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Unbound fraction of fluconazole and linezolid in human plasma as determined by ultrafiltration: Impact of membrane type

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ABSTRACT

Ultrafiltration is a rapid and convenient method to determine the free concentrations of drugs in plasma. Several ultrafiltration devices based on Eppendorf cups are commercially available, but are not validated for such use by the manufacturer. Plasma pH, temperature and relative centrifugal force as well as membrane type can influence the results. In the present work, we developed an ultrafiltration method in order to determine the free concentrations of linezolid or fluconazole, both neutral and moderately lipophilic antiinfective drugs for parenteral as well as oral administration, in plasma of patients. Whereas both substances behaved relatively insensitive in human plasma regarding variations in pH (7.0–8.5), temperature (5–37 °C) or relative centrifugal force (1000–10.000 x *g*), losses of linezolid were observed with the Nanosep Omega device due to adsorption onto the polyethersulfone membrane (unbound fraction 75% at 100 mg/L and 45% at 0.1 mg/L, respectively). No losses were observed with Vivacon which is equipped with a membrane of regenerated cellulose. With fluconazole no differences between Nanosep and Vivacon were observed. Applying standard conditions (pH 7.4/37 °C/1000 x *g*/20 min), the mean unbound fraction of linezolid in pooled plasma from healthy volunteers was 81.5 ± 2.8% using Vivacon, that of fluconazole was 87.9 ± 3.5% using Nanosep or 89.4 ± 3.3% using Vivacon. The unbound fraction of linezolid was 85.4 ± 3.7% in plasma samples from surgical patients and 92.1 ± 6.2% in ICU patients, respectively. The unbound fraction of fluconazole was 93.9 ± 3.3% in plasma samples from ICU patients.

1. Introduction

Antiinfective drugs such as linezolid, an antibiotic for serious gram-positive infections [1], or fluconazole, a still first line antifungal drug [2], are neutral, moderately lipophilic drugs for parenteral as well as oral administration. Antiinfective drugs are commonly evaluated on the basis of pharmacokinetic/pharmacodynamic (PK/PD) indices, all of which are based on a comparison of its plasma concentrations and the minimal inhibitory concentration (MIC) against the pathogen. As only the free drug is responsible for the pharmacological activity, all PK/PD indices should refer to the unbound (non-protein bound) fraction of the drug [3]. Ultrafiltration is a simple and popular method to determine the free, non-protein bound fraction of a drug in plasma. Ultrafiltration has several advantages over other methods such as equilibrium dialysis, ultracentrifugation or solid-phase microextraction. The method is technically simple, rapid and inexpensive, and it is also suitable for unstable drugs [4]. However, the experimental variables such as pH, temperature, relative centrifugal force and the specific ultrafiltration device can influence the results and should be validated [5,6]. Moreover, apart from Centrifree no device is recommended by the manufacturer for the determination of the free drug in plasma. Differing results for the unbound fraction of fluconazole with either polysulfone or cellulose filters [7] prompted us to examine the protein binding of fluconazole and linezolid in human plasma using our standard ultrafiltration device Nanosep Omega [5] (exhibiting a modified polyethersulfone membrane) as well as alternative devices.

2. Materials and methods

2.1. Drugs and chemicals

Standard substance linezolid was obtained from Pfizer, Berlin, Germany, fluconazole from Sigma-Aldrich, Steinheim, Germany. Stock solutions of the antiinfectives were prepared at 1 g/L in

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water and stored in aliquots at −70 °C. Zyvoxid 2 mg/mL solution for infusion (Pfizer, Berlin, Germany) or Fluconazol B. Braun 2 mg/mL solution for infusion (B. Braun, Melsungen, Germany) could equivalently be used for calibration. Tween 80 was obtained from Sigma-Aldrich, Munich, Germany, all other chemicals were obtained from Merck, Darmstadt, Germany. Water was purified using an Arium basic Ultrapure Water System (Sartorius Stedim, Göttingen, Germany). Plasma (LiHeparin) or serum (albumin concentration 42–44 g/L) was obtained from healthy volunteers and pooled. The term plasma is used consistently in the text, as plasma or serum could be used equally.

2.2. Determination of total and free drug

2.2.1. Sample preparation

For validation purposes, pooled plasma from healthy volunteers was spiked with 5% aqueous dilutions of linezolid or fluconazole (final concentrations 100, 30, 10, 3, 1, 0.3, 0.1 mg/L). For the determination of total drug, 100 μL plasma was mixed with 100 μL 7% perchloric acid (10 mL perchloric acid 70% mixed with 90 mL water), incubated for 15 min at 4 °C and centrifuged (2 min/10,500 x *g*) to separate the precipitated proteins. An aliquot of 1–2 μL was injected into the HPLC. The determination of free drug was performed as described previously [5]. To determine the dependence of protein binding on concentration, pH, temperature or relative centrifugal force (RCF), 1500 μL plasma was buffered with 50 μL 3 M potassium phosphate pH 7.45, 3 M TRIS-HCl, pH 8.08 or pH 8.62, and the pH was fine adjusted with concentrated HCl or 10 M NaOH to 6.90–6.95 (target pH 7.0), 7.25–7.30 (target pH 7.40), 8.05–8.10 (target pH 8.0) and 8.55–8.60 (target pH 8.5), respectively. An aliquot of 285 μL buffered plasma was transferred to the ultrafiltration device and spiked with 15 μL of aqueous linezolid or fluconazole dilutions. The solution was mixed and incubated in the centrifuge at 37 °C for 10 min while running at 100 × *g*. No filtrate was obtained during this time period. Characteristics of the ultrafiltration devices and the ultrafiltration process are listed in Table 1. To assess the amount of non-specific binding to the ultrafiltration device, solutions of the drugs in 0.1 M potassium phosphate buffered saline pH 7.4 (PBS) were treated identically except that the centrifugation time was shorter. The recovery (%) was calculated from the concentrations measured in the ultrafiltrate (C_{UF}) and PBS donor samples (C_{PBS}) using the equation: Rec. = C_{UF}/C_{PBS} * 100 (%).

The following centrifugal filter devices were used: Amicon Ultra 10, Centrifree 30 K, Microcon 10 K (Millipore, Bad Schwalbach, Germany), Vivacon 500 Hydrosart 10/30 K (Sartorius Stedim Biotech, Göttingen, Germany) or Nanosep Omega 10 K (VWR, Ismaning, Germany). Centrifugation was performed using a Heraeus Multifuge 1 L-R with swing-out rotor (Thermo Fisher Scientific, Braunschweig, Germany) for Centrifree devices, or an Eppendorf 5417R centrifuge with fixed angle rotor F45-30-11 (Eppendorf, Hamburg, Germany) for the other devices which all are based on Eppendorf tubes.

2.2.2. Chromatography

HPLC was carried out on a Prominence Modular LC20 system equipped with a variable wavelength UV detector LC-20A and a LC-20 solution data management system (Shimadzu, Duisburg, Germany). The separation system consisted of a Nucleoshell RP 2.7 μm 100 × 3 mm (Macherey-Nagel, Düren, Germany) analytical column (linezolid) or a Luna PFP 3 μm 150 × 4.6 mm (Phenomenex, Aschaffenburg, Germany) column (fluconazole). The eluent was 50 mM sodium dihydrogen phosphate/acetonitrile, pH 6.0–6.3, 75:25 (v/v, linezolid) or 80:20 (v/v, fluconazole). The analytical columns were protected with a 4 × 3 mm guard column filled with Nucleoshell RP 18 2.7 μ (column protection system, Macherey-Nagel, Düren, Germany). The flow-rate was 0.4 mL/min (linezolid) or 1.0 mL/min (fluconazole), the retention times were 2.5 min and 7.0 min, respectively. The column temperature was kept at 40 °C. The UV-detector was set to 254 nm (linezolid) or 210 nm (fluconazole). The injection volume was 1 to 10 μL. The assays were validated according to the relevant EMA guideline [8]. Regarding the determination of total fluconazole, the recovery from plasma was 100.7 ± 3.0%. The linearity has been proven from 100 to 0.1 mg/L (R > 0.9998) with intra- and inter-assay imprecision ≤6% and accuracy of 99.8%. The corresponding values for total linezolid were: recovery 100.7 ± 1.9%, coefficient of linear correlation (100–0.1 mg/L) R > 0.9996, intra- and inter-assay imprecision <3%, accuracy 100.5%. The lowest concentration on the calibration curve (0.1 mg/L) was taken as LLOQ.

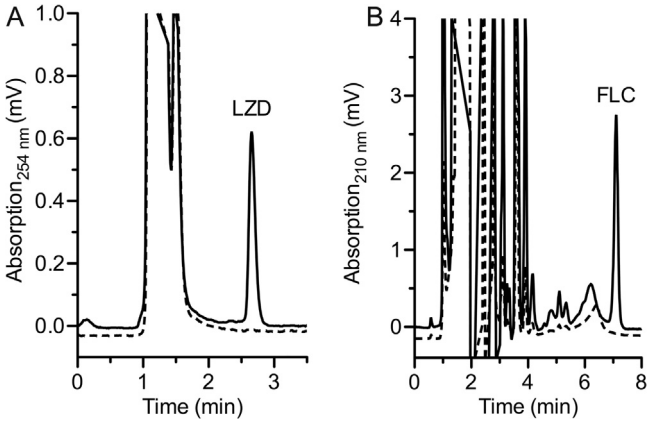

Fig. 1. Chromatograms of free (A) linezolid or (B) fluconazole in plasma of healthy volunteers spiked with linezolid 0.8 μg/mL or fluconazole 3.0 mg/L. The dashed lines indicate blank plasma. Injection volume (A) 1 μL or (B) 10 μL.

2.3. Statistical analysis

GraphPad Prism 6.0 for MacOSX (GraphPad Software, La Jolla, CA, USA) was used for calculating nonparametric or parametric descriptive statistics and Pearson's correlation coefficient R. Least square analysis of dilution series was performed using the weighting factor $1/y^2$. Data are presented as mean (± standard deviation when n > 3), median or range, as appropriate.

3. Results

3.1. Chromatography

The validation data of precision and accuracy were reproduced in routine work using one point calibration at mid-level concentration. Imprecision and in-accuracy, based on in-process cofntrol samples, were <3% for total linezolid (20 and 2 mg/L) and <2% for total fluconazole (30 and 3 mg/L), respectively. Processed samples of linezolid as well as of fluconazole (total and free) were stable at least for 24 h at room temperature or 48 h at 6 °C. The in-process precision of the determination of free linezolid or fluconazole was assessed using spiked pool plasma from healthy volunteers analyzed with each run. The intra-assay precision was not further examined as precision between duplicates was 1% in preliminary experiments. The inter-assay precision of free linezolid was 2.6% (unbound fraction 78.2 ± 2.0%, n = 18) and 2.1% of free fluconazole (unbound fraction 89.7 ± 1.9%, n = 10), respectively. Accuracy and linearity cannot be specified exactly, as the true unbound fraction of the drugs in plasma is not known and protein binding can be concentration dependent. Representative chromatograms of spiked plasma samples are depicted in Fig. 1.