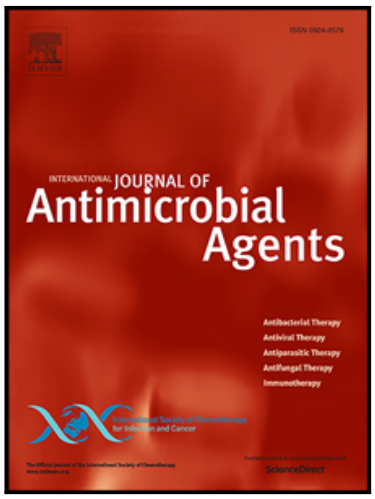

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Investigation of unbound colistin A and B in clinical samples using a mass spectrometry method.

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Abstract

Colistin, used as a last resort drug, has a narrow therapeutic range that justifies therapeutic drug monitoring. Few data are available in the literature concerning the *in vivo* unbound fraction of colistin. The objectives of this study were to develop a method to isolate unbound colistin in clinical samples by ultrafiltration and to quantify it. The association between unbound colistin and biological parameters (total protein, albumin, alpha-1-acid glycoprotein, and creatinine) was investigated. The measured ranges were 0.036-7.160 mg/L for colistin A and 0.064-9.630 mg/L for colistin B. The process of isolation and determination of unbound colistin was applied to clinical samples (n=30) within 40 minutes and no non-specific binding was observed during the ultracentrifugation step. The median unbound fractions of colistin measured were 34.3% (12.8%-51.0%) and 53.4% (27.0%-77.8%) for colistin A and colistin B, respectively. High inter-individual biological variation of binding was observed for colistin A and B which was not explained by the biochemical parameters studied. The method developed could be useful to improve outcomes for patients.

Keywords: unbound colistin; LC-MS/MS; clinical samples; ultracentrifugation

1. Introduction

Colistin is mainly used as a last resort antibiotic in cystic fibrosis [1, 2] and critically ill patients [3, 4]. These populations have altered pharmacokinetic (PK) characteristics due to their disease state that result in unpredictable colistin plasma levels. Moreover, colistin has a narrow therapeutic range, causing nephrotoxicity and neurotoxicity [5, 6]. Thus, therapeutic monitoring is useful to optimize dosing in these populations. However, this is complicated by the fact that colistin is administrated as an inactive prodrug, colistin methanesulfonate (CMS), which is hydrolysed *in vivo* into its microbiologically active form. Moreover, there is no method available for assaying unbound colistin in clinical samples. The majority of data on protein binding by colistin were established in spiked

plasma samples and were based on an equilibrium dialysis method [7-10]. The length of time required for this extraction process is not compatible with determination of unbound colistin in clinical samples due to the *in vitro* conversion of CMS into colistin. Therefore, we have developed a separation method based on ultrafiltration to measure unbound colistin levels in clinical samples. In order to develop this method, we have considered the important issues of matrix effect and the non-specific binding of colistin to container surfaces. We also tested this new method in clinical samples by using it to measure unbound and total colistin concentrations in the plasma of patients receiving intermittent perfusion of CMS sodium. The correlation between unbound and total colistin, as well as the potential influence of several biochemical parameters, was investigated.

2. Materials and methods

2.1. Chemicals

Colistin sulfate, CMS, polymyxin B (internal standard [IS]), tigecycline 98% HPLC grade, and trifluoroacetic anhydride (TFA) were purchased from Sigma-Aldrich (Bornem, Belgium). Acetonitrile (ACN) and formic acid 99% (FA) were obtained from Biosolve (Valkenswaard, The Netherlands) and were all mass spectrometry grade. Ultrapure water was obtained by means of a Milli-Q water purification system (Millipore, Brussels, Belgium).

All tubes employed were polypropylene to avoid drug adsorption. Polypropylene vials for LC-MS/MS were purchased from Agilent (Diegem, Belgium).

2.2. HPLC and mass spectrometry (MS)

Chromatographic separation was carried out using a 1260 Infinity HPLC system (Agilent Technologies, Diegem, Belgium) equipped with a binary pump and an autosampler thermostatically controlled at 15°C. The MS/MS detection was performed using an Agilent Technologies 6490 Triple Quad LC-MS/MS with a Jet Stream electrospray ionization source.

2.3. Biochemical parameters

The assays for total protein, albumin, alpha-1-acid glycoprotein, and creatinine were performed on a Modular Analytics P800 instrument (Roche Diagnostics GmbH, Mannheim, Germany) with Roche Diagnostics kits (TP, ALB plus, α1-acid glycoprotein, and CREA).

2.4. Clinical samples

Samples were collected from 30 patients infected with multidrug-resistant Gram-negative bacteria and treated with intravenous infusion of CMS (Colistineb®, Teva Pharma, Belgium). The regimen was a loading dose of 300 mg colistin base activity (CBA) (approximately 9 million IU) followed by 330 mg CBA (approximately 5 MIU) every 12 hours. Patient characteristics are detailed in Table 1. Blood samples were collected before the next dose of colistin, in heparinized tubes, transported on ice, and centrifuged immediately. The supernatants were stored at -80°C until assayed. Biochemical parameters were measured on the leftover volume of samples. The local ethical committee approved the study: P2015/374.

2.5. Total colistin assay

The protocol was based on the work published by Jansson *et al.* [11] with some modifications. The description of the method and the validation process are presented in the supplemental files section. The main modifications were the use of a water/ACN gradient for the mobile phase, a precipitation step, and the recovery of the residue obtained after evaporation at room temperature without acidic conditions. Maintenance of a neutral pH during the whole process inhibited the *in vitro* conversion of CMS into colistin.

2.6. Unbound colistin assay

Unbound colistin was separated by ultrafiltration using a 30 kDa Centrifree device (number 4104, Merck Millipore, Overijse, Belgium). The assay of unbound concentrations was set up as follows.

2.6.1. Method development

2.6.1.1. Matrix effect

One mL of colistin-free plasma from 6 different patients not treated with colistin was ultrafiltrated (35 min, room temperature (RT), 4000 rpm, fixed angle of 45°) to obtain 200 µL of ultrafiltrate. This was spiked at two levels of concentration (1 and 10 mg/L of total colistin base A+B) and added to 100 µL of colistin-free plasma pool and 50 µL of IS. Proteins were precipitated with 1200 µL of ACN and pelleted by centrifugation (10 min, 4°C, 15,000 rpm). Then, 1200 µL of supernatant were recovered and evaporated under nitrogen at room temperature. The residue was reconstituted with 100 µL of 95/5 (v/v) ultrapure water/ACN, vortex-mixed, and centrifuged (10 min, 4°C, 15,000 rpm). The supernatant was transferred into a vial and 5 µL were injected into the LC-MS/MS system. The measured colistin/IS area ratio was compared to that obtained with the extraction protocol of calibration standards (200 µL of plasma treated with 800 µL of ACN; see supplementary material). The (aqueous phase/ACN] ratio was the same in the two assays.

2.6.1.2. Non-specific binding

Tigecycline was used as IS solely for this experiment. The assessment of colistin losses by non-specific binding to the Centrifree device was performed at a concentration of 2.5 mg/L of total colistin base A+B (spiked plasma). Three devices were used for this experiment. The first one (i) served as a control (without pre-treatment). The second (ii) and the third (iii) ones were pretreated with polymyxin B solutions (10 and 100 mg/L). Polymyxin B was selected to saturate the sites involved in potential non-specific binding of colistin because it has the same physicochemical characteristics as colistin. After the pre-treatment solution was discarded, 1 mL of sample was introduced in the devices (i), (ii), and (iii). Total colistin (C_{total}) was determined in the sample compartment before centrifugation. The unbound colistin ($C_{unbound}$) was measured in the ultrafiltrate compartment after centrifugation and after discarding the first ultrafiltrate from devices (ii) and (iii) (3 min of centrifugation, RT, 4000 rpm, fixed angle of 45°). The results were expressed in terms of ratio

(unbound fraction) to normalize the results. The aim of these three precautions was to avoid variability in the results between the two conditions (with and without pre-treatment) caused by a dilution of the spiked plasma by trapped pre-treatment solution in devices (ii) and (iii). The ratios were compared between the three conditions.

2.6.2. Determination of unbound colistin in plasma samples

A total of 800 µL to 1 mL of plasma were introduced into the Centrifree device. Then, a 35 min centrifugation step (RT, 4000 rpm, fixed angle of 45°) was performed. Next, 200 µL of ultrafiltrate were transferred to an Eppendorf tube containing 100 µL of colistin-free plasma pool. Then, 50 µL of IS and 1200 µL of ACN were added. The mix was centrifuged (10 min, 4°C, 15,000 rpm). Then, 1200 µL of supernatant were recovered and evaporated under nitrogen at RT. The residue was reconstituted with 100 µL of 95/5 (v/v) ultrapure water/ACN, vortex-mixed, and centrifuged (10 min, 4°C, 15,000 rpm). The supernatant was transferred to a vial and 5 µL were injected into the LC-MS/MS system. Measurement of colistin in the ultrafiltrates provided the $C_{unbound}$ of colistin A and $C_{unbound}$ of colistin B.

The repeatability of this protocol was assessed in plasma spiked at two concentration levels (1 and 5 mg/L of total colistin base A+B) in triplicate.

2.7. Statistical analysis

The results were analysed with Analyse-it, version 3.80 (Analyse-it Software, Leeds, United Kingdom). The Shapiro-Wilk normality test was used to assess the distribution of continuous variables. The differences between paired groups were evaluated by the paired t-test (Gaussian distribution) or the Wilcoxon signed rank sum test (non-Gaussian distribution). The correlations were estimated using the Pearson (r; Gaussian distribution) or Spearman (r_s; non-Gaussian distribution) coefficient. A p-value <0.05 was considered statistically significant. Parametric results are expressed as means and ranges and non-parametric results as medians and ranges.

3. Results

3.1. Total colistin assay

The results of the validation are presented in the supplemental files section. The linearity ranges were 0.036-7.160 mg/L for colistin A and 0.064-9.630 mg/L for colistin B. The coefficient of variation of intermediate precision was below 11%.

3.2. Unbound colistin assay

3.2.1. Method development

3.2.1.1. Matrix effect

The $[(Colistin/IS)_{ultrafiltrate}]/[(Colistin/IS)_{plasma}]$ ratio was calculated to determine the recovery (%) between the two matrices. The recovery for colistin A was 101.1% (95.7%-111.4%) at 1 mg/L and 102.4% (94.8%-106.4%) at 10 mg/L; for colistin B it was 100.2% (98.1%-104.1%) at 1 mg/L and 114.8% (104.4%-118.7%) at 10 mg/L, demonstrating the absence of a matrix effect. Therefore, the linearity range of the total colistin assay (see supplemental files section) was applicable for the unbound colistin assay: 0.036-7.160 mg/L for colistin A and 0.064-9.630 mg/L for colistin B.

3.2.1.2. Non-specific binding

Losses of colistin in the Centrifree device were evaluated by comparing the colistin unbound fraction in spiked plasma obtained from a device without pre-treatment and from 2 devices in which their potential binding sites were neutralized by a solution of 10 or 100 mg/L of polymyxin B. The unbound fractions were 20% (17-20%) for colistin A and 37% (35-38%) for colistin B in the three conditions, indicating that there was no significant binding of colistin to the device.

3.2.2. Repeatability of the determination of unbound colistin

This was evaluated in spiked samples. The imprecision of $C_{unbound}$ was 12% for colistin A and 7% for colistin B for a total concentration of 1 mg/L, and 4% for colistin A and 3% for colistin B for a total concentration of 5 mg/L.

3.3. Determination of unbound fraction of colistin in clinical samples

The evaluated protocol was then used to determine unbound colistin concentrations in 30 clinical samples in which the range of total colistin was 0.36-4.98 mg/L for colistin A and 0.20-2.05 mg/L for colistin B. The median of unbound colistin was 0.44 mg/L (0.05-2.19 mg/L) for colistin A and 0.21 mg/L (0.08-1.13 mg/L) for colistin B, corresponding to unbound fractions of 34.3% (12.8%-51.0%) and 53.4% (27.0%-77.8%) for colistin A and colistin B, respectively. A significant correlation was observed between the total and unbound concentrations for both colistin A and B (colistin A, r_s=0.95; colistin B, r_s=0.81; Figure 1) but not between unbound fractions and biological parameters like total protein, albumin, alpha-1-acid glycoprotein, or creatinine (colistin A, r or r_s <0.20; colistin B, r or r_s <0.30). Likewise, no correlation was observed between the unbound fraction and C_{total} of colistin (colistin A, r_s=0.16; colistin B, r_s=-0.17; Figure 2).

4. Discussion

We have developed a simple LC-MS/MS method for colistin quantification before (C_{total}) and after ultrafiltration ($C_{unbound}$) that allows the determination of the unbound colistin fraction. In comparison with other published methods, our protocol displayed several advantages. First, the isolation step for unbound colistin, requiring 800 µL of sample only, was relatively short, about 40 min, preventing the conversion of CMS into colistin. Second, the analytical range validated fulfills clinical expectations [3, 4, 9]. Third, the extraction procedure with ACN was less expensive, faster, and easier than procedures that use an SPE cartridge [7, 12-14]. Fourth, the precipitation step and the reconstitution of the dry residue without acidic conditions prevented the conversion of CMS into colistin [14, 15]. For the development of the unbound colistin assay, the first major point to investigate was the ability of the method to quantify colistin in a mix of ultrafiltrate and plasma because the initial protocol was developed for plasma. The recoveries in the two matrices were in a close agreement. Therefore, the

matrix effect was considered to be non-significant and plasma standards were used for the calibration curves and the analytical performance of the total colistin assay (see supplemental files section) was applicable for the unbound colistin assay.

The second major point to investigate was the non-specific binding of colistin to the Centrifree device. The results obtained showed that the device was reliable for the quantification of unbound colistin concentration in clinical samples without pretreatment of the device.

Thirty clinical samples were analysed and demonstrated substantial inter-individual variation in the unbound colistin fraction. The mean values of the unbound fraction (34.3% for colistin A and 53.4% for colistin B) were comparable with those reported in the literature [7-9, 12, 16-17] for spiked samples after equilibrium dialysis or ultrafiltration (colistin A: 30-42%, colistin B: 43-60%), but higher than those observed in an *in vivo* microdialysis study (2.8%-14.1% [10]). The low unbound colistin fraction observed in this study may be explained by non-specific binding of colistin which is likely to occur in a dialysis device. Furthermore, the study was performed on healthy volunteers receiving a single infra-therapeutic dose of CMS, possibly associated with higher protein binding.

A good linear relationship between unbound and total colistin in clinical samples suggests that no saturation of protein binding occurs at therapeutic concentrations. Such a correlation was observed in the study of Dudhani *et al.* [8] but not with the protocol of Cheah *et al.* where the study was performed in mice [17]. This difference might explain the absence of a relationship between unbound and total colistin as a high level of protein binding of colistin (>90%) was observed in mice. Therefore, it might be difficult to highlight a concentration-dependent relationship at therapeutic levels because the unbound colistin values were very low.

In our study, we observed high inter-individual variation in unbound colistin concentrations. The differences could not be explained by albumin and alpha-1-acid glycoprotein levels (potential colistin binding proteins), nor by serum creatinine levels (an important covariate in the population PK/PD model of Garonzik *et al.* [4]). Nation *et al.* observed the same variability in their study (unbound fraction of colistin A+B: 49% ±11%) [16]. Other individual factors might explain this variability such as

co-administration of other drugs competing for protein binding sites or patient comorbidities. These factors need to be further explored. Our data suggest that adjustment of the drug dosage on the basis of total concentrations (as is current practice) might not be as appropriate as expected and that monitoring of unbound concentrations could be more informative. A new challenge would thus be to establish targets for unbound colistin which could improve the outcome of patients.

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Declarations

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Competing Interests: None

Ethical Approval: The local ethical committee has approved the study: P2015/374.

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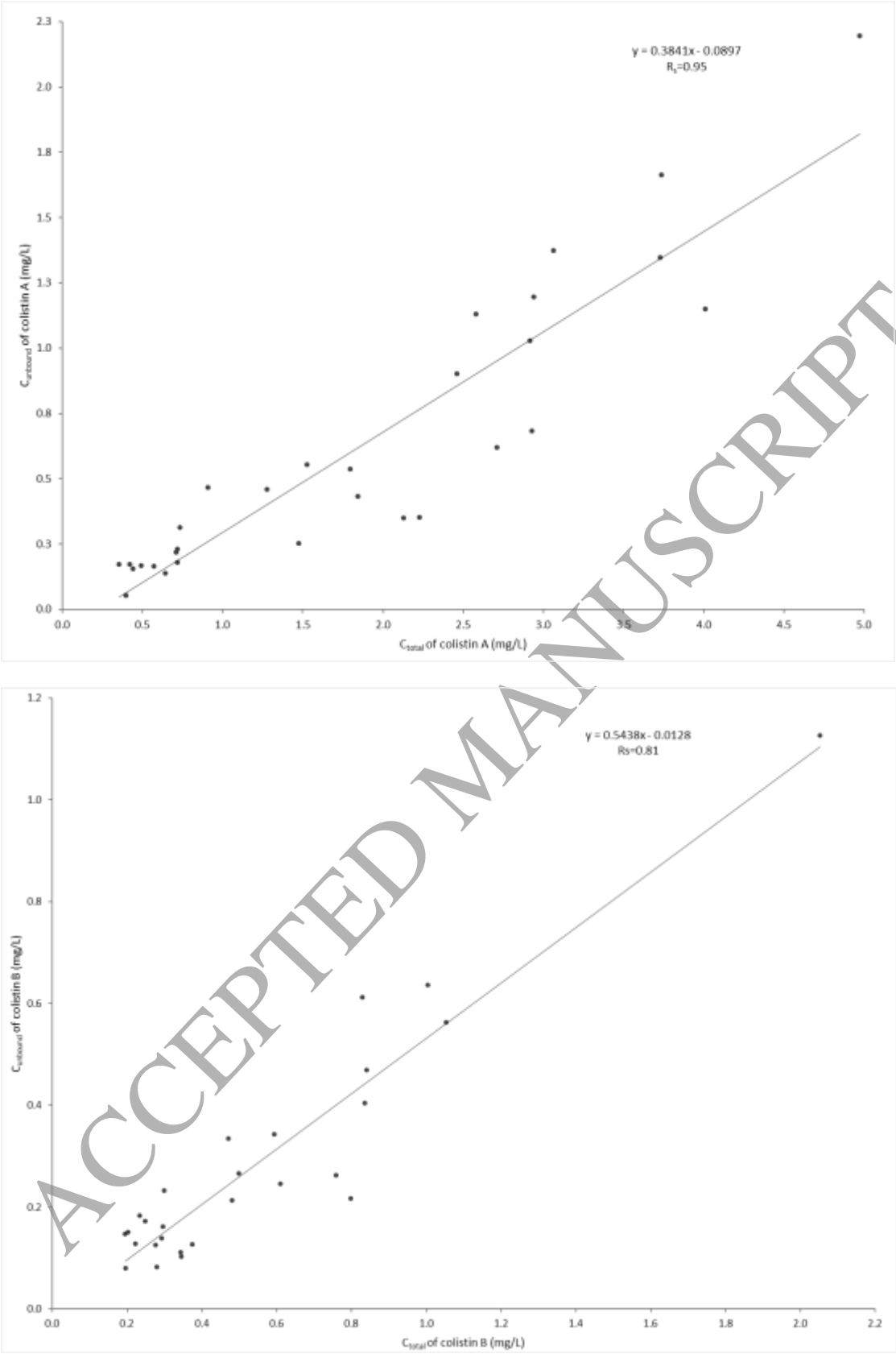

Figure 1. Relation between $C_{unbound}$ and C_{total} for colistin A and for colistin B.

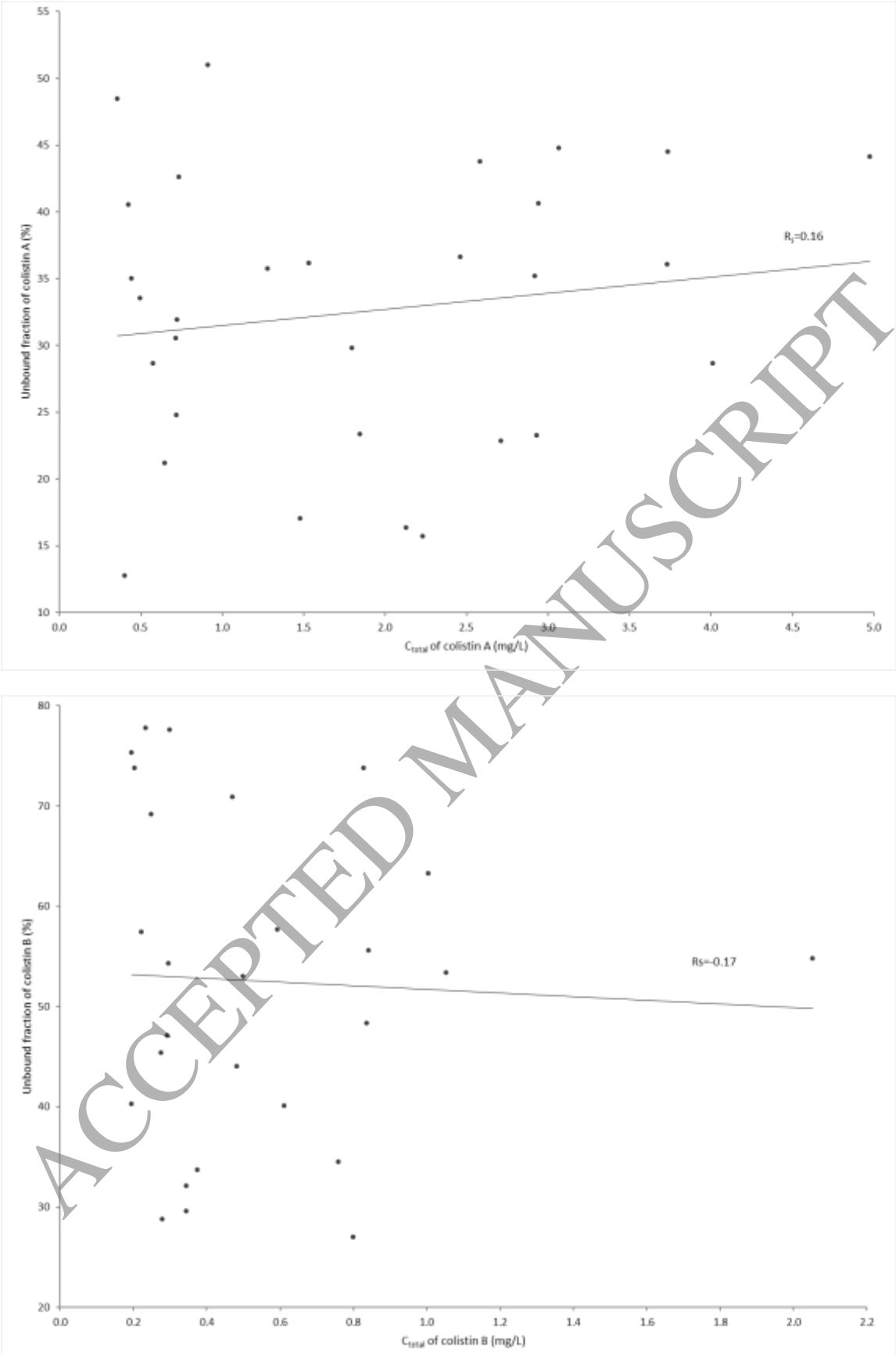

Figure 2. Inter-individual variation of the unbound fraction of colistin A and colistin B.

Table 1

Characteristics of patients receiving Colistineb®.

N	Gender	Age	Unit	CF[1] patient	Source of bacteremia	Strain	Other treatments	Total protein (g/L)	Albumin (g/L)	A-1-AG[2] (g/L)	Creatinine (mg/dL)	GFR[3] (mL/min/1.73 m²)
1	F	26	ICU[4]	Yes	Pneumonia	*P. aeruginosa*	azithromycin, piperacillin, tacrolimus, vancomycine	43	17	191	1.1	71
2	F	33	ICU[4]	Yes	Pneumonia	*P. aeruginosa*	azithromycin, piperacillin, tacrolimus	64	28	183	0.5	132
3	F	38	ICU[4]	Yes	Pneumonia	*A. xylosoxida*	meropenem, minocycline, paracetamol, piperacillin, tacrolimus	53	22	214	0,3	147
4	M	41	Nephrology	No	Cholecystitis	*P. aeruginosa*	cyclosporine, meropenem	51	15	75	0.4	138
5	M	44	Oncology	No	Wound infection	*P. aeruginosa*	cefepime, meropenem	53	20	229	1.1	78
6	M	70	Outside patient					41	19	179	NP[5]	NP[5]
7	M	70	Outside patient					71	33	218	NP[5]	NP[5]
8	F	57	ICU[4]	No	Origin unknown	*A. baumanii*	meropenem	69	31	139	0.4	125
9	M	43	Nephrology	No	Acute cholecystitis	*P. aeruginosa*	meropenem	59	18	115	0.6	121
10	M	69	ICU[4]	No	Angiocholitis	*K. pneumoniae*	amikacin, meropenem, tacrolimus	47	25	178	1.9	34
11	F	41	Oncology	No	Pneumonia	*K. pneumoniae*	meropenem	65	28	271	0.4	137
12	M	68	Nephrology	No	Urinary tract infection	*E. coli*	cyclosporine, clarithromycin, trimethoprim/sulfamethoxazole	52	14	122	0.8	14
13	M	70	ICU[4]	No	Pneumonia	*P. aeruginosa*	aztreonam, meropenem	45	26	139	0.7	99
14	F	62	Outside patient					60	33	157	NP[5]	NP[5]
15	F	74	Gast	No	Angiocholiti	*E. cloacae*	meropenem	59	25	174	1.2	46

			rology		s	*complex*	, tigecycline				
16	F	57	Nephrology	No	Origin unknown	*A. baumanii*	meropenem	75	31	151	0.4	128
17	M	67	ICU[4]	No	Pneumonia	*P. aeruginosa*	meropenem, tobramycin	63	23	214	0.6	102
18	M	51	ICU[4]	No	Pneumonia	*A. baumanii*	meropenem, tacrolimus, vancomycin	53	23	182	0.7	107
19	M	61	ICU[4]	No	Pneumonia	*P. aeruginosa*	amikacyn, fosfomycin, meropenem, vancomycin	51	22	191	0.5	112
20	M	50	ICU[4]	No	Pneumonia	*P. aeruginosa*	amikacyn, cefepime, vancomycin	57	31	102	0.5	118
21	M	73	Neurology	No	Pneumonia	*A. baumanii*	meropenem, phenytoin	59	22	260	0.8	90
22	M	62	Outside patient					54	33	279	NP[5]	NP[5]
23	M	52	ICU[4]	No	Pneumonia	*P. aeruginosa*	amikacin, cefepime, vancomycin	55	36	92	0.5	122
24	M	65	ICU[4]	No	Pneumonia	*P. aeruginosa*	meropenem, tobramycin	63	24	234	1.1	65
25	M	69	ICU[4]	No	Pneumonia	*P. aeruginosa*	aztreonam, piperacillin	32	12	117	1.6	41
26	M	69	ICU[4]	No	Pneumonia	*S. maltophilia*	amikacin, ceftazidime, meropenem, vancomycin	47	30	156	0.4	111
27	M	67	ICU[4]	No	Pneumonia	*P. aeruginosa*	ceftazidime	54	21	167	2.2	29
28	M	41	Nephrology	No	Osteitis	*P. aeruginosa*	meropenem	52	15	86	0.3	147
29	F	57	ICU[4]	No	Pneumonia	*K. pneumoniae*	meropenem	68	28	158	0.3	131
30	F	71	Gastrology	No	Angiocholitis	*K. pneumoniae*	meropenem	61	29	121	1.1	50

[1]Cystic fibrosis

[2]Alpha-1-acid glycoprotein

[3]Glomerular filtration rate (CKD-EPI formula)

[4]Intensive care unit

[5]Not performed