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Correlation between free and total vancomycin serum concentrations in patients treated for Gram-positive infections

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ABSTRACT

Routine therapeutic drug monitoring (TDM) reports only total vancomycin (VAN) concentrations, although protein binding varies and it is generally accepted that only free VAN is active. The aims of this study were to examine the correlation between free and total VAN concentrations in order to estimate whether free VAN levels can be predicted based on its total concentration. A high-performance liquid chromatography (HPLC) method was set up and validated (against routine laboratory immunoassays) for measurement of free [ultrafiltration (Centrifree®); cut-off 30 kDa] and total [solid-phase extraction (Oasis® MCX cartridge)] VAN in serum. Samples ($n = 65$) from patients ($n = 15$) treated by continuous infusion were analysed. There was a wide variation in free to total VAN ratios [range 12–100%; mean $63.6 \pm 25.8\%$, with 59 values falling outside the 95% confidence interval (57.3–69.9%); median 70.2%]. The correlation between free and total VAN was poor ($R^2 = 0.55$). Artefacts such as pH variation of sera could be excluded. Both intrapatient and interpatient variabilities were large and no correlation could be made with patients' clinical conditions. Total VAN concentration is not predictive of free VAN concentration, suggesting that actual determination of free VAN might be recommended as an improved method of TDM.

1. Introduction

Despite the recent introduction of new antistaphylococcal drugs, vancomycin (VAN) remains widely used to treat infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and other β-lactam-resistant Gram-positive cocci [1–3]. However, the potential rise in minimum inhibitory concentrations (MICs) of VAN in target organisms [4,5] makes it increasingly critical to adjust its dosage in order to ensure adequate concentrations in blood and other infected areas as well as to avoid undue toxicity [6–8]. Moreover, only the total fraction of VAN is routinely measured and taken into consideration for dosage adjustment in clinics [7], even though it is known that, as for most antibiotics [9], it is probably the free fraction of VAN that is critical both for diffusion into infected areas [10,11] and for binding to its bacterial target [12,13]. In recent recommendations [7] it was stated that free drug levels could be predicted based on an average binding value of ca. 50%. An original study concluded that there was a satisfactory correlation between free and total VAN concentrations in patients' serum, with a mean value for the free fraction of $41.9 \pm 14.1\%$ [14], apparently justifying this approach. Yet other studies have pointed out an important variability in VAN protein binding not only between animals and man but also between volunteers and patients and between patients [15–18]. Because the importance of optimising VAN therapy as effectively as possible has been advocated by many authors in difficult-to-treat patients for the reasons stated above (see, e.g., [7,19–22]), we decided to re-examine to what extent free and total drug concentrations are correlated. To this effect, we used samples from a population of patients receiving VAN in our institutions for suspected or documented Gram-positive infection and for whom therapeutic drug monitoring (TDM) was routinely performed under close supervision by a clinical pharmacist. A preliminary account of the findings has appeared previously [23].

2. Materials and methods

2.1. Materials

VAN and cefuroxime (CXM) (used as internal standard) were obtained from GlaxoSmithKline S.A. (Genval, Belgium) as the commercial products Vancocin® 500 and Zinacef®, respectively, registered for clinical usage in Belgium and complying with the

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provisions of the European Pharmacopoeia (>90% purity). All products used for high-performance liquid chromatography (HPLC) analysis were of HPLC grade and were obtained as follows: acetonitrile and methanol from BioSolve® (Westford Chemical Corporation, Westford, MA); sodium acetate anhydrous from Acros Organics (Thermo Fisher Scientific Inc., Waltham, MA); glacial acetic acid, orthophosphoric acid 85% and hydrochloric acid fuming 37% from E. Merck AG (Darmstadt, Germany); and pure formic acid from Riedel-de Haën (Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany). Oasis® MCX solid-phase extraction cartridges [average pore size 80 Å; average particle size 30 μm; surface functionality (sulfonic acid substituents $-SO_3H$) 1.0 meq/g; sorbent mass 1 cc] and analytical columns [Atlantis® dC18 (150 mm × 4.6 mm) and Symmetry Shield® RP18 (150 mm × 4.6 mm)] were from Waters Corp. (Milford, MA), and ultrafiltration devices (Centrifree®; cut-off 30 kDa) were from Millipore Corp. (Billerica, MA).

2.2. Sera

Commercial human serum (from AB donors) used for setting up the methods was purchased from Lonza Ltd. (Basel, Switzerland). Clinical samples were from hospitalised patients undergoing treatment with VAN at two of our university hospitals (Cliniques Universitaires UCL de Mont-Godinne, Yvoir, Belgium, and Cliniques Universitaires Saint-Luc, Brussels, Belgium) in general internal medicine, intensive care, orthopedy and haematology wards and for whom TDM was ordered by the attending physician as part of their normal care. Material used for the present study was obtained as leftovers from samples after transfer and use by the clinical laboratories of the participating institutions and was maintained at −20 °C until analysis.

2.3. Sample preparation and HPLC assay of free and total vancomycin

An HPLC assay was used to ensure maximal accuracy and also because none of the routine laboratory methods for VAN determination are validated for assay of the free drug. The following methods were devised, based partly on a method describing the extraction of total VAN from serum [24] and the behaviour of CXM chosen as internal standard [25]. For total VAN, thawed samples were subjected to solid-phase extraction by passage through Oasis® MCX cartridges conditioned with 1 mL of methanol followed by 1 mL of water. Samples (500 μL) were mixed with 500 μL of water, 30 μL of orthophosphoric acid 85% and 10 μL of CXM (1 g/L). Following low-speed centrifugation, 1 mL was loaded on the cartridge and completely drawn through under light vacuum (typically 2 mmHg). After washing with 1 mL of 0.1 N HCl and twice with 100 μL of methanol, VAN and CXM were desorbed with 1 mL of methanol containing 5% ammoniac and the eluate was immediately neutralised with 36 μL of HCl 37%. Following evaporation under airflow at room temperature, the residue was reconstituted in 250 μL of 70 mM sodium acetate buffer (pH 5.0) for HPLC analysis (buffer mobile phase). The latter was performed on an Atlantis® dC18 column using stepwise gradient elution at a flow rate of 1 mL/min unless otherwise stated with the buffer mobile phase and the elution phase (acetonitrile/methanol/0.1% formic acid 63:27:10 v/v/v) being varied from 95–5 (2 min), 70–30 (3 min), 60–40 (21 min; 0.5 mL/min) and 5–95 (2 min). Detection was made at 280 nm using a diode array detector with analysis of the absorption spectrum (200–400 nm) for positive identification of VAN and CXM. The concentration of VAN was calculated by integration of the peak area ratio between VAN and CXM, based on standard calibration curves. For free VAN, 500 μL of sample was subjected to ultrafiltration using Centrifree® tubes by centrifugation at 2000 × *g* (3153 rpm) for 30 min at 4 °C (5810R Eppendorf centrifuge; Eppendorf AG, Hamburg, Germany). The filtrate (200 μL) was mixed with 2 μL of CXM (0.5 g/L) and used as such for HPLC analysis. The latter was performed with a Symmetry Shield® RP18 using a mobile phase made of 70 mM sodium acetate buffer (pH 5.0) and acetonitrile/methanol (70:30 v/v) mixed to form a stepwise gradient exactly as for total VAN except that the 60–40 step was run for 16 min only and at 1 mL/min.

All analyses were carried out with a Waters 2690 Separations Module, equipped with two pumps, a degassing line and a thermostated autosampler, connected with a Waters 996 photodiode array detector and operated with the Millenium32® software (Waters Corp.). Baselines were visually inspected and were manually adapted when necessary. The typical intraday coefficients of variation for total VAN were 6.8% at 4.4 mg/L, 0.2% at 15.7 mg/L and 0.2% at 26.8 mg/L, and for free VAN were 12.5% at 2.7 mg/L, 2.3% at 9.9 mg/L and 2.5% at 15.1 mg/L.

2.4. Comparison with routine laboratory methods

Samples were analysed independently by two established laboratory methods: a particle-enhanced turbidimetric inhibition immunoassay (PETINIA) performed with a Dimension® Xpand® Plus instrument (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany), which was used for both free (after ultrafiltration) and total VAN measurements; and a fluorescence polarisation immunoassay (FPIA) performed with an Abbott AxSYM Instrument (Abbott Diagnostics, Abbott Park, IL) for total VAN measurements.

2.5. Patients and clinical data

Patients were from two teaching hospitals and were treated for suspected or proven Gram-positive infection by an organism susceptible to VAN and for whom the use of β-lactams was considered inappropriate. Pertinent clinical, microbiological and biological data were obtained by retrospective analysis of the corresponding medical files. All patients included in the modelling analysis were from a single institution (Cliniques Universitaires UCL de Mont-Godinne) and received VAN by continuous infusion for documented Gram-positive infection. No patient received haemodialysis or haemofiltration during the treatment period.

2.6. Ethical considerations

The present study was part of a larger study aiming at evaluating the impact of the supervision of TDM by a clinical pharmacist and was approved by the ad-hoc ethical committee of the leading clinical centre for this study (Cliniques Universitaires UCL de Mont-Godinne, Yvoir, Belgium).

2.7. Statistics

Descriptive statistics and linear regression analyses were made using GraphPad Prism® software (GraphPad Software, San Diego, CA). Modelling was performed using JMP 7.0® (SAS Institute Inc., Cary, NC).

3. Results

3.1. Validation of the assay methods

Commercial serum samples were spiked with known amounts of VAN to set up the methods and to determine the recovery of the antibiotic. Fig. 1 shows two typical chromatograms obtained from the serum of a patient treated with VAN and analysed for free

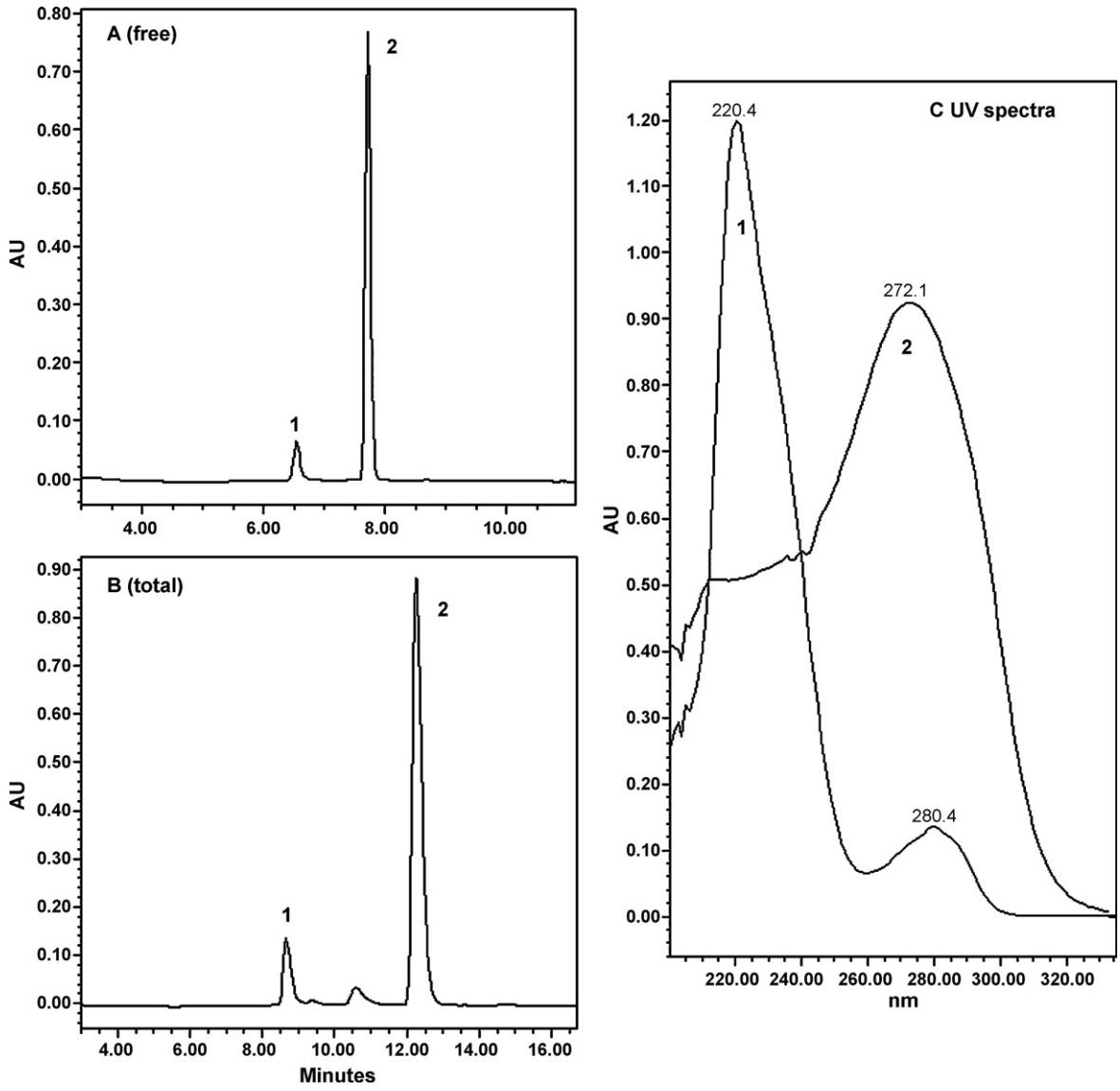

Fig. 1. Typical chromatograms of (A) free and (B) total vancomycin from a clinical sample. **1**, vancomycin; **2**, cefuroxime (internal standard). (C) Identification by ultraviolet spectra of vancomycin (**1**) and cefuroxime (**2**).

(Fig. 1A) and total (Fig. 1B) VAN content, together with the identification of VAN and CXM based on their absorption spectra (Fig. 1C). The method allowed for unambiguous detection of VAN with a retention time of ca. 6.5 min (free) and 8.5 min (total), respectively, and well separated from the internal standard (CXM) in both situations with a limit of quantification of 1.6 mg/L (total) and 0.3 mg/L (free), a linearity of the response up to 300 mg/L (free and total), intraday variation coefficients of ≤8.3% (total) and ≤14.8% (free) and interday variation coefficients of ≤9.6% (free) and ≤15.4% (free) for three concentrations (7.5, 25 and 45 mg/L). VAN recoveries (free and total) tested at three concentrations (10, 20 and 40 mg/L) were between 98.2% (lowest) and 103.9% (highest) of the nominal value. No or little disturbing interferences were noted for most of the samples analysed.

Fig. 2 shows the correlation between the HPLC method and the clinical laboratory method (PETINIA) for both free and total VAN using clinical samples selected for a drug content spanning the entire meaningful clinical range (3–35 mg/L). The correlation between the two methods was satisfactory ($R^2 > 0.95$), but with slopes around 0.8 and a slight divergence of zero values (up to 1.9 mg/L). As a further validation, total VAN concentrations were compared between HPLC and another clinical method (FPIA), with a correlation coefficient (R^2) of 0.83, a slope of 0.82 and a deviation of the origin at 2.2 mg/L.

A potential influence of pH on the extent of protein binding of VAN was examined as follows. First, the pH of all samples was measured and it was observed that it could vary between 7.4 and 8.1. The values of free fraction observed for eight clinical samples with pH values spanning this range were then compared, but no correlation between binding and pH was seen. In parallel, samples from one batch of commercial VAN-free serum were spiked with VAN at 10, 20 or 30 mg/L, adjusted to pH 7.1, 7.4 and 8.1, and then processed for measurement of free and total VAN. The mean value of free VAN was 40.7 ± 4.4% for all samples ($n = 25$) with no significant effect of pH or concentration taken individually [$P \geq 0.2$, one-way analysis of variance (ANOVA)] or globally ($P \geq 0.2$, one-way ANOVA).

3.2. Determination of free/total vancomycin concentration ratios in clinical samples

First, the free and total VAN concentrations were measured using the HPLC method in 65 samples obtained from 15 patients

treated with VAN in a single institution and receiving the drug by continuous infusion (with a total concentration target set at 27 mg/L) for 1–43 days (mean 19 ± 10 days). The results of this analysis are presented in Fig. 3 as (i) the percentage of free VAN with respect to total VAN (upper part) and (ii) the correlation between the free and total concentrations of VAN in each individual sample. There was a clear variation of the total concentration despite the mode of administration used (continuous infusion), which will be analysed elsewhere. Within the context of the present paper, the main observations are that: (i) the mean value for percentage of free VAN was close to 65%, which is higher than usually considered [7]; (ii) there was a considerable spread of the individual values, which increased almost continuously from as low as 12% to 100% (samples with values >100% are within the error margin of the assay); and (iii) only a weak correlation could be established between the free and total concentrations for each of the samples, with the majority of the data falling outside of the 95% confidence interval. Similar conclusions could be reached if the values obtained by HPLC for both free and total concentrations were corrected for discrepancy between HPLC and PETINIA determinations using the equations shown in Fig. 2.

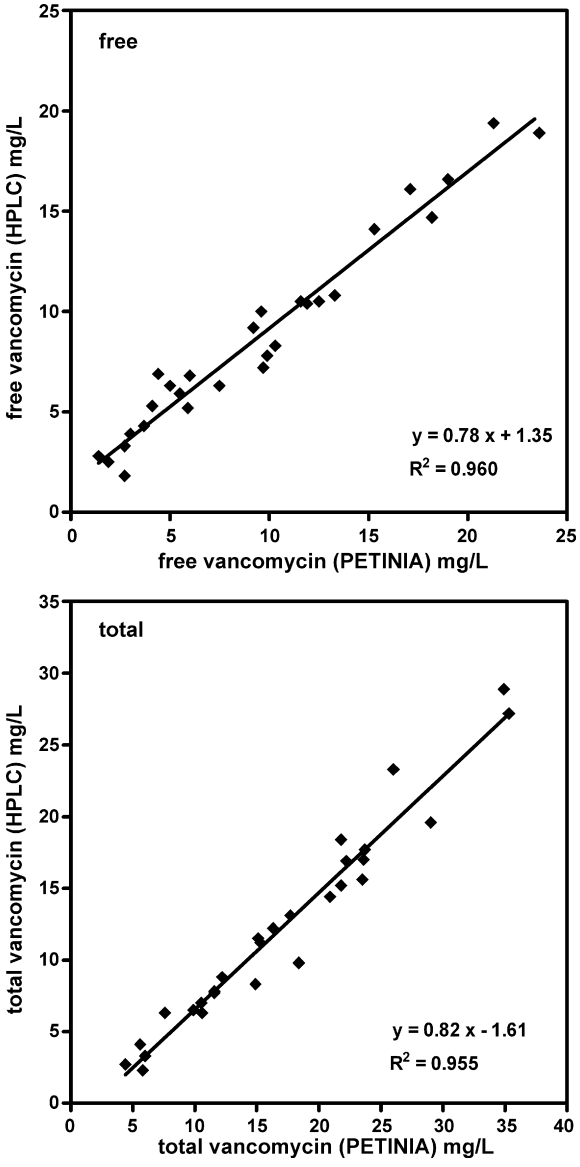

Fig. 2. Correlation between the vancomycin serum concentration (upper panel, free; lower panel, total) as determined by high-performance liquid chromatography (HPLC) (ordinate) and by the routine clinical laboratory assay [particle-enhanced turbidimetric inhibition immunoassay (PETINIA)] (abscissa).

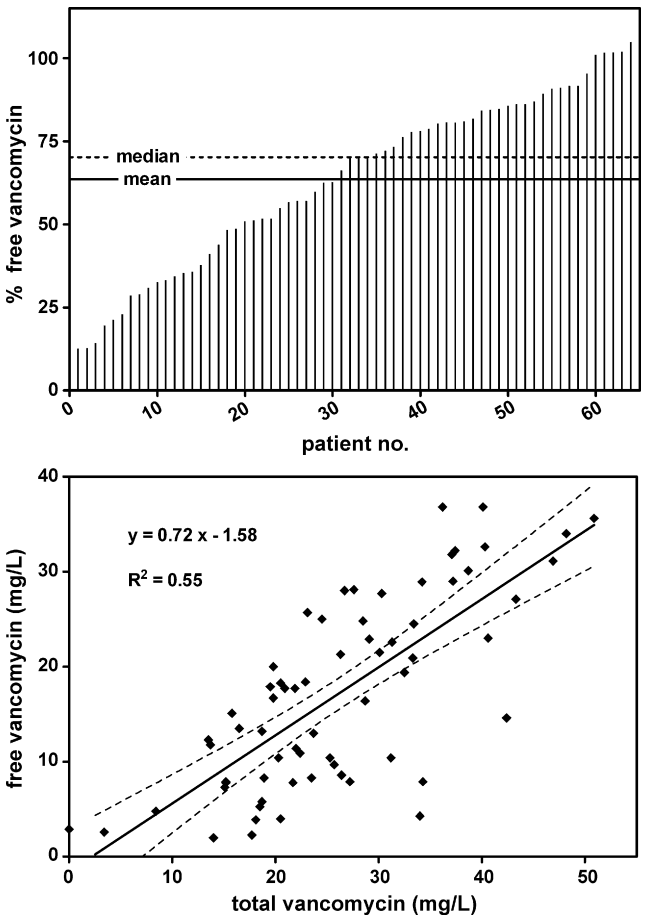

Fig. 3. Free and total vancomycin concentration in 65 samples from 15 patients receiving vancomycin by continuous infusion. Upper panel: % of free vancomycin in individual samples ranked by increasing value with mean (solid line) and median (dotted line). Lower panel: correlation between free and total vancomycin concentrations for each individual sample with 95% confidence interval (dotted lines).

Two approaches were used to try to gain insight into this apparent lack of correlation between free and total VAN concentrations. In the first approach, we examined whether binding was influenced by the actual total concentration of VAN, disclosing potential saturation. No significant correlation between the free/bound percentage ratio and the total concentration could be demonstrated (regression equation $y = 0.29x + 56$; $R^2 = 0.012$; $P = 0.37$). In the second approach, samples were stratified (i) by patients (for whom at least four independent samples could be assayed) and (ii) by medical environment and underlying pathology [intensive care (trauma and severe sepsis), haematology (post-chemotherapy fever) and orthopedy (trauma)]. Fig. 4 shows that (i) the intrapatient variation in percent protein binding was very large (28.6–87%; mean 50.1%), however, interpatient variability was even larger ($P < 0.01$, two-way ANOVA) and (ii) the medical environment and corresponding main underlying pathology was without apparent effect. In a second step, a logistical regression model was applied using patients' available clinical data (gender, age, main diagnostic, co-morbidities, co-administration of other antibiotics, administration of immunosuppressors, total protein levels, creatinine level, white blood cell counts and C-reactive protein level) to try to relate the free fraction level to one or several of these parameters, but without success ($P > 0.05$ for all conditions univariate or multivariate). Of note, the patient population analysed did not include burns patients, patients with insufficient protein diet or patients suffering from nephrotic

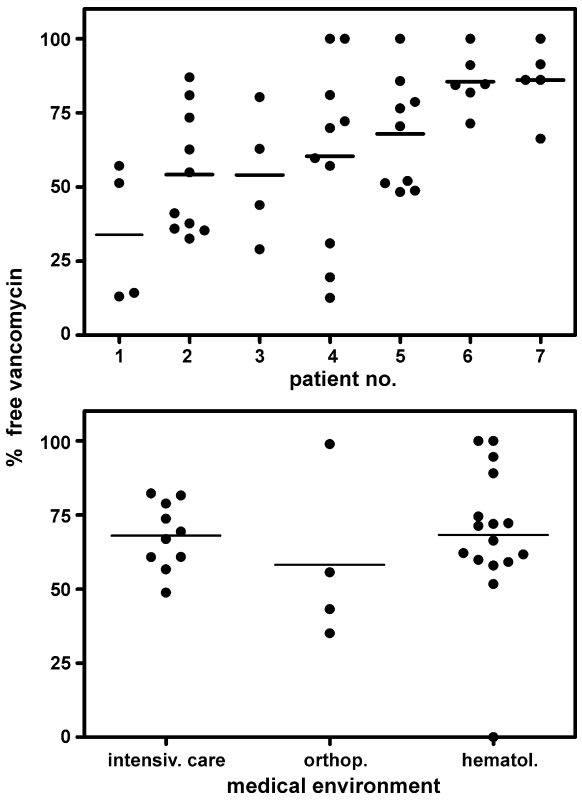

Fig. 4. Variation of free vancomycin fraction: individual data and mean (horizontal bar). Upper panel: stratification of samples by individual patients for whom four or more samples were available. Lower panel: stratification of samples by hospitalisation ward (with different main underlying pathology) using one single sample by patient obtained during treatment {the difference between the three groups is not significant [$P = 0.68$ by one-way analysis of variance (ANOVA); $P = 0.37$ by Kruskal–Wallis test (non-parametric ANOVA)]}.

syndrome, severe hepatic dysfunction or other clinical situation in which serum protein content could have been qualitatively grossly abnormal.

4. Discussion

The present study shows that the free fraction of VAN can vary considerably in samples obtained from patients treated with this drug, irrespective of their main medical situation, not only between patients but also for an individual patient during treatment. Previous studies have already found a large variation in VAN protein binding, with ranges from 7.9% to 71% [14], 23% to 59% [18] and 3.7% to 47% [26]. The present study extends over these observations by showing that (i) this variability can be even larger than suspected and (ii) the free fraction is only poorly related to the total drug concentration, making predictions very hazardous. This is in apparent contrast to the conclusion of a previous study [14] where free and total VAN concentrations were claimed to be correlated. This study analysed a similar number of samples ($n = 62$) from patients ($n = 12$) also suffering from infection. Close analysis of the raw data of this study, however, shows a coefficient of determination (R^2) of 0.67, which was improved to 0.90 (and presented as such in the abstract) by use of orthogonal regression and suppression of one sample with a high protein binding value. We did not apply such corrections here because the main point of this study was not so much about getting population information but to examine how the total VAN concentration of a given sample could safely and reliably predict the corresponding free drug concentration. Also, most samples used by Ackerman et al. [14] were apparently drawn over a short period of time, whereas ours were obtained over the whole duration of treatment, giving more chance for patient and treatment factors to exert a disturbing effect and thereby being more representative of the true clinical situation. Finally, in contrast to the study of Ackerman et al., we did not exclude patients on the basis of age, sepsis, hypotension or trauma, which are common situations encountered in VAN-treated patients.

Variability of the free to total VAN concentration ratios, and the ensuing lack of predictability of the true free level from total level determinations, has been ascribed to lack of control of pH during separation of the unbound and bound drug [27]. This artefact could be ruled out here as our validation study did not evidence a variation of binding due to pH (within the range of values observed in our clinical samples) at clinically meaningful VAN concentrations. The serum protein binding characteristics of VAN have been studied in detail and found to be predominantly related to albumin and immunoglobulin A (IgA) serum content [28,29]. In our study, no correlation could be made with total serum protein content. IgA could not be specifically assayed as the study was retrospective and non-interventional, but no patient had evidence of myeloma or other gross pathology involving IgA.

The significance of the present data, together with the observation made by others regarding the variability in the free fraction, with respect to the activity of VAN needs to be underlined. Indeed, several studies indicate that a critical threshold of drug exposure [pharmacodynamically expressed as the 24-h area under the concentration–time curve divided by the MIC (AUC_{24h}/MIC) [30] or, in recent guidelines, as minimal trough levels in the case of discontinuous administration [7]] must be met to ensure clinical success in staphylococcal infections. Because it is the free VAN concentration that probably matters most in this context (see discussion in [7,31]), reporting total levels may be insufficient and even misleading. The same could also apply to the use of TDM values for prevention of nephrotoxicity (see [32] for an example with continuous infusion), since it may develop via a tubular secretion mechanism [33] that ought to be primarily related to the free rather than the total drug concentration. More systematic assay of free VAN concentration could, therefore, be of interest to improve our knowledge of VAN pharmacodynamics/pharmacokinetics and help in better assessing which parameter and which serum concentration values are associated with successes and failures or with toxicity. Conversely, it could be argued that the variability in the therapeutic and toxicological responses and the often claimed difficulties in linking the results of TDM with clinical outcomes [34] may find its origin in the unpredictability of free VAN levels from total levels as described here.

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Competing interests: None declared.

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