma at a time, it is planned to construct an instrument for parallel measurements. This is extremely useful for drug development purposes where hundreds of compounds are often synthesized by automatisation. The work is already in progress.

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