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Short Communication

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

D. Fage[a,*], G. Deprez[a], F. Wolff[a], M. Hites[b], F. Jacobs[b], F. Van Bambeke[c], F. Cotton[a]

[a] Department of Clinical Chemistry, LHUB-ULB, Brussels, Belgium
[b] Department of Infectious Diseases, Hôpital Erasme, Brussels, Belgium
[c] Louvain Drug Research Institute UCL, Brussels, Belgium

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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 regarding 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 min 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 B, respectively. High interindividual biological variation of binding was observed for colistin A and B that was not explained by the biochemical parameters studied. The method developed could be useful to improve outcomes for patients.

1. Introduction

Colistin is mainly used as a last-resort antibiotic in patients with cystic fibrosis [1,2] and critically ill patients [3,4]. These populations have altered pharmacokinetic characteristics owing to their disease state that results in unpredictable colistin plasma levels. Moreover, colistin has a narrow therapeutic range, causing nephrotoxicity and neurotoxicity [5,6]. Thus, therapeutic drug monitoring is useful to optimise 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 to 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 owing 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 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% high-performance liquid chromatography (HPLC)-grade and trifluoroacetic anhydride were purchased from Sigma-Aldrich (Bornem, Belgium). Acetonitrile (ACN) and formic acid 99% were obtained from Biosolve B.V. (Valkenswaard, The Netherlands) and were all mass spectrometry (MS)-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 liquid chromatography–tandem mass spectrometry (LC-MS/MS) were purchased from Agilent Technologies (Diegem, Belgium).

* Corresponding author. Tel.: +32 2 555 82 82.
E-mail address: david.fage@erasme.ulb.ac.be (D. Fage).

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2.2. High-performance liquid chromatography (HPLC) and mass spectrometry (MS)

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

2.3. Biochemical parameters

Assays for total protein, albumin, alpha-1-acid glycoprotein (α_1AGp) 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, Antwerp, Belgium). The regimen was a loading dose of 300 mg colistin base activity (CBA) [ca. 9 million International Units (mIU)] followed by 330 mg CBA (ca. 5 MIU) every 12 h. Characteristics of the 30 patients are detailed in Table 1. Blood samples were collected in heparinised tubes before the next dose of colistin, were transported on ice and were centrifuged immediately. The supernatants were stored at −80 °C until analysis. Biochemical parameters were measured on the leftover volume of samples. The study was approved by the Ethics Committee of Hôpital Erasme (Brussels, Belgium).

2.5. Total colistin assay

The protocol was based on the work published by Jansson et al. [11] with some modifications. A description of the method and the validation process are presented in the Supplementary material. The main modifications were the use of a water/ACN gradient for the mobile phase, a precipitation step, and 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. A volume of 1 mL of colistin-free plasma from six 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 concentrations (1 mg/L and 10 mg/L of total colistin base A+B) and was added to 100 µL of a colistin-free plasma pool and 50 µL of IS. Proteins were precipitated with 1.2 mL of ACN and were pelleted by centrifugation (10 min, 4 °C, 15000 rpm). Then, 1.2 mL of supernatant was recovered and was evaporated under nitrogen at RT. The residue was reconstituted with 100 µL of 95/5 (v/v) ultrapure water/ACN, was vortex-mixed and was centrifuged (10 min, 4 °C, 15000 rpm). The supernatant was transferred into a vial and 5 µL was injected into the LC-MS/MS system. The measured colistin/IS area ratio was compared with 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. 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 device (i) served as a control (without pre-treatment). The second (ii) and third (iii) devices were pre-treated with polymyxin B solutions (10 mg/L and 100 mg/L, respectively). 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 into devices (i), (ii) and (iii). Total colistin (C_{total}) was determined in the sample compartment before centrifugation. Unbound colistin ($C_{unbound}$) was measured in the ultrafiltrate compartment following 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 normalise 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 0.8–1 mL of plasma was introduced into the Centrifree® device. A 35-min centrifugation step (RT, 4000 rpm, fixed angle of 45 °) was then performed. Next, 200 µL of ultrafiltrate was transferred to an Eppendorf tube containing 100 µL of a colistin-free plasma pool and then 50 µL of IS and 1.2 mL of ACN were added. The mix was centrifuged (10 min, 4 °C, 15000 rpm) and then 1.2 mL of supernatant was recovered and evaporated under nitrogen at RT. The residue was reconstituted with 100 µL of 95/5 (v/v) ultrapure water/ACN, was vortex-mixed and was centrifuged (10 min, 4 °C, 15000 rpm). The supernatant was transferred to a vial and 5 µL was injected into the LC-MS/MS system. Measurement of colistin in the ultrafiltrates provided the $C_{unbound}$ of both colistin A and colistin B.

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

2.7. Statistical analysis

The results were analysed using Analyse-it v.3.80 (Analyse-it Software, Leeds, UK). The Shapiro–Wilk normality test was used to assess the distribution of continuous variables. 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 of <0.05 was considered statistically significant. Parametric results are expressed as mean and range and non-parametric results as median and range.

3. Results

3.1. Total colistin assay

The results of the validation are presented in the Supplementary material. 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 <11%.

Table 1
Characteristics of patients ($n = 30$) receiving Colistineb®.

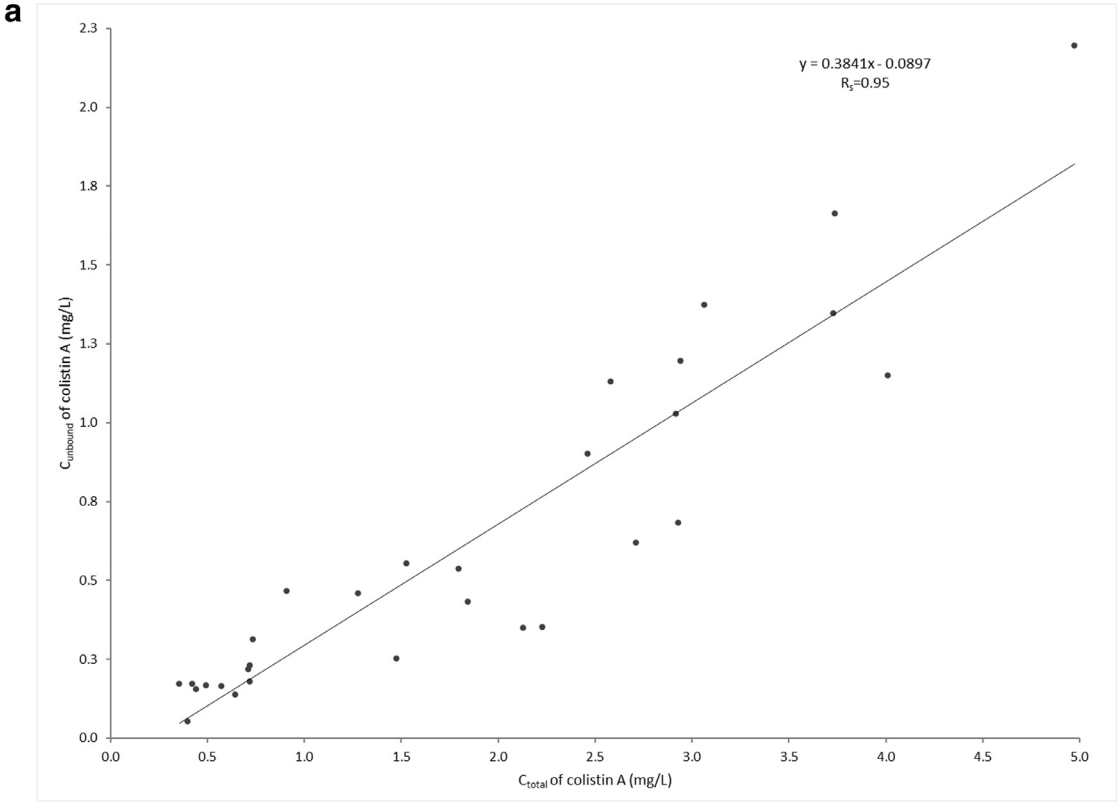
Sex	Age (years)	Unit	CF patient	Source of bacteraemia	Isolate	Other treatments	Total protein (g/L)	Albumin (g/L)	α_1AGp (g/L)	Creatinine (mg/dL)	GFR (mL/min/1.73 m^2)
F	26	ICU	Yes	Pneumonia	*Pseudomonas aeruginosa*	Azithromycin, piperacillin, tacrolimus, vancomycin	43	17	191	1.1	71
F	33	ICU	Yes	Pneumonia	*P. aeruginosa*	Azithromycin, piperacillin, tacrolimus	64	28	183	0.5	132
F	38	ICU	Yes	Pneumonia	*Achromobacter xylosoxidans*	Meropenem, minocycline, paracetamol, piperacillin, tacrolimus	53	22	214	0.3	147
M	41	Nephrology	No	Cholecystitis	*P. aeruginosa*	Cyclosporine, meropenem	51	15	75	0.4	138
M	44	Oncology	No	Wound infection	*P. aeruginosa*	Cefepime, meropenem	53	20	229	1.1	78
M	70	Outpatient					41	19	179	NP	NP
M	70	Outpatient					71	33	218	NP	NP
F	57	ICU	No	Origin unknown	*Acinetobacter baumannii*	Meropenem	69	31	139	0.4	125
M	43	Nephrology	No	Acute cholecystitis	*P. aeruginosa*	Meropenem	59	18	115	0.6	121
M	69	ICU	No	Angiocholitis	*Klebsiella pneumoniae*	Amikacin, meropenem, tacrolimus	47	25	178	1.9	34
F	41	Oncology	No	Pneumonia	*K. pneumoniae*	Meropenem	65	28	271	0.4	137
M	68	Nephrology	No	Urinary tract infection	*Escherichia coli*	Cyclosporine, clarithromycin, SXT	52	14	122	0.8	14
M	70	ICU	No	Pneumonia	*P. aeruginosa*	Aztreonam, meropenem	45	26	139	0.7	99
F	62	Outpatient					60	33	157	NP	NP
F	74	Gastrology	No	Angiocholitis	*Enterobacter cloacae* complex	Meropenem, tigecycline	59	25	174	1.2	46
F	57	Nephrology	No	Origin unknown	*A. baumannii*	Meropenem	75	31	151	0.4	128
M	67	ICU	No	Pneumonia	*P. aeruginosa*	Meropenem, tobramycin	63	23	214	0.6	102
M	51	ICU	No	Pneumonia	*A. baumannii*	Meropenem, tacrolimus, vancomycin	53	23	182	0.7	107
M	61	ICU	No	Pneumonia	*P. aeruginosa*	Amikacin, fosfomycin, meropenem, vancomycin	51	22	191	0.5	112
M	50	ICU	No	Pneumonia	*P. aeruginosa*	Amikacin, cefepime, vancomycin	57	31	102	0.5	118
M	73	Neurology	No	Pneumonia	*A. baumannii*	Meropenem, phenytoin	59	22	260	0.8	90
M	62	Outpatient					54	33	279	NP	NP
M	52	ICU	No	Pneumonia	*P. aeruginosa*	Amikacin, cefepime, vancomycin	55	36	92	0.5	122
M	65	ICU	No	Pneumonia	*P. aeruginosa*	Meropenem, tobramycin	63	24	234	1.1	65
M	69	ICU	No	Pneumonia	*P. aeruginosa*	Aztreonam, piperacillin	32	12	117	1.6	41
M	69	ICU	No	Pneumonia	*Stenotrophomonas maltophilia*	Amikacin, ceftazidime, meropenem, vancomycin	47	30	156	0.4	111
M	67	ICU	No	Pneumonia	*P. aeruginosa*	Ceftazidime	54	21	167	2.2	29
M	41	Nephrology	No	Osteitis	*P. aeruginosa*	Meropenem	52	15	86	0.3	147
F	57	ICU	No	Pneumonia	*K. pneumoniae*	Meropenem	68	28	158	0.3	131
F	71	Gastrology	No	Angiocholitis	*K. pneumoniae*	Meropenem	61	29	121	1.1	50

CF, cystic fibrosis; α_1AGp, alpha-1-acid glycoprotein; GFR, glomerular filtration rate (CKD-EPI formula); ICU, intensive care unit; NP, not performed; SXT, trimethoprim/sulfamethoxazole.

3.2. Unbound colistin assay

3.2.1. Method development

3.2.1.1. Matrix effect. The $[(\text{colistin/IS})_{\text{ultrafiltrate}}]/[(\text{colistin/IS})_{\text{plasma}}]$ 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 Supplementary material) 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.



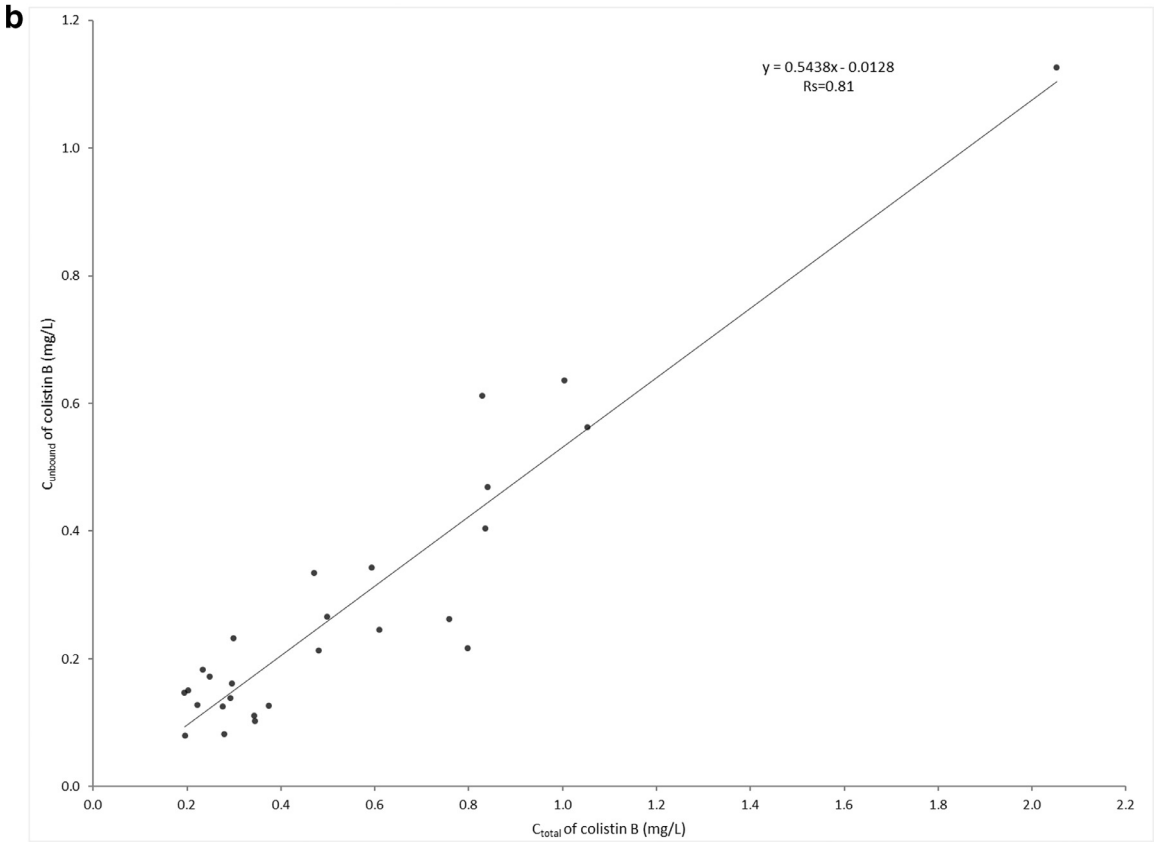


Fig. 1. Relationship between unbound colistin ($C_{unbound}$) and total colistin (C_{total}) for (a) colistin A and (b) colistin B.

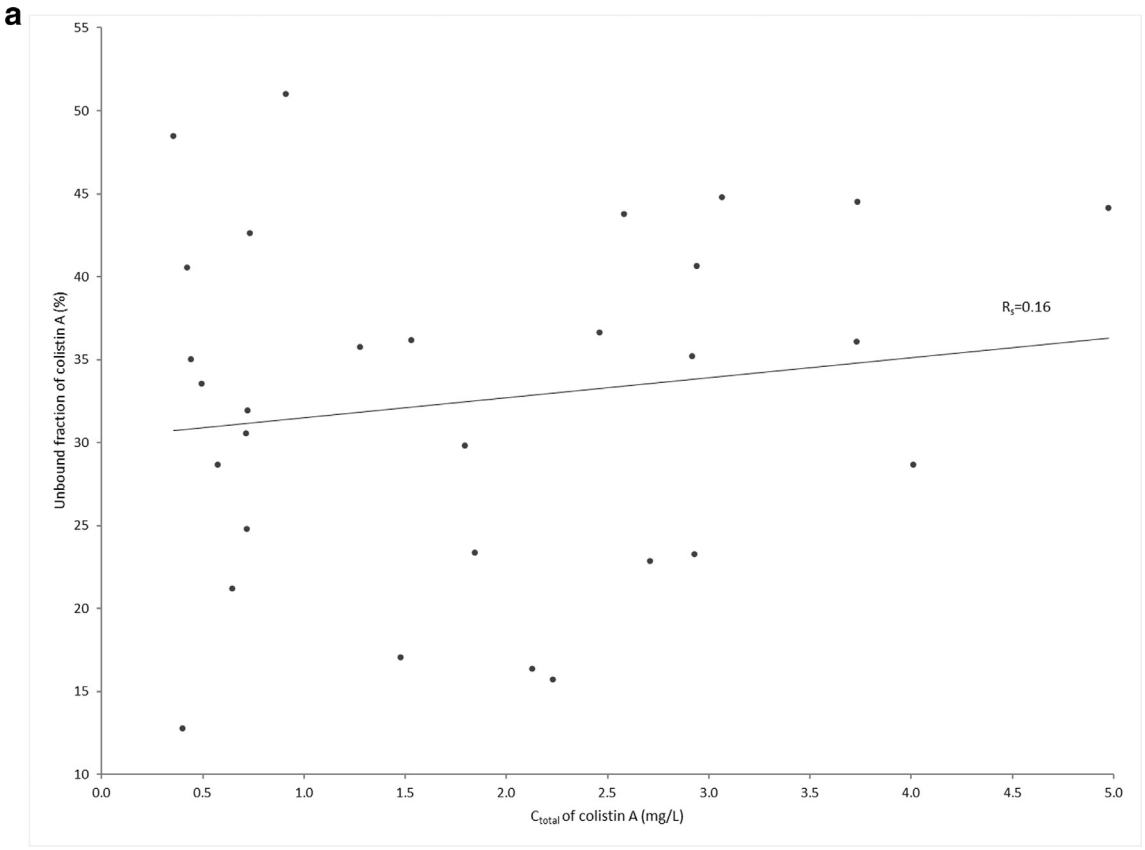


Fig. 2. Interindividual variation of the unbound fraction of (a) colistin A and (b) colistin B.

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 two devices in which their potential binding sites were neutralised by a solution of 10 mg/L or 100 mg/L 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

The repeatability of the determination of unbound colistin 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 the 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 unbound colistin concentration 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 B, respectively. A significant correlation was observed between the total and unbound concentrations both for colistin A and B (colistin A, $r_s = 0.95$; colistin B, $r_s = 0.81$) (Fig. 1) but not between unbound fractions and biological parameters such as total protein, albumin, α_1AGp 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, $r_s = -0.17$) (Fig. 2).

4. Discussion

We have developed a simple LC-MS/MS method for colistin quantification before (C_{total}) and after ($C_{unbound}$) ultrafiltration that allows the determination of the unbound colistin fraction. In comparison with other published methods, this protocol displays several advantages. First, the isolation step for unbound colistin, requiring 800 µL of sample only, was relatively short (ca. 40 min), preventing the conversion of CMS into colistin. Second, the analytical range validated fulfils clinical expectations [3,4,9]. Third, the extraction procedure with ACN was less expensive, faster and easier than procedures that use a solid phase extraction (SPE) cartridge [7,12–14]. Fourth, the precipitation step and reconstitution of the dry residue without acidic conditions prevented the conversion of CMS into colistin [14,15].

For 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 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 the Supplementary material) 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 pre-treatment of the device.

A total of 30 clinical samples were analysed and demonstrated substantial interindividual variation in the unbound colistin fraction. 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 that 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 subtherapeutic 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 the current study, high interindividual variation in unbound colistin concentrations was observed. The differences could not be explained by albumin or α_1AGp levels (potential colistin binding proteins) nor by serum creatinine level (an important covariate in the population pharmacokinetic/pharmacodynamic 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 co-morbidities. These factors need to be further explored. The current 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 that could improve the outcome of patients.

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Competing interests

None declared.

Ethical approval

This study was approved by the Ethics Committee of Hôpital Erasme (Brussels, Belgium) [P2015/374].

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.10.017.

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

1. Materials and methods

1.1. High-performance liquid chromatography (HPLC) and mass spectrometry (MS)

1.1.1. Chromatographic conditions

The column was an X-Terra RP18 3.5 μm (3.9 × 100 mm) (Waters, Zellik, Belgium) thermostated at 37 °C. The mobile phase, consisting of 0.1% (v/v) formic acid (FA) in water (mobile phase A) and 0.1% (v/v) FA in acetonitrile (ACN) (mobile phase B), was delivered at a flow rate of 0.6 mL/min according to the following gradient: 0 min, 95/5 (A/B); 1 min, 70/30 (A/B); 5 min, 63/37 (A/B); 5.1 min, 15/85 (A/B); 7 min, 15/85 (A/B); and 7.1 min, 95/5 (A/B). After each run, the column was re-equilibrated for 3 min. To avoid carryover, the autosampler needle was washed for 30 s with 90/10/1 (v/v/v) ACN/water/trifluoroacetic acid after each injection. Two solvent blanks 95/5 (A/B) were injected between calibration standards (CS) and validation standards (VS) and between VS and samples.

1.1.2. MS/MS condition

MS/MS detection was performed in positive ion mode. The source temperature was 325 °C and the gas flow rate was 10 L/min. The nebuliser pressure was 40 psi. The sheath gas temperature and flow rate were, respectively, 350 °C and 9 L/min. Ions were analysed by multiple-reaction monitoring (MRM) using the following transitions: 585.5 → 241.3 for colistin A; 578.5 → 227.3 for colistin B; and 602.5 → 241.2 for polymyxin B.

1.2. Total colistin assay

1.2.1. Pre-validation study: characterisation of bulk colistin powder

Colistin powder used for the CS and the VS is mainly a mixture of colistin A and colistin B in unknown and variable proportions depending on the lot of the product. The purity of the powder and the proportion of the two major compounds were established by HPLC-UV. An Atlantis T3 3 μm (3.0 × 150 mm) column (Waters) was used with a mobile phase of phosphoric acid at pH 2.4 (mobile phase A) and ACN (mobile phase B). Detection was carried out at 210 nm with an Agilent 1200 Infinity HPLC System (Agilent Technologies, Diegem, Belgium). The mobile phase was delivered in isocratic mode (80% A/20% B) at a flow rate of 0.6 mL/min during 20 min. A volume of 20 μL of an aqueous solution of colistin at 500 mg/L was injected.

1.2.2. Preparation of calibration standards and validation standards

Polymyxin B was used as internal standard (IS). Stock solutions of colistin base A+B and polymyxin B at 1000 mg/L were prepared in ultrapure water and were stored at –80 °C. Three series of CS and VS

used to validate colistin quantification in heparinised plasma were prepared in plasma pools with independent stock solutions at final concentrations of 0.05, 0.1, 1, 10, 15 and 20 mg/L of total colistin base A+B. Plasma pools were obtained by mixing leftover heparinised plasma samples from patients who were not treated with colistin. The absence of colistin in these pools was confirmed by MS analysis. Aliquots of plasma pools were stored at –20 °C before use for CS and VS preparation.

1.2.3. Extraction protocol of calibration standards, validation standards and samples

The protocol was based on the work published by Jansson et al. [1] with some modifications. To 200 μL of CS, VS or sample, 50 μL of IS working solution (aqueous solution of polymyxin B at 5 mg/L) and 800 μL of ACN were added. The resulting mixture was vortex-mixed for 2 min and was centrifuged for 15 min at 15 000 rpm and 4 °C. Then, 800 μL of supernatant was evaporated under nitrogen at room temperature. The residue was reconstituted with 100 μL of 95/5 (v/v) ultrapure water/ACN, was vortex-mixed and was centrifuged for 10 min at 15 000 rpm and 4 °C. The supernatant was transferred to a vial and 5 μL was injected into the liquid chromatography–tandem mass spectrometry (LC-MS/MS) system. A chromatogram of colistin in human plasma is shown in Supplementary Fig. S1.

1.2.4. Method validation

1.2.4.1. Recovery

To evaluate the recovery, three concentrations (0.1, 1 and 10 mg/L of total colistin base A+B) were analysed. For each level, three colistin-free plasma samples were spiked with colistin and IS *before* the precipitation step and three colistin-free plasma samples were spiked with IS *before* and with colistin *after* the precipitation step. They were processed according to the protocol described in Section 1.2.3.

1.2.4.2. Ion suppression

Ion suppression was assessed qualitatively through a post-column infusion. At a flow rate of 0.6 mL/min, a 5 mg/L colistin solution in 95/5 (v/v) ultrapure water/ACN was introduced directly into the MS/MS detector through a T-piece. Extracts from six different colistin-free plasma samples and the pool used to prepare CS and VS were injected into the chromatographic column and were driven into the MS/MS detector through the same T-piece.

1.2.4.3. Accuracy profiles

The accuracy profiles approach was used to validate the method. The acceptance limits were fixed at 30% according to the regulatory requirements, and the risk of having future results falling outside those limits was set at 5%. Six levels of CS ranging from 0.05 mg/L to 20 mg/L of total colistin base A+B were analysed three times a day during 3 days. VS were analysed independently at the same levels, four times during 3 days. Trueness, precision, accuracy, limit of quantification and linearity were assessed.

1.2.4.4. Stability studies and in vitro conversion of colistin methanesulfonate (CMS) into colistin

The long-term stability of stock solutions and VS was assessed at –80 °C during 3 months. Short-term stability of colistin in spiked plasma samples (0.1, 1 and 10 mg/L of total colistin base A+B) was studied under three freeze–thaw cycles.

To evaluate conversion of CMS into colistin during the extraction process, three colistin-free plasma samples were spiked with CMS base at a final concentration of 5 mg/L. The appearance of colistin was monitored by LC-MS/MS in MRM mode. The absence of CMS conversion was also assessed after three freeze–thaw cycles.

The stability of the extracted samples (0.1, 1 and 10 mg/L for total colistin base A+B; 5 mg/L for CMS base) in the autosampler was assessed in triplicate for each concentration, over 5 h, with one injection per hour.

2. Results of total colistin assay

2.1. Colistin powder composition

The purity and the proportion of colistin A and B in the bulk powder were established with five injections of an aqueous solution of colistin. The purity of colistin was 87.7% (85.9–88.4%) with proportions of 35.8% (35.6–36.2%) colistin A and 64.2% (63.8–64.4%) colistin B.

2.2. Method validation

2.2.1. Recovery

The recovery rates were 51.3% (50.8–52.0%) at 0.1 mg/L, 53.8% (52.7–54.1%) at 1 mg/L and 62.9% (62.1–63.3%) at 10 mg/L for colistin A, and 51.8% (51.7–53.3%) at 0.1 mg/L, 50.0% (49.9–50.4%) at 1 mg/L and 65.3% (63.5–65.6%) at 10 mg/L for colistin B.

2.2.2. Ion suppression

Before injection of a colistin-free plasma extract, the m/z signal of colistin and IS were stable in the MS/MS detector. In the case of ion suppression due to proteins, surfactants or phospholipids mainly, the signal lost intensity. With the present chromatographic conditions, ion suppression occurred before the elution of colistin A, colistin B and IS.

2.2.3. Accuracy profiles

The trueness, expressed by the relative bias, the precision including the repeatability and the intermediate precision, as well as the linearity results are shown in Supplementary Table S1. The method allowed the quantification of total colistin with a known trueness and precision in the following dosing ranges: 0.100–16.790 mg/L for colistin A+B, with 0.036–7.160 mg/L for colistin A and 0.064–9.630 mg/L for colistin B.

2.2.4. Stability studies and in vitro conversion of CMS into colistin

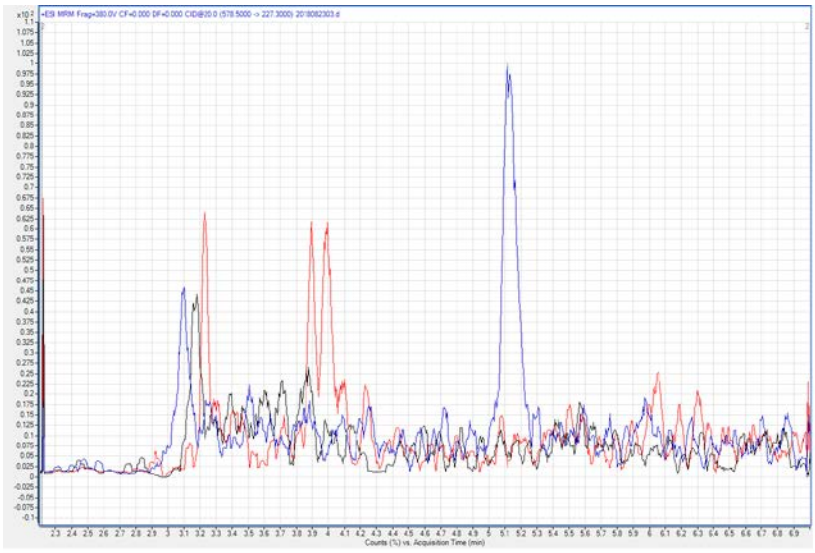
The VS were stable at –80 °C for ≥3 months. After three freeze–thaw cycles, there was no significant degradation of colistin at the various levels assessed (bias <6% for colistin A and <7% for colistin B) and no significant in vitro conversion of CMS into colistin.

After extraction of plasma spiked with CMS, the intensity of the m/z colistin signal, detected by MRM, was below the limit of quantification.

The extracted samples containing colistin were stable for ≥5 h (bias <7% for colistin A and <8% for colistin B) and no in vitro conversion of CMS into colistin was observed for extracted samples containing CMS.

Reference

[1] Jansson B, Karvanen M, Cars O, Plachouras D, Friberg LE. Quantitative analysis of colistin A and colistin B in plasma and culture medium using a simple precipitation step followed by LC/MS/MS. J Pharm Biomed Anal 2009;49:760–7. doi: 10.1016/j.jpba.2008.12.016.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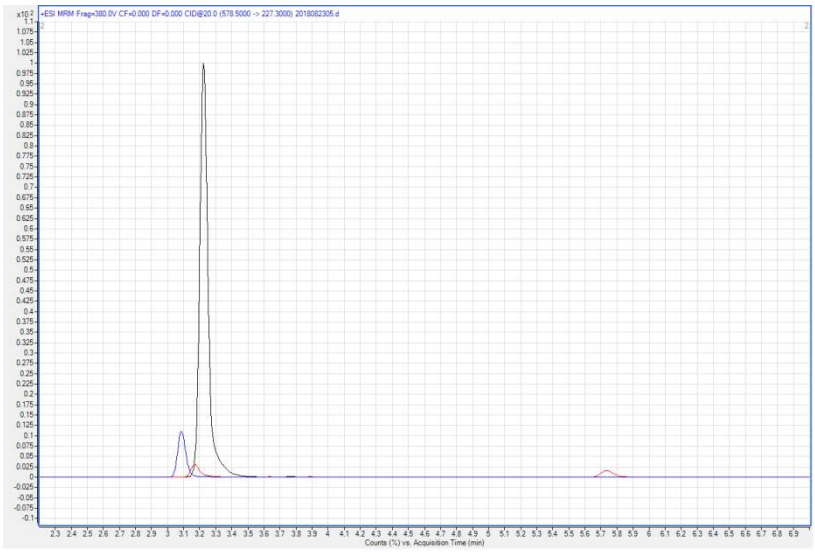
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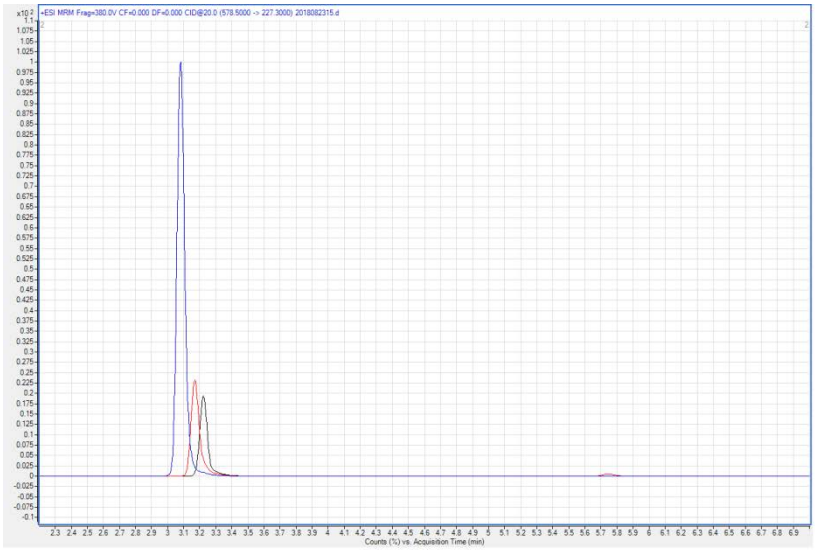
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Counts (%) vs. Acquisition Time (min)

Supplementary Fig. S1. Typical chromatogram of colistin A (black line), colistin B (blue line) and polymyxin B (red line) in (A) colistin-free plasma, (B) at the limit of quantification and (C) in a patient treated with colistin.

Supplementary Table S1

Criteria of analytical performance (trueness, precision and linearity) obtained for plasma total colistin base A and B measurement. Validation standards were analysed at six different concentrations. Calibration curves were built using a 1/*X* weighted quadratic regression

Total colistin base A					Total colistin base B				
Target concentration (mg/L)	Mean (mg/L)	Relative bias (%)	CV intermediate precision (%)	Lower–upper limit calculated (%)	Target concentration (mg/L)	Mean (mg/L)	Relative bias (%)	CV intermediate precision (%)	Lower–upper limit calculated (%)
0.018 [a]	0.019	3.7	21.7	–48.0 to 55.5	0.032 [a]	0.030	–5.5	27.7	–71.8 to 60.9
0.036	0.033	0.0	5.9	–14.5 to 14.4	0.064	0.056	–0.1	4.1	–10.0 to 9.8
0.358	0.367	2.9	3.7	–6.0 to 11.8	0.642	0.682	6.2	5.0	–6.1 to 18.6
3.580	3.549	–0.9	7.1	–17.9 to 16.2	6.420	6.138	–4.4	8.3	–24.4 to 15.6
5.370	5.424	1.0	9.0	–20.7 to 22.8	9.630	9.410	–2.3	10.7	–27.8 to 23.2
7.160	7.001	–2.2	8.0	–21.5 to 17.1	12.800 [a]	10.972	–14.5	13.6	–47.1 to 18.0
	Slope 0.986		95% CI (0.974–0.999)			Slope 0.970		95% CI (0.955–0.985)	

CV, coefficient of variation; CI, confidence interval.

[a] Level not acceptable (total error was above the accuracy limit).