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SHORT COMMUNICATION

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Determination of free clindamycin, flucloxacillin or tedizolid in plasma: Pay attention to physiological conditions when using ultrafiltration

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Abstract

Pharmacokinetic/pharmacodynamic indices of anti-infective drugs should be referenced to free drug concentrations. In the present study, clindamycin, flucloxacillin and tedizolid have been determined in human plasma by HPLC–UV. The drugs were separated isocratically within 3–6 min on a C_{18} column using mixtures of phosphate buffer–acetonitrile of pH 7.1–7.2. Sample treatment for the determination of total drug concentrations in plasma included extraction/back-extraction (clindamycin) or protein precipitation (flucloxacillin, tedizolid). The free drug concentrations were determined after ultrafiltration. An ultrafiltration device with a membrane consisting of regenerated cellulose proved to be suitable for all drugs. Maintaining a physiological pH was crucial for clindamycin, whereas maintaining body temperature was essential for tedizolid. The methods were applied to the analysis of total and free drug concentrations in clinical samples and were sufficiently sensitive for pharmacokinetic studies and therapeutic drug monitoring.

KEYWORDS

HPLC–UV, lincosamide, oxazolidinone, penicillin, protein binding, therapeutic drug monitoring

1 | INTRODUCTION

Clindamycin (CLI) is a lincosamide antibiotic with similar mechanism of action to macrolides, e.g. clarithromycin. It is highly effective against Gram-positive and -negative anaerobic pathogens, as well as Gram-positive aerobes (e.g. Cho et al., 2005). Flucloxacillin (FXN) is an isoxazolyl penicillin with particular activity against penicillinase-forming staphylococci (e.g. Chin et al., 2018). Among many other indications, both drugs are used for long-term treatment (several weeks) of difficult-to-treat infections such as osteomyelitis, in which optimal exposure to the antibiotic drug is of great importance. Tedizolid (TZD) is a second-generation oxazolidinone for the treatment of skin and skin structure infections with a focus on Gram-positive pathogens including methicillin-resistent *Staphylococcus aureus* and vancomycin-resistent enterococci (e.g. Stainton et al., 2018). The chemical structures are depicted in Figure S1. The plasma protein binding of CLI https://www.pfizer.de/fileadmin/produktdatenbank/pdf/003271_freigabe.pdf, varies between 60 and 94% (for a summary of product characteristics see Sobelin Solubile, Pfizer Pharma, Berlin, Germany), the protein binding of FXN https://www.gelbe-liste.de/produkte/Fluclox-Stragen-1-g-Pulver-zur-Herstellung-einer-Injektions-bzw-Infusionsloesung_484867/fachinformation, in healthy subjects is 92–96% (SPC Fluclox Stragen, Stragen Pharma GmbH, Köln, Germany) and that of TZD https://www.gelbe-liste.de/produkte/

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Sivextro-200-mg-Pulver-fuer-ein-Konzentrat-zur-Herstellung-einer-Infusionsloesung_883940/fachinform, is 70–90% (SPC Sivextro, Merck Sharp & Dohme, Haarlem, The Netherlands).

The pharmacokinetic/pharmacodynamic indices of antibiotics such as area under the concentration–time curve/minimum inhibitory concentration (AUC/MIC) and time above minimum inhibitory concentration (T > MIC) should be referenced to the unbound fraction (f_u) of the drug, and the degree of protein binding should be stated in such a way that the free concentration of the drug can be readily calculated (Mouton, Dudley, Cars, Derendorf, & Drusano, 2005). Unfortunately, the free concentrations cannot reliably be calculated from total concentrations in particular for drugs with high levels of protein binding (Chin et al., 2018). The importance of developing accurate, fast and affordable bioanalytical methods for measuring free drug concentrations has been emphasized recently (Musteata, 2017). Ultrafiltration fulfils these requirements, but the type of membrane, temperature, pH and relative centrifugal force can significantly influence the results (Nilsson, 2013). The aim of the present study was to establish HPLC–UV methods for the determination of CLI, FXN and TZD in human plasma with focus on the determination of the free, pharmacologically active drug.

2 | EXPERIMENTAL

2.1 | Drugs and chemicals

Clindamycin-HCl (purity 96.8%) was obtained from Fagron (Barsbüttel, Germany). Flucloxacillin (Fluclox Stragen 2 g, one vial contains 2.176 g flucloxacillin sodium monohydrate equivalent to 2.00 g FLX) was obtained from Stragen Pharma (Köln, Germany), Clarithromycin (CLR, pharmaceutical secondary standard, purity 100%) was obtained from Sigma-Aldrich (Steinheim, Germany). Tedizolid 10 mM stock solution in DMSO (purity 97%) was obtained from Biozol Diagnostics (Eching, Germany). Methyl *tert*-butylether was obtained from Carl Roth (Karlsruhe, Germany), Acetonitrile, methanol (HPLC gradient grade), the other chemicals and Nanosep Omega 10 K ultrafiltration devices were obtained from VWR (Darmstadt, Germany). Vivafree 500 30 K centrifugal filters, which are identical to Vivacon 500 30 K (Sartorius, Göttingen, Germany), but manufactured as CE-registered *in vitro* diagnostic medical devices, and recommended for the determination of free drug in plasma, were obtained from Vivaproducts Inc. (Littleton, MA, USA). HPLC-grade water was produced with an Arium basic water purification system (Sartorius, Göttingen, Germany). Blank plasma (anticoagulant heparin) was obtained from healthy volunteers.

2.2 | Stock solutions, calibrators and quality control samples

Stock solutions of CLI (5 g/L) and FXN 5 (g/L) were prepared in water. CLR (300 mg/L; internal standard, IS, for the determination of CLI) was prepared by dissolving 6.00 mg CLR in 10 ml methanol and filling up to 20 ml with water. The TZD was obtained as a 10 mM solution in Dimethyl sulfoxide (DMSO). Working standards (STD) and quality controls (QC) were prepared in plasma for the analysis of total drug. For the analysis of free drug, dilutions were prepared in saline as a surrogate for ultrafiltrate. Owing to the poor solubility of TZD in water/saline, predilutions were prepared in DMSO and further diluted 1:40 or higher in saline. The final concentrations are listed in Table S1.

2.3 | Sample treatment for total drug analysis

Sample treatment for the determination of total CLI in plasma was performed by liquid–liquid extraction/back-extraction adapting a published method (Gatti et al., 1993). That fpr total FXN included protein precipitation with acetonitrile and removing acetonitrile by extraction into dichloromethane (Kratzer et al., 2019 and references therein) and that for TZD included deproteinization of 100 μl plasma with 200 μl 7% perchloric acid (for details see Supporting Information).

2.4 | Determination of free drug concentrations, unbound fraction and nonspecific binding

Sample treatment for the determination of the free plasma concentrations was performed by ultrafiltration as previously described (Kratzer, Liebchen, Schleibinger, Kees, & Kees, 2014). In brief, 10 μl 3 M potassium phosphate, pH 7.43 ± 0.02, was pipetted into the ultrafiltration device and mixed with 300 μl plasma. The sample was incubated for 10 min at 100***g***/37°C (centrifuge 5417R, Eppendorf, Hamburg, Germany), and then centrifuged for 20 min at 1000***g***/37°C. An aliquot of the ultrafiltrate was injected onto the column. The f_u was calculated as follows: f_u (%) = $C_{free}/C_{total} \times 100$. The influence of temperature, pH and relative centrifugation forces (RCF) on the protein binding was investigated as described previously (Kratzer, Kees, & Dorn, 2016). To assess nonspecific binding (NSB) to ultrafiltration membranes, 400 μl CLI 3 mg/L, FXN 30 mg/L and TZD 0.5 mg/L in saline were ultrafiltrated at 37°C/1000***g*** for 3–5 min and the ultrafitrate (120–150 μl) was analyzed. NSB was calculated as NSB (%) = $(1 - C_{ultrafiltrate}/C_{upper\ container}) \times 100$.

2.5 | Chromatography

The HPLC consisted of a Shimadzu Prominence modular system fromwith a quaternary solvent pump LC-20 AD, degasser DGU-20A3R, autosampler SIL-20 AC HT (set to 6°C, with 25°C for TZD), column oven CTO-20 AC (set to 40°C), SPD-M30A photodiode array detector equipped with cells of 10 or 80 mm optical path length and system controller CBM-20A. Alternatively, an integrated LC-2040 3D Nexera-i PDA HPLC system was used. Both systems utilize the LabSolutions workstation for evaluation (all from Shimadzu, Duisburg, Germany). The detection wavelengths

were 205 nm (CLI), 220 nm (FXN) and 300 nm (TZD). Separation was performed isocratically using a Nucleoshell RP_{18} column (2.7 µ, 100 × 3 mm, Macherey-Nagel, Düren, Germany) preceded by a column protection system (Nucleoshell RP_{18} 2.7 µm, 4 × 3 mm). The mobile phases were mixtures of 20 mM sodium phosphate–acetonitrile with pH 7.1–7.25. The flow rate was 0.4 ml/min. For details see the Supporting Information (Figures S2–S5).

2.6 | Method characteristics and statistical analysis

The methods were validated with respect to linearity, precision and accuracy. The lowest standard on the calibration curve was taken as LLOQ. Accuracy and precision were assessed by comparing the back-calculated concentrations of the standards with the nominal concentrations, and by in-process QCs. Regarding the determination of free concentrations in clinical samples, spiked plasma samples were included as QCs in each run of unknown samples to assess inter-assay precision. The intra-assay precision was not further examined as precision between duplicates was 1% in preliminary experiments. Note that accuracy cannot be tested as the true unbound concentration is unknown (Nilsson, 2013). The weighting factor $1/y^2$ was used for linear regression. Prism 7 (GraphPad Software, La Jolla, CA, USA) was used for calculating statistics.

2.7 | Clinical samples

Clindamycin and TZD were determined in serial plasma samples from healthy volunteers after short infusion of CLI phosphate equivalent to 900 mg CLI or 200 mg TZD phosphate. Flucloxacillin was determined in remains of plasma from routine blood sampling in patients receiving FXN 12 g/day as a continuous infusion. Plasma was separated by centrifugation (10 min, 3800*g*), snap frozen at −20°C and than stored at −80°C until analysis. Ethical approval was obtained from the institutional ethics committee of the Medical University of Vienna (CLI, reference no. 1608/2017, EudraCT no. 2017-002216-14; TZD, reference no. 2251/2018, EudraCT no. 2018-004743-23) and the University of Regensburg, Germany (FXN, reference no. 18-1020-101).

3 | RESULTS AND DISCUSSION

3.1 | Method characteristics

The linearity of the assays in plasma/saline has been shown down to 0.1/0.03 mg/L (CLI and FXN) and 0.03/0.005 mg/L (TZD), respectively. Precision (0.84–4.2%) and accuracy (96–107%) were well within the predefined limit of 15% (Table S1) (FDA, 2018). Precision and accuracy of the determination of total drugs in clinical samples based on in-process QCs were similar (Table 1). As outlined in the Experimental section, the precision of the determination of free drug in clinical samples was assessed by analyzing a spiked plasma pool with each run. The values of f_u in such QC samples are listed in Table 1. The selectivity was sufficient to analyse CLI, FXN and TZD in plasma and ultrafiltrate within a run time of 3–6 min. Additional peaks compared with the plasma of healthy volunteers were observed in some clinical samples, which however did not interfere (Figure S2–S5). The present methods for the analysis of CLI or FXN are faster than previous HPLC–UV methods, and exhibit comparable or higher sensitivity (Cho et al., 2005; Zhou, Ruan, Yuan, Jiang, & Xu, 2007). Regarding TZD, there is one publication using HPLC–UV and several publications using LC–MS/MS (Housman et al., 2012; Iqbal, 2016; Sahre et al., 2012; Stainton et al., 2018). However, detailed information is provided only in a study in rats using LC–MS/MS (Iqbal, 2016). The present method is more sensitive than the previous HPLC–UV method (Sahre et al., 2012). The effective run-time of 3 min is not essentially longer than the aforementioned LC–MS/MS method. The sample preparation is simpler and the use of an internal standard is not necessary (Iqbal, 2016).

3.2 | Nonspecific binding and plasma protein binding

Ultrafiltration has become the preferred method for the determination of free drug concentrations at least in the clinical setting, being faster (minutes vs. hours) than equilibrium dialysis, which is often regarded as a reference method (Nilsson, 2013). Nonspecific binding to the ultrafiltration membrane is considered as a major disadvantage. From an adsorption perspective, the regenerated cellulose membranes (e.g. Centrifree or Microcon from Merck Millipore,

TABLE 1 In-process validation data of the determination of total clindamycin (CLI), flucloxacillin (FXN) and tedizolid (TZD) in plasma and inter-assay variation of the unbound fraction (f_u, %) of the drugs in a plasma pool from healthy volunteers

	Clindamycin		Flucloxacillin		Tedizolid	
Concentration (mg/L)	8.0	1.0	100	5.0	4.0	0.2
CV_{intra} (%)	2.1	3.8	0.84	3.9	1.8	1.7
CV_{inter} (%)	2.1	2.9	1.8	2.4	4.2	4.2
Accuracy (%)	99.6	96.2	107.0	107.1	98.0	98.9
f_u (%)	15.8, 13.4, 18.9	8.38, 8.23, 9.70	4.4, 3.4	3.1, 2.0	21.0 ± 0.5[a]	21.2 ± 1.3[a]

[a] n = 4.

Darmstadt, Germany and Vivacon/Vivafree) are considered to be preferable (Nilsson, 2013; Wang & Williams, 2013). In a previous study on betalactams and vancomycin, we evaluated different ultrafiltration devices and selected Nanosep Omega 10 K (modified polyethersulfone membrane) as it showed a higher flow rate than other ultrafiltration devices and comparable results to Centrifree 30 K at much lower costs (Kratzer et al., 2014). Using Vivafree, no NSB of CLI or FXN was observed and that of TZD was 10%. Using Nanosep, the NSB of CLI was 11%, that of FXN was 42% and that of TZD was as high as 97%. However, results in saline are only of limited relevance, as NSB is dramatically decreased in the presence of plasma owing to a protective effect of plasma components such as albumin (Wang & Williams, 2013). As depicted in Figure 1, the f_u of CLI observed in plasma was only marginally lower with Nanosep compared with Vivafree, i.e. both ultrafiltration devices seem to be suitable for the determination of free CLI. In contrast, the values of f_u of FXN and TZD were significantly lower with Nanosep compared with Vivafree, in particular at low concentrations. The threefold increase in the f_u of CLI between 0.3 and 30 mg/L can be attributed to the saturation of binding to α-1-acid glycoprotein, the primary binding protein for CLI (Kays, White, Gatti, & Gambertoglio, 1992). The slightly increased f_u at FXN 100 mg/L may indicate saturation of albumin binding. In agreement, an increased f_u of FXN was observed in patients with hypoalbuminaemia (Chin et al., 2018).

3.3 | Influence of temperature, pH and RCF

Ultrafiltration of antibiotics was performed at 5–6°C (because of stability concerns), at room temperature (for convenience) and at ≥10,000*g* (for time-saving).

RCF had a minor influence on the f_u of CLI or FXN (10–20% lower at 10,000*g* compared with 1000*g*); the f_u of TZD was unaffected (Table 2). The decrease in f_u at high RCF, referred to as the "pressure effect", is correlated with the molecular weight and becomes relevant at a molecular weight >500 Da, e.g. vancomycin (Kratzer et al., 2014).

Temperature had a distinctly different influence on the f_u of the drugs. Compared with at 37°C, the f_u of FXN was 13–15% lower at ambient temperature and at 5°C; the f_u of CLI was unchanged at ambient temperature and 40% higher at 5°C; the f_u of TZD was more than halved at 5°C.

The pH of plasma increases by 0.1–0.2 units already immediately after blood collection and centrifugation to separate the plasma, and can reach values >9 after long-term storage owing to evaporation of

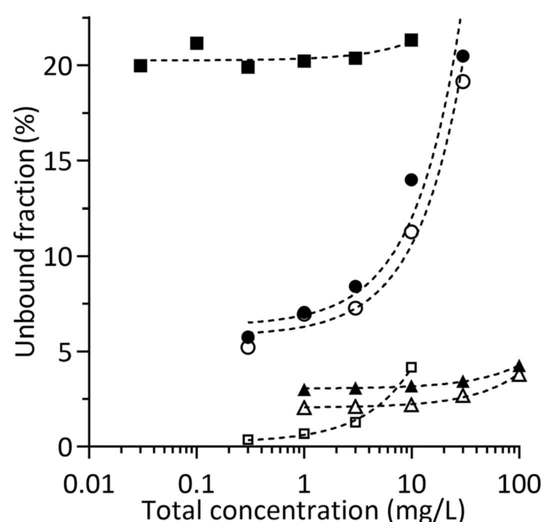

FIGURE 1 Plot of unbound fraction (f_u, %) vs. total concentration of tedizolid (squares), clindamycin (circles) and flucloxacillin (triangles) in human plasma as determined by ultrafiltration using a Vivafree 500 30 K (closed symbols) or a Nanosep Omega 10 K (open symbols)

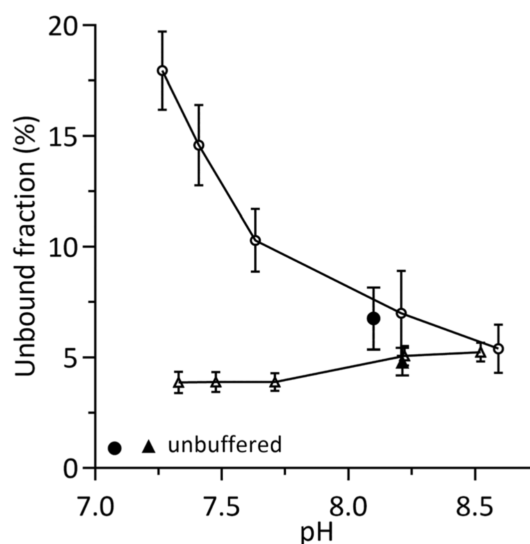

FIGURE 2 Plot of unbound fraction (f_u, %) of clindamycin 5 mg/L (circles) and flucloxacillin 30 mg/L (triangles) in human vs. pH (mean ± SD of n = 5–6). The mean value of the measured pH before/after ultrafiltration is plotted on the x-axis. Full symbols = unbuffered plasma

TABLE 2 Unbound fraction (f_u, %, mean of n = 2–4 assays) of CLI, FXN and TZD in pooled plasma at different temperatures and relative centrifugal force

Drug	Molecular weight	Concentration (mg/L)	1000*g*/37°C	10,000*g*/37°C	1000*g*/22°C	1000*g*/5°C
CLI	425.0	5	15.8	12.7	16.6	22.1
FXN	453.9	30	3.74	3.38	3.07	3.16
TZD	370.3	1	20.7	20.3	15.1	7.44

CO_2 (Nilsson, 2013). The f_u of TZD was 20.5 ± 0.6% in plasma buffered to pH 7.4 and nearly identical (19.8 ± 0.4%) in unbuffered plasma (pH of the plasma sample prior to ultrafiltration 7.9–8.1, after ultrafiltration 8.4–8.7). Therefore, the influence of pH was not further investigated. In contrast, the f_u of CLI was nearly halved at pH >8 compared with the physiological pH of 7.4. The f_u of FXN was constant at pH 7.3–7.7 and 20–25% higher at pH >8 (Figure 2).

The f_u of TZD (about 20%) was within the range reported by the manufacturer (SPC Sivextro, Merck Sharp & Dohme, Haarlem, The Netherlands) and in agreement with a very recent publication using Centrifree (Stainton et al., 2018), but nearly double the value published previously where ultrafiltration was performed at 10°C (Housman et al., 2012). The pronounced slope of the f_u of CLI at physiological values is in good agreement with the pK_a of 7.55, i.e. small changes of pH have a strong effect on the lipophilicity of CLI and thus on the protein binding (Nilsson, 2013). The distinctly higher f_u of FXN at pH >8 may be linked to the conformational change of albumin (N–B transition) around the physiological pH (Zeitlinger et al., 2011). The bias of the f_u of FXN in unbuffered plasma can probably be kept small or may be negligible if fresh plasma is used.

3.4 | Concentrations of free drug in clinical samples

The methods proved to be sensitive enough to describe the concentration–time course of CLI in plasma of healthy subjects following intravenous infusion of CLI phosphate, equivalent to 900 mg CLI or of TZD following intravenous infusion of 200 mg TZD phosphate. The lowest (total/free) observed concentrations were: CLI 0.914/0.0802 mg/L after 8 h or TZD 0.115/0.0236 mg/L after 24 h. Flucloxacillin was determined in plasma of six ICU patients treated with continuous infusion of FXN 12 g/day. The free concentrations varied between 4.67 and 39.1 mg/L and the total concentrations between 52.2 and 205 mg/L. The median (range) f_u was 11.1% (5.6–19.1%), i.e. the f_u was higher than in spiked plasma of healthy subjects in agreement with previous results in ICU patients (Chin et al., 2018).

4 | CONCLUSION

The described HPLC–UV methods allow the fast and precise determination of total and free CLI, FXN and TZD for pharmacokinetic studies or therapeutic drug monitoring. Maintaining physiological conditions during ultrafiltration is recommended: in particular pH for the analysis of free CLI and body temperature for the analysis of free TZD.

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DECLARATIONS OF INTEREST

The authors declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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