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Determination of free vancomycin, ceftriaxone, cefazolin and ertapenem in plasma by ultrafiltration: Impact of experimental conditions

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ABSTRACT

Ultrafiltration is a rapid and convenient method to determine the free concentrations of drugs. In the present work, we aimed to develop an ultrafiltration method which is appropriate for routine determination of the free fraction of vancomycin and highly protein bound beta-lactams such as ertapenem, ceftriaxone and cefazolin in plasma from intensive care unit patients. Different filter types and experimental conditions (molecular weight cut-off, centrifugal force and time, pH, temperature) were evaluated and found to have influence on the result. In the final protocol, serum or plasma was buffered to pH 7.4–7.5, ultrafiltered at 1000 × *g* at 37 °C for 20 min using Nanosep Omega 10 K filters and subsequently analysed for the antibiotics by RP-HPLC with UV detection. The data from our investigation suggest to aim physiological conditions, i.e. 37 °C and pH 7.4, and low to moderate relative centrifugal forces in order to get reliable results. With regard to the chromatographic separation, modulation of the pH in the range of 2.5–7.0 allows to determine several beta-lactams isocratically and/or to avoid interferences by co-administered drugs.

1. Introduction

It is generally accepted that the pharmacodynamic effect of a drug is related to the exposure of a patient to the free concentration of the drug at its action site rather than its total concentration [1]. This is of particular importance in antibiotic therapy, where total plasma concentrations are traditionally related to the minimal inhibitory concentration (MIC) which however is determined in protein free culture medium. The clinical relevance of plasma protein binding changes particularly in ICU patients has been addressed in a recent review [2]. Equilibrium dialysis is accepted as reference method to determine free drug in vitro [3]. However, ultrafiltration has become more popular due to much shorter time required: <30 min vs. up to 24 h. Ultrafiltration is conducted under the assumptions that: (1) the drug does not bind to the membrane; (2) there is no leakage of plasma protein through the membrane; (3) the equilibrium constant does not change as the protein is gradually concentrated during the separation process; and (4) the membrane is equally permeable to the drug and water [3]. Accordingly, several variables may have influence on the results. The Millipore Centrifree (formerly Amicon Centrifree MPS-1) ultrafiltration device is traditionally used, but newer and less expensive devices have become available based on 1.5 mL Eppendorf tubes. Although designed to concentrate protein or RNA solutions, these devices have also been used to determine free concentrations of drugs including beta-lactams in human plasma or serum [4–10]. In preliminary experiments, we determined the free fraction of vancomycin in plasma of ICU patients to 47% using 14,000 × *g* and room temperature. However, re-analysis at 1000 × *g* with the centrifuge thermostated at 25 °C yielded a free fraction of 63%. This prompted us to investigate the influence of experimental conditions such as filter device, pH, temperature, centrifugation time and relative

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centrifugal force (RCF) on the free drug concentration with focus on vancomycin and highly protein bound beta-lactam antibiotics such as ceftriaxone, cefazolin and ertapenem.

2. Materials and methods

2.1. Chemicals, drugs and instruments

The following centrifugal filter devices were used: Amicon Ultra-0.5 mL Ultracel 10 K/30 K, Centrifree YM-30 (Millipore, Bad Schwalbach, Germany), Vivaspin 500 PES 10 K/30 K, Vivacon 500 Hydrosart 10 K/30 K (Sartorius Stedim Biotech, Göttingen, Germany), and Nanosep Omega PES 10 K/30 K (VWR, Ismaning, Germany). Centrifugation was performed using a Sorvall Super T21 centrifuge with fixed angle rotor SL-50T (Thermo Fisher Scientific, Dreieich, Germany) for Centrifree devices, or an Eppendorf 5417R centrifuge with fixed angle rotor F45-30-11 (Eppendorf, Hamburg, Germany) for all other filter devices. The HPLC columns were Kinetex C18 2.6 μm 100 mm × 3 mm (Phenomenex, Aschaffenburg, Germany), XBridge C18 2.5 μm 30 mm × 4.6 mm, XBridge BEH C18 2.5 μm 50 mm × 3 mm (Waters, Eschborn, Germany) or Nucleoshell RP 2.7 μm 50 mm × 4 mm (Macherey-Nagel, Düren, Germany), preceded by a column protection system (Nucleoshell RP 2.7 μm 4 mm × 3 mm, Macherey-Nagel, Düren, Germany). Vancomycin hydrochloride from *Streptomyces orientalis* and human serum albumin (HSA) essentially fatty acid free were obtained from Sigma–Aldrich, Steinheim, Germany. Vancomycin (Vancomycin Eberth 1g), ertapenem (Invanz 1g), ceftriaxone (Ceftriaxon Kabi 2g) cefazolin (Cephazolin Fresenius Kabi 2g) were obtained from the respective pharmaceutical company. All other chemicals were obtained from E. Merck, Darmstadt, Germany. Water was purified using an Arium basic water purification system (Sartorius Stedim, Göttingen, Germany).

2.2. Ultrafiltration

Blank serum or heparin plasma was obtained from healthy volunteers and stored at −70 °C. The samples were thawed in ice water, mixed and centrifuged (3800 × *g*, 10 min) prior to ultrafiltration in order to remove possibly precipitated fibrin. Solutions of vancomycin in HSA were prepared in isoosmotic sodium phosphate buffer of pH 7.4. Stock solutions were prepared at 2–5 g/L in water and stored in aliquots at −70 °C. Serum, plasma or HSA was spiked with the antibiotics at the ratio of 1:50. Centrifuge rotor and chamber were pre-warmed or cooled to the desired temperature before ultrafiltration. According to the manufacturers' manual, a pre-conditioning time of approximately 1 h is necessary. To check the rotor temperature, two 1.5 mL Eppendorf tubes filled with glycerine were centrifuged, and the actual temperature of glycerine was measured before and after the run. The thus determined temperature did not differ more than 1 °C from the nominal value, as indicated on the display. In the final protocol, 300 μL of plasma or serum was buffered with 10 μL 3 M potassium phosphate of pH 7.5 (resulting pH 7.3–7.35; vancomycin: 10 μL of 0.69 M sodium phosphate, pH 6.8, resulting pH 7.4–7.5), centrifuged 10 min at 100 × *g* (conditioning) and 20 min at 1000 × *g* using a Nanosep Omega 10 K filter device. An aliquot of 1–5 μL ultrafiltrate was injected into the HPLC system.

2.3. Chromatography

Chromatography was performed on a Prominence Modular LC-20 series HPLC (Shimadzu, Duisburg, Germany) with photometric detection at 240 nm (vancomycin), 260 nm (cephalosporins) and 300 nm (ertapenem), respectively. Vancomycin was determined as previously described [11], the beta-lactams according to McWhinney [12] with minor modifications. The autosampler was cooled to 6 °C, the column temperature was maintained at 40 °C, and the flow rate was 0.4 mL/min. The analytical column was a Waters XBridge C18 BEH 2.5 μm (50 mm × 3 mm). The mobile phase was prepared by mixing 100 mM o-phosphoric acid and/or sodium dihydrogenphosphate with sodium hydroxide, as appropriate. Acetonitrile was added as organic modifier. The ratio buffer/acetonitrile (v/v) and apparent pH of the mixture was: for ertapenem 100:6, pH 6.2–6.3 or for ertapenem and cefazolin 100:15, pH 3.0–3.1, and for ceftriaxone 100:12, pH 2.55. Ertapenem and cefazolin eluted after 2.9–3.2 min and ceftriaxone after 2.4–2.6 min.

2.4. Quantification of protein

The protein concentration in ultrafiltrate was determined using a Bradford Protein Assay kit (Bio-Rad Laboratories, Munich, Germany) and a Tecan Sunrise microplate reader (Tecan, Crailsheim, Germany).

2.5. Statistical analysis

The free fraction was calculated in the spiked samples as the percentage ratio of drug concentration in the ultrafiltrate to the nominal concentration. GraphPad Prism version 6 for MacOSX (GraphPad Software, La Jolla, CA, USA) was used for calculating descriptive statistics.

3. Results and discussion

3.1. Selection of the ultrafiltration device

Vancomycin was used as model drug to test different devices (Table 1), as much work has been published on this compound using different ultrafiltration devices and conditions with very different results (cf. [11]). The ultrafiltration devices were compared with the traditionally used Centrifree device at 1000 × *g* and 2000 × *g* which is the maximum recommended RCF for the Centrifree device. Amicon Ultra-0.5 filter devices were excluded, because they did not produce sufficient filtrate at 2000 g/25 °C. Moreover, the free fraction of vancomycin was low, when high RCFs were used as applied e.g. for beta-lactams [4,7–10,13]. We reproduced the observation of lowered free drug at high RCFs with the Nanosep filter devices. The measured free fraction in plasma samples spiked with vancomycin 25 mg/L decreased significantly from about 73% at 1000 × *g* to 45% (−60%) at 10,000 × *g*. This phenomenon has been previously described and referred to as "pressure effect" [14], i.e. the permeability of drug and water through the membrane differ with increasing RCF and molecular weight. The effect was less pronounced with ertapenem or ceftriaxone (decrease by 30%, data not shown) exhibiting a molecular weight of 476–555 D compared with 1450 D for vancomycin. Obviously, the potential advantage of the newer filter devices to be used at high speed [4,7–10,13] cannot be exploited without severe alteration of the results. Because the magnitude of the effect is dependent on the molecular weight of the solute, a generally upper limit for RCF cannot be specified. However, a maximum RCF of 2000 × *g* and a fixed angle rotor is recommended in agree with the Centrifree user manual where the "pressure effect" is described under "polarisation control". As most applications were performed using this device, alternative devices should be validated against Centrifree as reference method.

The Nanosep Omega 10 K filter device was selected for the following reasons: The free fraction of vancomycin was independent of molecular weight cut-off (MWCO) and comparable with the Centrifree device at lower costs. Moreover, it can be used in microcentrifuges with fixed-rotors of high capacity. A sufficient