Protein-Binding Characteristics of Voriconazole Determined by High-Throughput Equilibrium Dialysis

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ABSTRACT: Plasma protein binding (PPB) can possibly alter the already variable pharmacokinetics of voriconazole. Voriconazole PPB was determined only once, being 58%, according to equilibrium dialysis (ED). We investigated voriconazole PPB more in detail, with a convenient and newer high-throughput ED assay (HT-ED), in human blank plasma spiked with voriconazole and in plasma from intensive care unit (ICU) patients treated with voriconazole. HT-ED was conducted in a 96-well plate, setup against phosphate-buffered saline. Voriconazole concentrations were measured by liquid chromatography–tandem mass spectrometry. The median PPB was 47.6% [interquartile range (IQR) 45.3%–50%] *in vitro*, and 49.6% (IQR 42.5%–52.5%) in ICU samples ($p = 0.35$), and is not depending on total voriconazole concentration (0.7–11.2 mg/L, $p = 0.65$). The drug mainly binds to albumin (25.5 ± 5.1%), and to a lesser extent to α-1-acid glycoprotein (AAG; 4.8 ± 1.2%). The HT-ED assay can be performed at 37°C or 25°C ($p = 0.44$) and in batch: PPB variations during freeze–thaw cycles ($p = 0.13$) and during frozen storage up to 12 months ($p = 0.10$) were not clinically relevant. Voriconazole PPB is approximately 50%, according to HT-ED. As albumin and AAG only account for approximately 30% of total voriconazole PPB, other plasma components could influence PPB and therefore efficacy or toxicity because of variations in unbound fractions. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:2565–2570, 2014

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INTRODUCTION

Since the launch of voriconazole, the first-line treatment for invasive aspergillosis,[1] its extreme inter- and intraindividual variability in exposure has been a challenge in setting an adequate therapy regimen.[2–11] Several factors influencing voriconazole plasma concentrations have been identified over the last few years, such as saturable metabolism by CYP2C19, CYP2C9 and CYP3A4,[11–13] CYP2C19 polymorphism,[14,15] drug accumulation in liver disease,[16] influence of age on its metabolism,[9] coadministration with CYP2C19- and CYP3A4-interacting comedication,[9] i.v. to oral switch,[9,12,17,18] and impact of food,[19,20] all resulting in large intra- and interpatient variability. Plasma protein binding (PPB) of voriconazole has, however, never been investigated as a factor causing variations in voriconazole plasma concentrations. Yet, this would be interesting, as only unbound drug concentrations exert pharmacological activity. For drugs with linear pharmacokinetics, hypoalbuminemia only seems to have relevant clinical impli-

cations in case of high protein binding (>70%).[21] However, in drugs with nonlinear pharmacokinetics, such as voriconazole, an effect can probably be seen also for drugs with lower PPB, because elevated unbound concentrations, resulting from hypoalbuminemia, cannot be eliminated immediately because of saturated hepatic metabolism.

A first step in exploring the influence of hypoalbuminemia on voriconazole protein-binding characteristics is the development of a robust, reliable, and easy-to-use method for determining voriconazole-unbound and voriconazole-bound concentrations. Moreover, *in vitro* tests are needed to determine voriconazole PPB in a reliable manner, enabling us to reveal alterations in voriconazole PPB because of hypoalbuminemia in a future project.

The most common methods for investigating protein binding of drugs *in vitro* include equilibrium dialysis (ED), ultracentrifugation, and ultrafiltration.[22–25] ED is the method of choice in research settings, using a semipermeable membrane to separate unbound and bound fractions with subsequent analysis of drug concentrations.[22–25] It is typically considered to be the gold standard, minimizing nonspecific binding of compounds to the device and avoiding the need for large plasma volumes.[24,26] Voriconazole PPB was previously reported to be 58%, according

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to ED results in premarketing studies, using a Dianorm Dialyzer (Harvard Bioscience Inc., Holliston, Massachusetts).[27] No data are available about the impact of technical factors potentially influencing PPB of voriconazole, such as temperature during ED, mass balance, volume shift, freeze–thaw cycles, and frozen storage. Nowadays, newer high-throughput ED (HT-ED) assays are available[28,29] (such as HTDialysis, Gales Ferry, Connecticut; ThermoFisher Scientific, Waltham, Massachusetts; Spectrum Laboratories, Laguna Hills, California) that are more user-friendly and more accurate compared with former ED devices, because of even less nonspecific binding to perform ED.[24,29] HT-ED also allows minimal volume sampling in a clinical patient setting.[24,29]

In this study, a robust and easy-to-use HT-ED assay is tested for the determination of voriconazole PPB. Moreover, the influence of several technical factors during HT-ED on PPB characteristics of voriconazole was investigated more in detail, both *in vitro* as *in vivo*.

MATERIALS AND METHODS

Chemicals and Solutions

Voriconazole (0.35 kDa) was provided by Pfizer (New York) and was used to prepare a stock solution of 3493 mg/L in methanol (LC–MS grade from Acros Organics, Geel, Belgium). This stock solution was further diluted in phosphate-buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl, pH 7.4) to the appropriate concentrations for the different experiments. α-1-acid glycoprotein (AAG) from Sigma–Aldrich BVBA (Diegem, Belgium) was used to prepare PBS solutions of 1 g/L. Albumin solutions were prepared in PBS, at a concentration of 40 g/L, with Albunorm® (Octapharma, Benelux, Belgium), Albumin® 20% (CAF, Brussels, Belgium), and Flexbumin® 200 g/L (Baxter, Braine-l′Alleud, Belgium). All solutions were freshly prepared at the moment of the experiments.

HPLC-graded water was generated using a Milli-Q-water-purification system (Merck Millipore, Billerica, Massachusetts). Ethanol was bought from Prolabo VWR (Amsterdam, The Netherlands). Pooled blank lithium heparin plasma was collected from healthy volunteers. Samples from intensive care unit (ICU) patients, treated with voriconazole for at least 4 days, were collected into lithium heparin tubes without gel separator, after approval by the local Ethical Committee and signing of the informed consent form. After centrifugation (10 min, 1500*g*), plasma was stored at −20°C until further analysis.

HT-ED Followed by Liquid Chromatography-Tandem Mass Spectrometry

Procedure

High-throughput ED was performed using a Teflon 96-well ED device and dialysis membranes (molecular weight cut-off 12–14 kDa) from HTDialysis (model HTD96b). Prior to use, the dialysis membranes were soaked for 60 min in 100% PBS, followed by 20 min in PBS with 20% ethanol. Before placing the membranes in the ED device, membranes were rinsed thoroughly with Milli-Q water so that they could be separated. In each well, 150 µL PBS buffer was loaded in the dialysate side against 150 µL investigational solution at the other side of the semipermeable membrane, through which only unbound molecules diffuse until equilibrium. Equilibration was carried

out during shaker incubation (150 rpm) (model Edmund Bühler GmbH; type KS-15 control; Glasatelier Saillart, Meerhout, Belgium) at 37°C (Edmund Bühler GmbH; TH15; Glasatelier Saillart), whereas the device was covered with an adhesive sealing film (HTDialysis), preventing evaporation of carbon dioxide (CO_2) and pH changes. After reaching the equilibrium, solutions from both compartments were stored at −20°C until analysis.

Determination of unbound and bound voriconazole concentrations was carried out using a previously in-house developed and validated liquid chromatography–tandem mass spectrometry (LC–MS/MS) method, with a LLOQ of 0.2 mg/L, between-run precision, expressed as coefficient of variation, below 12% and accuracy, expressed as a percentage of the theoretically added concentration, between 89% and 109%. Linearity was demonstrated from 0.06 to 20.0 mg/L.[30]

Calculation of PPB of Voriconazole

The percentage of unbound voriconazole was calculated using the following formula[22]:

$$UF\% = \frac{[\text{voriconazole}]_{PBS}}{[\text{voriconazole}]_{plasma}} \times 100$$

in which UF% stands for the percentage of unbound voriconazole. [Voriconazole]$_{PBS}$ and [voriconazole]$_{plasma}$ represent the concentration of voriconazole at equilibrium, in the buffer compartment and plasma compartment, respectively.

Factors Influencing PPB During HT-ED

Time to equilibrium was determined using human blank plasma, spiked with voriconazole at three clinically relevant concentrations (1.75, 3.5, and 14 mg/L). Voriconazole concentrations and unbound fractions were determined after 1, 2, 4, and 6 h of equilibration.

Mass balance was checked to rule out nonspecific adsorption of voriconazole to the device, by comparing a total voriconazole concentration of 3.4 mg/L in the plasma samples before HT-ED and the sum of the recovered voriconazole amounts in the buffer and plasma compartment after HT-ED.

All the latter *in vitro* tests were conducted using human blank plasma spiked with different concentrations of voriconazole (0.7–1.4–2.8–5.6–11.2 mg/L). The influence of temperature on PPB was investigated by conducting the HT-ED at 25°C and at 37°C. Variations in PPB of voriconazole because of frozen storage at −20°C were investigated up to 12 months. The influence of 1–3–5 freeze–thaw cycles on PPB was also investigated and the pH during ED, during frozen storage, and during freeze–thawing was measured with a pH meter, model Consort C830 (Consort BVBA, Turnhout, Belgium).

For every experiment, including time to equilibrium, HT-ED analyses were conducted at least in triplicate. Except when investigating the influence of temperature on ED, all experiments were performed at 37°C.

Voriconazole PPB determined in blank plasma spiked with voriconazole was compared with the PPB measured in plasma from critically ill patients treated with voriconazole.

In Vitro-Binding Characteristics of Voriconazole

Binding characteristics of voriconazole to albumin and AAG were investigated at 37°C in PBS solutions with 40 g/L albumin
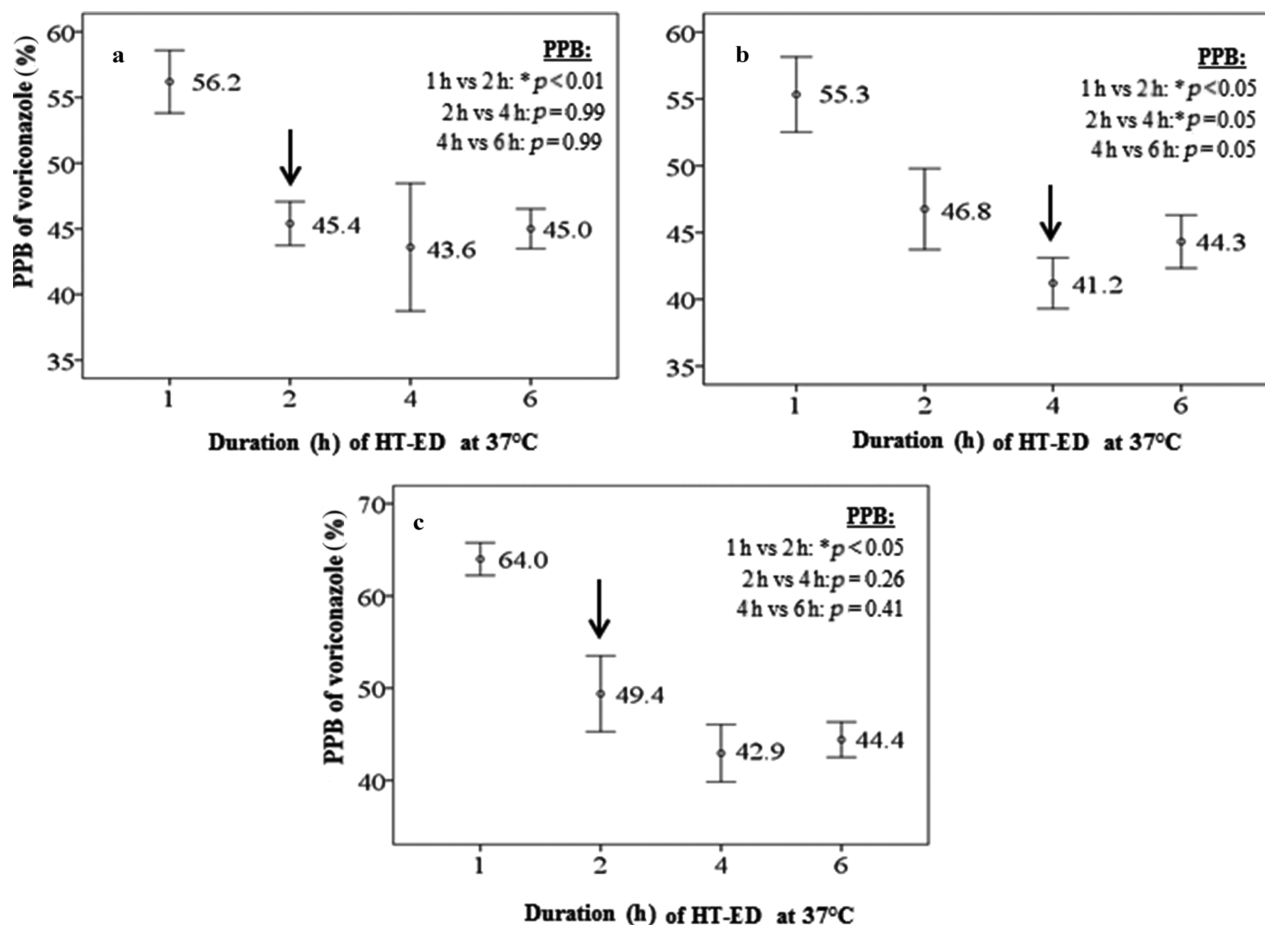
Figure 1. Time to equilibrium (h) for a total voriconazole concentration of 1.7 mg/L (a), 3.5 mg/L (b), and 14 mg/L (c) in human blank plasma. ED was performed at 37°C during shaker incubation. Unbound and bound voriconazole concentrations were determined in triplicate for each time point with the HT-ED device followed by LC–MS/MS.[30] Based on the graph combined with the *p* values, equilibrium time (arrow) was set at 2 h (a and c), and 4 h (b). *Significant difference.

(Albumin® 20% 10 mL; CAF), 1 g/L AAG (Sigma–Aldrich BVBA) or both, spiked with a fixed voriconazole concentration of 3.5 mg/L.

The potential differences in PPB of voriconazole associated with different commercial brands of human albumin were evaluated at 37°C by adding voriconazole (3.5 mg/L) to 40 g/L albumin in PBS, using Albunorm® (Octapharma), Albumin® 20% (CAF), and Flexbumin® 200 g/L (Baxter).

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 for Windows (SPSS Inc. 2011, Chicago, Illinois). Depending on the distribution of data, median, and interquartile range (IQR) or mean and SD were calculated and the appropriate tests were used (unpaired *t*-test or Mann–Whitney U test; one-way ANOVA with post-hoc Tukey analysis or Kruskal Wallis test, repeated measures ANOVA test, Linear Mixed Models, or Friedman's test; Regression Analysis). $p < 0.05$ was considered significant. The Bonferoni correction was used as multiple-comparison correction.

RESULTS

Equilibrium dialysis at 37°C during shaker incubation revealed an equilibration time of 2 h with 1.7 and 14 mg/L and 4 h,
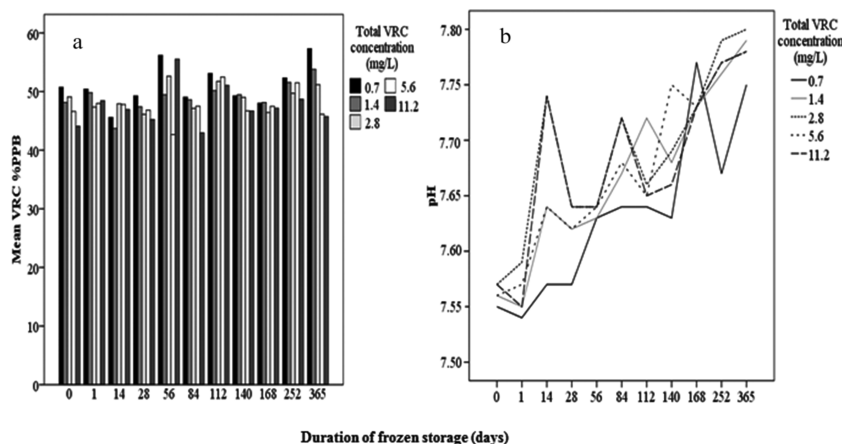
with 3.5 mg/L total voriconazole concentrations (Fig. 1). On the basis of these results, an equilibrium time of 4 h was applied in all following experiments. Mass balance was confirmed, using an initial voriconazole concentration of 3.4 mg/L: the sum of voriconazole concentration in buffer and plasma compartment after 4 h of equilibrium at 37°C was 3.3 ± 0.007 mg/L, which is within the margins of the analytical variance of the LC–MS/MS method [95% confidence interval (3.2 mg/L; 3.6 mg/L)].

Plasma protein binding did not vary with different voriconazole concentrations at 37°C ($p = 0.65$) (Table 1) and PPB was not influenced significantly when HT-ED was performed at 25°C versus 37°C ($p = 0.44$), nor during 1–3–5 freeze–thaw cycles, irrespective of total voriconazole concentration ($p = 0.13$) (Fig. 2a). No clinically significant changes in PPB were seen during the period of frozen storage ($p = 0.10$; data not shown). The sum of voriconazole concentration in PBS and in plasma remained stable overtime and did not differ from the concentrations of freshly prepared voriconazole in plasma solutions up to 1 year of frozen storage ($p = 0.96$), indicating the stability of voriconazole in both fluids.

pH increased during ED at 37°C, from 7.38 (freshly prepared plasma) to 7.68 (HT-ED during 6 h), with a mean difference of 0.26 ± 0.032 pH units compared with 7.38 ($p < 0.001$). An increasing trend, with 0.0006 units per day ($p < 0.001$) was

Table 1.   Voriconazole (VRC) plasma protein binding (PPB) determined with HT-ED at 37°C during 4 h with human blank plasma spiked with different total VRC concentrations (mg/L)

PPB (Mean ± SD)	Total VRC concentration (mg/L) spiked in human blank plasma					*p* value
	0.7	**1.4**	**2.8**	**5.6**	**11.2**	
	41.8 ± 5.3%	42.3 ± 5.9%	45.8 ± 2.0%	43.4 ± 1.0%	44.8 ± 0.8%	0.65

Figure 2.   (a) Variation in plasma protein binding (PPB) of voriconazole during frozen storage up to 12 months. At each time point, PPB was determined in triplicate for five different total voriconazole concentrations in human blank plasma. (b) Variation in pH value of human blank plasma during frozen storage up to 12 months. Mean pH increased from 7.54 to 7.80.

also seen in pH values during frozen storage, ranging from 7.54 in freshly spiked plasma to 7.80 after 12 months of storage (Fig. 2b).

In the *in vitro* experiment with albumin and AAG concentrations in PBS solution, voriconazole binds to albumin only for 25.5 ± 5.1% and to AAG for 4.8 ± 1.2%. No clear difference was seen in PPB when using Albunorm® 200 g/L (Octapharma), Albumine® 20% 10 mL (CAF), and Flexbumin® 200 g/L (Baxter) (33.4 ± 9.3%; 30.1 4.1%; 25.8 ± 5.4%, respectively; *p* = 0.23).

Finally, the median (IQR) PPB of all *in vitro* experiments with blank plasma spiked with voriconazole ($N = 235$) was 47.6% (45.3%–50%), which did not differ significantly ($p = 0.35$) from the median PPB in 20 plasma samples from 13 ICU patients [49.6% IQR 42.5%–52.5%)]. Median albumin and AAG concentrations in plasma of the ICU patients were 31.1 g/L (IQR 28.4–33.2 g/L) and 1.8 g/L (1.5–2.2 g/L), respectively. No exogenous albumin was administered within 1 month before sampling. The median total bilirubin plasma concentrations was 0.41 (IQR 0.28–1.69 mg/dL). The median number of voriconazole treatment days was 6 (IQR 4–14) and the median total voriconazole plasma concentration was 2.4 mg/L (IQR 1.5–4.5).

DISCUSSION

We have tested an easily workable HT-ED assay for determination of the PPB of voriconazole. To our knowledge, this is the first time that voriconazole PPB characteristics are investigated thoroughly, with a robust method, excluding factors that significantly influence the *in vitro* performance of the HT-ED assay. We found that voriconazole PPB could be determined accurately and robustly using HT-ED without significant influence of several factors such as environmental temperature

($p = 0.44$), freeze–thaw cycles ($p = 0.13$), and duration of frozen storage at $-20°C$ up to 12 months ($p = 0.10$).[31]

Moreover, the Teflon material of the 96-well plate prevents nonspecific adsorption of drug, as illustrated by the mass balance experiment.[28] Volume shift, because of osmotic pressure, is minimal because equilibrium is already reached within 4 h.[23,32] Because the stock solutions in methanol were first diluted in PBS buffer, the amount of solvent in the ED device was minimized to 0.02%–0.32%, preventing influence from solvent addition on the protein-binding equilibrium.[23,33]

In all experiments, voriconazole PPB was independent of total voriconazole concentration, ranging from 0.7 to 14 mg/L. Although the reference range of plasma voriconazole concentrations is set at 1 or 2–5.5 mg/L, often subtherapeutic (below 1 mg/L) and supratherapeutic (above 5.5 mg/L) plasma concentrations are seen in patients treated with voriconazole.[4,34] Because of the nonlinear pharmacokinetics, even plasma concentrations of 10–20 mg/L can be measured in some patients. This concentration independency can partly be explained because normal therapeutic voriconazole plasma concentrations (1 or 2–5.5 mg/L[4,34]) are many times smaller than the average plasma albumin concentration (±35–50 g/L),[35] implying nonsaturated albumin binding.

The fluctuations in pH during ED and during frozen storage seen in this project are most likely irrelevant, although it is often suggested that they occur because of CO_2 evaporation,[36] possibly inducing conformational changes to plasma proteins, resulting in an altered binding profile.[37,38] However, albumin conformational change only occurs at pH above 8.[37] A change in degree of ionization of voriconazole because of pH changes is also not applicable, because voriconazole behaves as a weak base with a pKa value of 1.76.[39] The variations in PPB during frozen storage for different total voriconazole concentrations are most likely not relevant in clinical practice, because they are all fluctuating around 50%.

The median PPB, both in blank plasma spiked with voriconazole [47.6% (IQR 45.3%–50%)] as in plasma from ICU patients treated with voriconazole [49.6% (42.5%–52.5%)] differed from 58%, as determined several years ago in the premarketing study.[27] Despite the fact that these differences in PPB might not lead to major changes in pharmacokinetics insights of voriconazole, we believe that the difference should primarily be evaluated from a technical point of view and that our reported PPB is more accurate because of an improvement of the study design.[27] First, and most importantly, the median *in vitro* PPB in our study was the result of 235 measurements, and was for the first time in literature, confirmed in plasma coming from 13 ICU patients treated with voriconazole. In the premarketing study,[27] time to equilibrium was set at 2 h, based on only one experiment with human plasma spiked with one concentration of voriconazole (i.e., 1 mg/L) in triplicate, which differs from our experiment where time to equilibrium was determined to be 4 h at several different clinically relevant voriconazole concentrations. Second, the Dianorm Dialyzer was rotated at 15 rpm during equilibration, compared with shaker incubation at 150 rpm in our experiment, which could have influenced time to equilibrium. Differences in thickness and pore diameter of the membranes used in the Dianorm Dialyzer compared with our assay could have influenced the diffusion of unbound voriconazole molecules and therefore time to equilibrium. Roffey et al.[27] used a volume of 1 mL plasma and buffer, compared with only 150 μL at both sides of the membrane in the HT-ED device. Third, in the premarketing study, a 0.1M phosphate buffer was used, which can possibly cause more volume shift because of a higher osmolarity compared with the PBS solution with 0.012M phosphate used in the HT-ED device. Volume shift can alter the results, because in the calculations as mentioned in the *Materials and Methods* section of our manuscript, an equal volume in both compartments is assumed.

Fourth, as liquid scintillation counting, which is the analytical method used by Roffey et al.,[27] is more prone to measurement variations, it might have resulted in lower precision and accuracy compared with our LC–MS/MS method,[30] possibly explaining in part the differences in protein binding of voriconazole between the two assays.

It is not completely clear whether these differences could have influenced the results; on the contrary, they cannot be neglected.

No information about mass balance is available for the Dianorm Dialyzer method.

To our knowledge, this is also the first study investigating which plasma components could be responsible for the total PPB of voriconazole. According to our results, albumin seems to be the main contributor to voriconazole PPB, although in literature, basic drugs have shown to bind preferably to AAG.[40] However, because only a PPB of approximately 30% was observed *in vitro* when combining clinically relevant concentrations of albumin and AAG, we believe that other components in human plasma are involved in voriconazole PPB, such as lipoproteins and α-, β-, γ-globulins.[23,40–43] The clinical relevance of these observations should be further explored.

As albumin was found to be the predominant protein determining voriconazole PPB, we were interested in evaluating the potential differences in binding capacities of albumin from different commercial brands. The impact of the presence of different stabilizers and suppliers has been a matter of debate before.[44] On the basis of our results, voriconazole PPB

remained stable *in vitro*, irrespective of the commercial brand of albumin ($p = 0.23$). Furthermore, the method described in this manuscript can be used in future clinical studies investigating our hypothesis about the influence of hypoalbuminemia on voriconazole protein-binding characteristics, despite its moderate protein binding.

The disadvantages of HT-ED, being its time-consuming nature (± 2 h of preparation and 4 h of equilibration) and the costs of the device, do not outweigh the advantages such as the possibility of batch analysis, the easy assembly, use and cleaning, the compatibility with multichannel equipment, and most important, the possibility of automatization.[28,29]

CONCLUSION

Voriconazole protein binding is approximately 50% in human plasma, according to an accurate, easy usable HT-ED method. As albumin is the main contributor to voriconazole PPB, this HT-ED-based method can be used in future studies to elucidate the influence of hypoalbuminemia on voriconazole pharmacokinetics and its relevance in clinical practice.

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