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Quantitative analysis of colistin A and colistin B in plasma and culture medium using a simple precipitation step followed by LC/MS/MS

Britt Jansson[a,*], Matti Karvanen[b], Otto Cars[b], Diamantis Plachouras[c], Lena E. Friberg[a]

[a] Division of Pharmacokinetics and Drug Therapy, Department of Biopharmaceutical Sciences, Biomedicum, Box 591, SE-751 24 Uppsala, Sweden
[b] Section of Infectious Diseases, Department of Medical Sciences, Uppsala University Hospital, SE-751 85 Uppsala, Sweden
[c] University of Athens, Medical School, 4th Dept. of Internal Medicine, University Hospital ATTICON, Rimini 1, GR-124 62 Haidari, Greece

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ABSTRACT

An analytical method for quantitation of colistin A and colistin B in plasma and culture medium is described. After protein precipitation with acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA), the supernatants were diluted with 0.03% TFA. The compounds were separated on an Ultrasphere C18 column, 4.6 mm × 250 mm, 5 μm particle size with a mobile phase consisting of 25% ACN in 0.03% TFA and detected with tandem mass spectrometry. The instrument was operating in ESI negative ion mode and the precursor–product ion pairs were *m/z* 1167.7 → 1079.6 for colistin A and *m/z* 1153.7 → 1065.6 for colistin B. The lower limit of quantification (LLOQ) for 100 μL plasma was 19.4 and 10.5 ng/mL for colistin A and B, respectively, with CV <6.2% and accuracy < ±12.6%. For culture medium (50 μL + 50 μL plasma), LLOQ was 24.2 and 13.2 ng/mL for colistin A and B, respectively, with CV <11.4% and accuracy < ±8.1%. The quick sample work-up method allows for determination of colistin A and B in clinical samples without causing hydrolysis of the prodrug colistin methanesulfonate (CMS).

1. Introduction

During the last decade, the world-wide increase in multidrug-resistance among Gram-negative bacteria has successively reduced the treatment options for infections caused by such pathogens. As established treatments are losing their effect, the polymyxins, mainly colistin (polymyxin E), is re-emerging as salvage therapy. Colistin came into use in the 1960s but was replaced in clinical practice by newer and less toxic broad spectrum antibiotics in the 1970s [1].

Chemically, the polymyxins consist of a decapeptide, whereof seven amino acid residues form a ring. Five of the diaminobutyric acid residues present free amines which are positively charged in physiologic conditions (Fig. 1). The tail of the peptide is connected to a fatty acid chain, e.g. 6-methyloctanoic acid in colistin A (polymyxin E_1) and 6-methylheptanoic acid in colistin B (polymyxin E2), the two major components of colistin preparations [2]. Thus, the molecule is amphipathic. Due to the toxic side effects reported in the 1960s and 1970s, colistin is reacted with formaldehyde and sodium bisulfite to yield an inactive prodrug, colistin methanesulphonate (CMS) [3]. CMS is hydrolyzed spontaneously in aqueous solutions into partially sulphomethylated derivatives and colistin base [4].

Due to the lack of specific and reliable methods for assay of colistin and its prodrug, little is known about their pharmacokinetic (PK) and pharmacodynamic (PD) properties.

The current dosage regimens are derived from experience in the 1970s, but is not based on a defined PD target attainment. Besides the need for a robust assay for determination of colistin in plasma samples, *in vitro* studies are crucial to unravel the PK/PD targets and to efficiently optimize dosage. It is therefore also of great importance to be able to analyze culture media samples from *in vitro* studies to ascertain drug concentrations during the study period of the experiments. One specific problem is that colistin is adsorbed to many different materials which are used in laboratory practice. If colistin is bound to, e.g. plastic ware used in the experiments, sample preparation or post-experiment sample handling, the efficacy of colistin may be misevaluated.

Another problem is that the degradation/hydrolysis of CMS to colistin is time, matrix and temperature-dependent [4,5] and when evaluating potential efficacy of CMS *in vitro* it is important to investigate if colistin is formed during the experiment and hence is the compound causing the antibiotic effect. In addition, in the analysis of plasma colistin concentrations following CMS administration, the time and pH for the work-up procedure may be critical to limit degradation of CMS to colistin and to avoid falsely high colistin concentrations at time points with high CMS concentrations.

* Corresponding author. Tel.: +46 18 471 4302; fax: +46 18 471 4003.
E-mail address: britt.jansson@farmbio.uu.se (B. Jansson).

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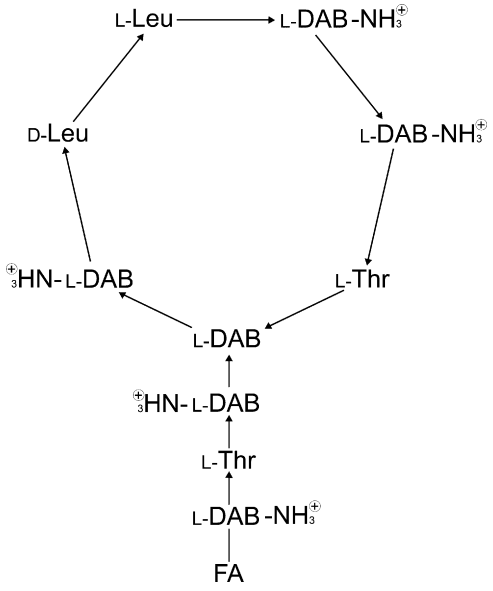

Fig. 1. Principal structure of colistin at physiologic pH. DAB, diaminobutyric acid; FA, fatty acid (e.g. 6-methyloctanoic acid).

Several techniques have been described for the quantification of colistin. Microbiological bioassays are simple and do not require expensive apparatus [6]. They are however less useful in assaying colistin in clinical samples as the CMS present in the sample will be hydrolyzed into colistin during the incubation, thus giving falsely too high concentrations. The use of high-performance liquid chromatography (HPLC) methods need time-consuming sample pretreatment including protein precipitation, solid phase extraction (SPE) and derivatisation [7,8]. Colistin has poor UV-absorption and no native fluorescence. When analyzing low concentrations the molecules had to be derivatised and the reagents used in the two examples above are 9-fluorenylmethyl chloroformate (FMOC-Cl) and ortho-phthalaldehyde (OPA).

The use of mass spectrometry for detection provides high selectivity and usually high sensitivity. One recently published method [9] describes a liquid chromatography tandem mass spectrometry (LC/MS/MS) method for analyzing biological fluids. The use of a narrow bore column and high content of organic modifier resulted in short retention times. This required clean samples and an extraction step had to follow the protein precipitation.

The aim of this study was to develop a robust and high sensitivity analysis method for determination of colistin A and colistin B in clinical plasma samples and samples from in vitro experiments in culture media that limit the degradation of CMS to colistin.

2. Experimental

2.1. Materials

Colistin sulfate salt and colistin methanesulfonate sodium salt were purchased from Sigma Chemicals (St. Louis, MO, USA). Acetonitrile (ACN) (LiChrosolve, gradient grade), trifluoroacetic acid (TFA) (Uvasol), sulphuric acid (analytical grade) and sodium hydroxide (1 M, Titrisol) were obtained from Merck (Darmstadt, Germany). The house deionized water was further purified with a Milli-Q Academic system Millipore (Bedford, MA, USA).

Blank human plasma was obtained from the University Hospital Blood Bank (Uppsala, Sweden) and the culture medium Mueller-Hinton broth was purchased from Difco BD (Franklin Lakes, NJ, USA).

2.2. LC/MS/MS method

The liquid chromatography was performed using a system with a LC-10AD*VP* pump (Shimadzu, Kyoto, Japan), a refrigerated autosampler, Triathlon 900 (Spark Holland, The Netherlands), and an analytical column Ultrasphere C18, 4.6 mm × 250 mm, 5 μm particle size (Beckman, Berkeley, CA, USA) with a guard column of the same material.

The detector was a triple quadrupole mass spectrometer Quattro Ultima (Waters, Milford, MA, USA) and MassLynx software version 4.0 was used for MS control and spectral processing.

The mobile phase, 25% ACN in 0.03% TFA, was pumped at a flow rate of 1 mL/min, generating a pressure of 120 bar. Before entering the source of the mass spectrometer, the flow was split to 0.25 mL/min. The samples were stored at 4 °C in the autosampler before 50 μL was injected on the column.

The detector was used in ESI negative ion mode. The measurements were made at 400 °C desolvation temperature and 130 °C source temperature. Cone gas (N_2) and desolvation gas (N_2) were held at 150 and 1000 L/h, respectively, and the collision gas (argon) pressure was 3×10^{-3} Torr. The capillary voltage was 3.60 kV, the cone voltage was 140 V and the collision energy was set to 40 eV.

Multiple reaction monitoring (MRM) was used, and the single charged precursor–product ion pairs were m/z 1167.7 → 1079.6 for colistin A and m/z 1153.7 → 1065.6 for colistin B.

The resolution was set at 1.5 u at half height for Q1 and Q3 and the dwell time for both channels was 1 s. The parameters were optimized, to provide the highest sensitivity, by direct infusion of 1 μg/mL colistin sulfate in mobile phase with a syringe pump operating at a flow rate of 200 μL/min.

2.3. Standard and quality control (QC) sample preparations

Two stock solutions of colistin sulfate were prepared in water to an approximate concentration of 1 mg/mL. One stock solution was used for the standards and the other for the QCs.

The percentage of colistin A and colistin B in the reference substance were estimated by using the method described in Ma et al. [9] with some modifications. In the current analysis method, the mobile phase consisted of 25% ACN in 0.03% TFA and the column used was a 3.9 mm × 300 mm μBondapak C18 with 10 μm particle size. The flow rate was 1 mL/min and 50 μL of a 100 μg/mL colistin sulfate solution was injected ($n = 5$).

The here used batch of colistin sulfate was estimated to contain 56.2% of A (CV = 0.7%) and 30.6% of B (CV = 0.7%). Correction for the sulfate content was made by using the average molecule weights for colistin sulfate (1403) and for colistin (1163) [10], which resulted in 46.6% colistin A and 25.4% colistin B.

Blank human plasma was spiked to eight standard samples in the range of 19.36–2420 and 10.52–1315 ng/mL for colistin A and B, respectively, and to three QC levels for colistin A (165.1, 550.2, and 1651 ng/mL) and for colistin B (89.70, 299.0, and 897.0 ng/mL).

The culture medium was mixed with an equal volume of human plasma to avoid adhesion. This mixture was spiked to seven standard samples in the range of 24.20–968.0 and 13.15–526.1 ng/mL for colistin A and B, respectively, and to three QC levels for colistin A (66.03, 330.1, and 660.3 ng/mL) and for colistin B (35.88, 179.4, and 358.8 ng/mL). The given concentrations above are of the analytes in the culture medium. All dilution steps for the plasma and the plasma–medium mix were carried out in 1.5 mL micro test tubes of polypropylene (PP) from Sarstedt (Nümbrecht, Germany). Stock solutions, plasma standards, medium standards and QCs were stored at −20 °C.

2.4. Sample collection

Blood samples were immediately chilled and thereafter centrifuged. The plasma was stored at −70 °C until analysis. Samples from *in vitro* experiments with culture medium (containing only colistin) were mixed with an equal volume of blank plasma in 1.5 mL PP micro test tubes from Sarstedt to avoid adsorption to the tube material and stored at −20 °C.

2.5. Sample preparation

The samples were thawed quickly in cold water in batches of not more than 12 samples. For the sample work-up, the 1.5 mL PP micro test tubes from Sarstedt were used.

A volume of 100 μL plasma or medium–plasma mix were precipitated with 200 μL ACN containing 0.1% TFA. The samples were vortex-mixed for 10 s and centrifuged for 5 min at 10,000 rpm

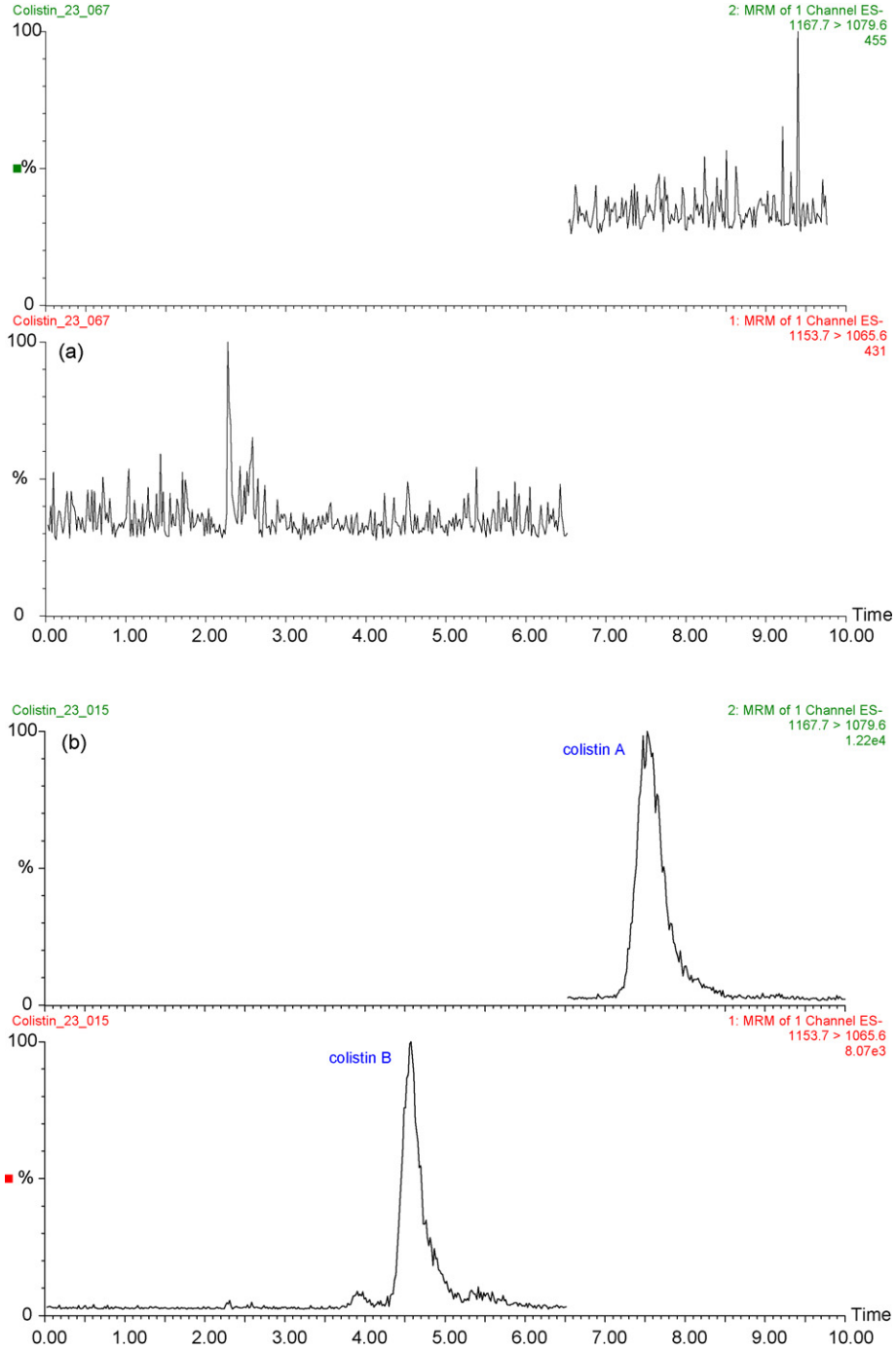

Fig. 2. Chromatograms of a blank plasma sample (a), a spiked plasma sample containing 193.6 ng/mL colistin A and 105.2 ng/mL colistin B (b), and a plasma sample obtained 30 min after intravenous administration of the first dose of 3 million Units CMS, where the found concentrations of colistin A and B were 67.94 and 22.36 ng/mL, respectively (c).

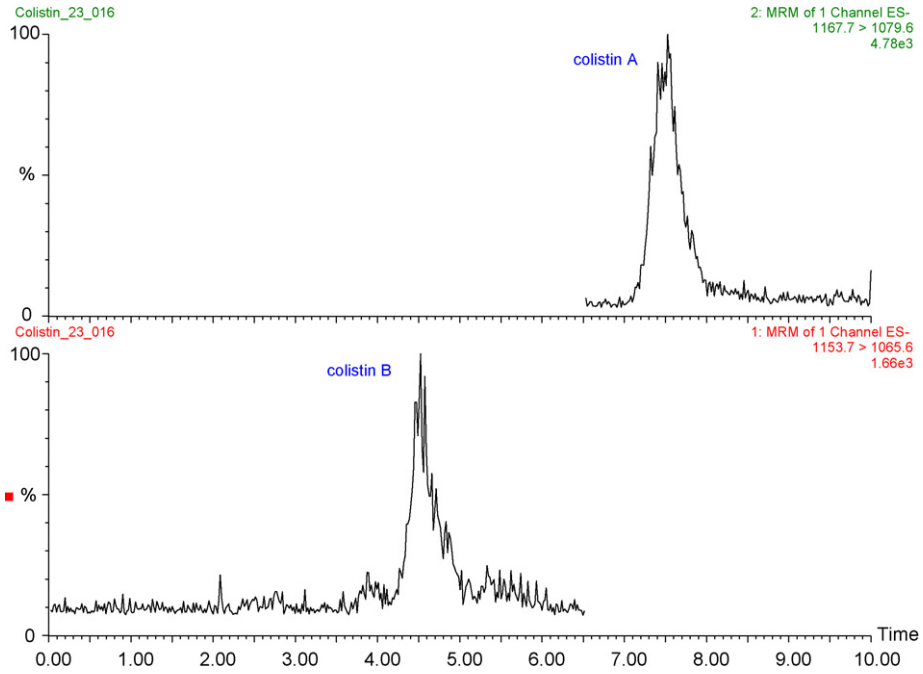

Fig. 2. (*Continued*)

(7200 × *g*) using a Force 7 centrifuge (Denver Instrument Company, USA).

Clean tubes were filled with 300 μL of 0.03% TFA before 200 μL of the supernatants were added. After vortex-mixing the samples were transferred to auto injector vials of PP and stored at 4 °C before injection.

2.6. Method validation

Calibration curves were constructed by linear regression of the peak areas (y) versus the added concentrations (x) with a weighting factor of $1/y$. The curves were not forced through the origin. The concentrations of QCs and unknown samples were calculated from the regression equations.

The intra-day precision and accuracy were determined by analyzing QC samples ($n = 6$) at each of the three concentrations during 1 day. The inter-day precision and accuracy were determined by analyzing the QCs in duplicate interspersed with unknown study samples at seven occasions. The precision was expressed as the coefficient of variation (CV) by calculating the standard deviation as a percentage of the mean concentration. Accuracy was determined as the percent deviation of analyzed concentration from the added concentration. The lower limit of quantification (LLOQ) was determined from the lowest concentration of samples ($n = 6$) that could be analyzed with CV < 15% and accuracy < ±15%. The extraction recovery was determined by comparing the slopes from a standard curve diluted in worked-up blank plasma (direct injection) with a standard curve in plasma subjected to the precipitating procedure. To measure the ion suppression caused by impurities in the precipitated plasma, the slopes from a standard curve in mobile phase and a curve diluted in worked-up blank plasma were compared.

2.7. Stability of colistin and CMS

2.7.1. Blood

EDTA blood was collected from two healthy volunteers. The blood was incubated at 37 °C before colistin and CMS were added to separate batches to obtain concentrations of 1494 ng/mL colistin and 3500 ng/mL CMS. After storage on ice-bath at 0 °C, in refrigerator at 4 °C and in room temperature at 22 °C for up to 60 min, the samples (in triplicate) were centrifuged for 5 min at 3000 rpm. The plasma was transferred to clean tubes and immediately stored in freezer until assay. As reference, the samples stored on ice-bath for 5 min were used.

2.7.2. Plasma

Blank human plasma was spiked resulting in concentrations of 747 ng/mL colistin and 2485 ng/mL CMS in separate batches. The samples were incubated up to 180 min at 4, 22 and 37 °C and thereafter stored in freezer until assay.

2.7.3. Culture medium

Blank medium–plasma mix was spiked with colistin to a medium concentration of 747 ng/mL. The samples were incubated up to 180 min at 4, 22 and 37 °C and thereafter stored in freezer until assay.

2.7.4. Plasma freeze/thaw

Plasma with colistin concentrations of 37.35, 373.5 and 3735 ng/mL and plasma with CMS concentrations of 350 and 3500 ng/mL were frozen at −20 °C for about 30 min and thereafter thawed in cold water for 5 min. The thawed samples were stored at room temperature for 10 min before refreezing. Three cycles were tested.

2.7.5. Stability of worked-up samples

Four study samples, collected 15–60 min after dose 1 ($n = 2$), dose 4 and dose 6 of 3 million Units CMS (Colistin, Norma, Greece) administration, were worked-up and placed in the refrigerated autosampler at 4 °C. Each sample was injected six times during 5 h.

2.8. Determination of CMS

CMS and partially sulfomethylated derivatives in plasma were hydrolyzed to colistin by using the method described by Li et al. [11] with some modifications. A volume of 20 μL sulphuric acid (1 M) was mixed with 150 μL plasma. After 15–20 min, 40 μL

sodium hydroxide (1 M) was added. Colistin standards and CMS controls were treated the same. Thereafter, the samples were precipitated with 400 μL ACN containing 0.1% TFA and 200 μL of the supernatants were diluted with 300 μL of 0.03% TFA before assay of the total colistin concentration. By subtraction of the colistin determined in the samples before hydrolysis from the colistin determined after the hydrolysis the CMS concentration could be calculated. Colistin standards were used for the quantification in ranges of 19.36–9680 and 10.52–5261 ng/mL for colistin A and B, respectively. Plasma containing CMS sodium in the range of 1230–18,450 ng/mL controlled the hydrolysis step.

3. Results and discussion

3.1. Analytical procedure

3.1.1. Analytical column

The Ultrasphere C18 column was primarily selected for running a UV method [7], but it was also shown to be suitable for the current method due to good peak performance and robustness, where the latter is of great importance when running a large number of samples from clinical studies.

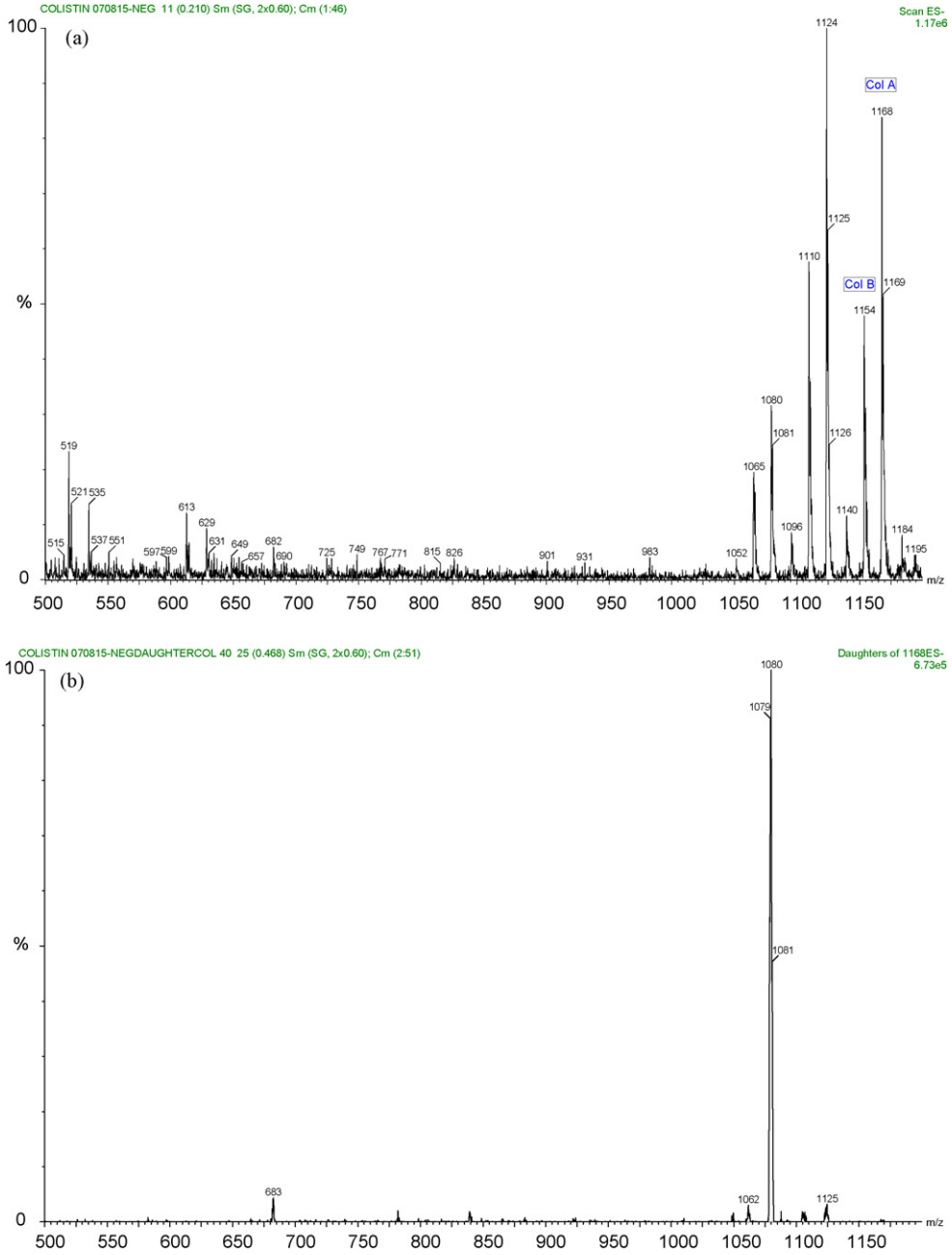

Fig. 3. Percursor ion mass spectrum of $[M-H]^-$ ions of colistin (a), product ion mass spectra of $[M-H]^-$ ions of colistin A at *m/z* 1168 (b), and $[M-H]^-$ ions of colistin B at *m/z* 1154 (c).

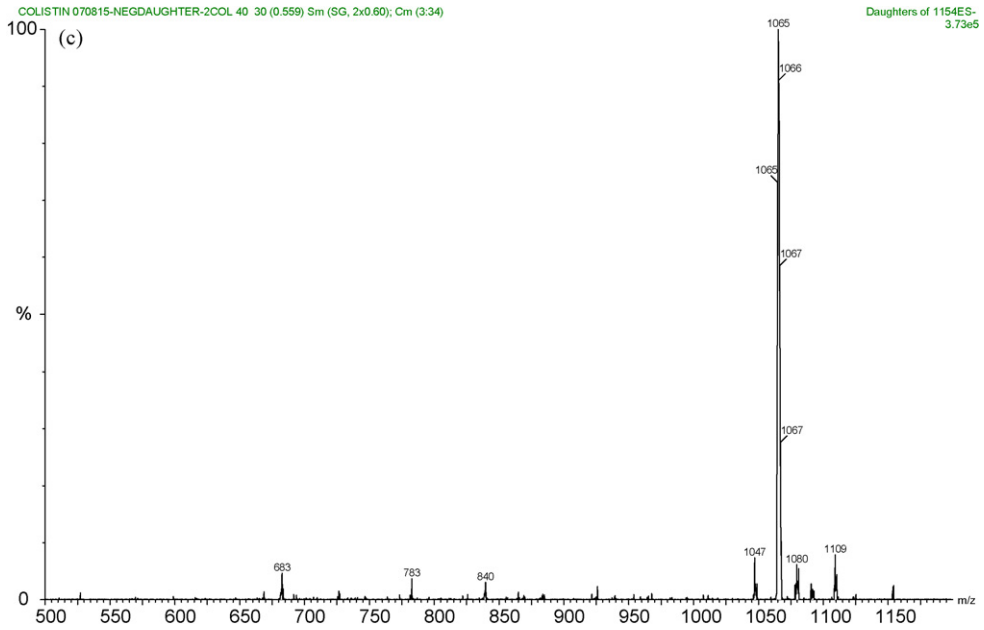

Fig. 3. (*Continued*)

3.1.2. Mobile phase

The selected mobile phase (25% ACN in 0.03% TFA) [5] resulted in separation between the front and the analytes, as well as separation between colistin A and B. The percentage of ACN and TFA were optimized to meet the requirements of the method for separation, satisfactory peak performance and high sensitivity. To get the highest possible sensitivity the TFA content had to be optimized. Evaluation of 0.02, 0.03, 0.04 and 0.08% TFA showed higher response the lower the percentage, as expected, but the peaks were tailing using 0.02%. A TFA concentration of 0.03% was therefore the best choice when taking into account both peak shape and response. The content of ACN (25%) was chosen to minimize ion suppression caused by the impurities in the precipitated samples. The retention times became 4.6 min for colistin B and 7.5 min for colistin A. Gradient runs were tested, but did not improve sensitivity, peak performance or run time.

No matrix effects of the impurities in plasma could be seen after the first 3 min (Fig. 2(a)). Chromatograms of a blank plasma sample, a spiked plasma sample, and a sample from a patient obtained 30 min after administration of the first dose of 3 million Units CMS, are presented in Fig. 2.

3.1.3. Mass spectrometry

The theoretical masses are 1155.76 Da for colistin B and 1169.77 Da for colistin A [7]. In full mass spectrum the molecules appeared single charged in negative ion mode when using TFA in the mobile phase (Fig. 3(a)). For both parent ions only one major daughter ion was seen after the fragmentation, and this improves the sensitivity (Fig. 3(b) and (c)). The response became also higher when detecting the two transitions in different time windows (Fig. 2). The chosen parameters gave the highest response for both colistin A and colistin B.

3.1.4. Sample preparation

One of the aims was to shorten the sample preparation time and thereby avoid hydrolysis of the prodrug CMS in clinical samples. Precipitation with ACN was the first choice but the peak areas became very small. A mix of 10% trichloroacetic acid (TCA) and methanol (50:50) [7,9] resulted in larger areas but the peaks were both fronting and tailing. By addition of TFA to ACN, the concentration of acid needed, for breaking the colistin–protein bonds, became as low as 0.1%. The supernatants were diluted to get an ACN content of about 25% matching the content in the mobile phase and to decrease the concentration of TFA to avoid hydrolysis of CMS. The sample work-up was performed at room temperature (<23 °C) and after thawing for 5 min in cold water, a batch of 12 samples could be proceeded within 10–15 min.

Table 1
Intra-and inter-day precision and accuracy of colistin A and colistin B in spiked human plasma samples.

Compound	Added conc., ng/mL	Intra-day				Inter-day			
		Found conc., ng/mL	CV, %	Accuracy, %	*n*	Found conc., ng/mL	CV, %	Accuracy, %	*n*
Colistin A	1651	1707	3.8	3.4	6	1655	3.5	0.2	13[a]
	550.2	610.8	4.2	11.0	6	548.1	3.1	−0.4	14
	165.1	164.0	4.4	-0.7	6	162.0	3.4	−1.9	14
	19.36	21.80	4.2	12.6	6				
Colistin B	897.0	918.3	3.5	2.4	6	895.5	3.3	−0.2	13[a]
	299.0	320.7	4.4	7.3	6	298.5	3.8	−0.2	14
	89.70	89.87	4.9	0.2	6	89.26	4.4	−0.5	14
	10.52	11.18	6.2	6.3	6				

Intra-day: Six replicates of each concentration level were processed according to the method at the same occasion.
Inter-day: Three quality controls were analyzed in duplicate together with study samples at seven different days.
[a] One sample is missing due to injection failure.

Table 2
Intra-day precision and accuracy of colistin A and colistin B in spiked medium–plasma mix.

Compound	Added conc., ng/mL	Intra-day			
		Found conc., ng/mL	CV, %	Accuracy, %	*n*
Colistin A	693.3	716.4	3.6	3.3	6
	346.7	363.4	8.5	4.8	6
	69.33	67.66	7.7	−2.4	6
	24.20	26.16	11.4	8.1	6
Colistin B	376.7	387.1	3.2	2.8	6
	188.4	192.3	7.4	2.1	6
	37.67	37.14	4.9	−1.4	6
	13.15	13.57	5.5	3.2	6

Six replicates of each concentration level were processed according to the method at the same occasion. The concentrations in medium are given.

3.2. Validation

The method was found to be linear in the investigated ranges. The coefficient of determination was on no occasion less than 0.995 for both substances. The weighing factor $1/y$ was chosen to give more importance to the lower standard points and thereby improve the curve fit for the lower part of the calibration curve.

For plasma the LLOQ was 19.4 and 10.5 ng/mL for colistin A and B, respectively with CV <6.2% and accuracy < ±12.6% and for culture medium the LLOQ became 24.2 and 13.2 ng/mL for colistin A and B, respectively, with CV <11.4% and accuracy < ±8.1%. The intra-day CVs for the QC samples were for plasma <4.9% and the accuracy ranged from −0.7 to 11.0%. For culture medium the CVs were <8.5% and the accuracy ranged from −2.4 to 4.8%. For plasma the inter-day CVs were <4.4% and accuracy ranged from −1.9 to 0.2% (Tables 1 and 2).

The ion suppression was calculated by dividing the slope from the curve in worked-up blank plasma with the slope from the curve in mobile phase. The result showed a decrease of the peak areas with 3.0% and 9.5% for colistin A and B, respectively.

To be able to only measure the recovery of colistin in sample preparation, it was necessary to have the same matrix for both the reference curve and the plasma curve. By dividing the slope from the plasma curve with the slope from the reference curve the recoveries of colistin A and B became 91.4% and 86.5%, respectively. All curves were linear with regression coefficients not less than 0.999.

3.3. Stability

Degradation of CMS in blood at sampling and in plasma during work-up procedure have a significant effect on the determined concentration of colistin in samples where CMS concentrations are high and colistin concentrations are low, such as at early time points following intravenous administration of CMS. The tests show that both time and temperature are important factors for the stability of CMS and also CMS in blood is more sensitive to degradation compared to plasma. Probably the substances do not partition into the red blood cells since the measured plasma concentration of colistin became in average 3307 ng/mL (added 1494 ng/mL). The EVF (erythrocyte volume fraction) was measured for the pooled blood to 55% and the plasma concentration for CMS was estimated to 7778 ng/mL (added 3500 ng/mL). After 30 min at 22 °C 3.9% of the CMS in blood and 0.5% of the CMS in plasma were hydrolyzed to colistin. After incubation of the plasma for 30 min at 37 °C the degradation of CMS became 4%. Profiles for the degradation of CMS are shown in Fig. 4. It is to recommend that blood samples are directly chilled and thereafter centrifuged as soon as possible. CMS in plasma is more stable than CMS in blood especially if kept at 4 °C (Fig. 4). High room temperatures must be avoided during sample work-up when CMS is present in the samples. Colistin was stable in blood, plasma and medium–plasma mix for the investigated time periods and temperatures (data not shown).

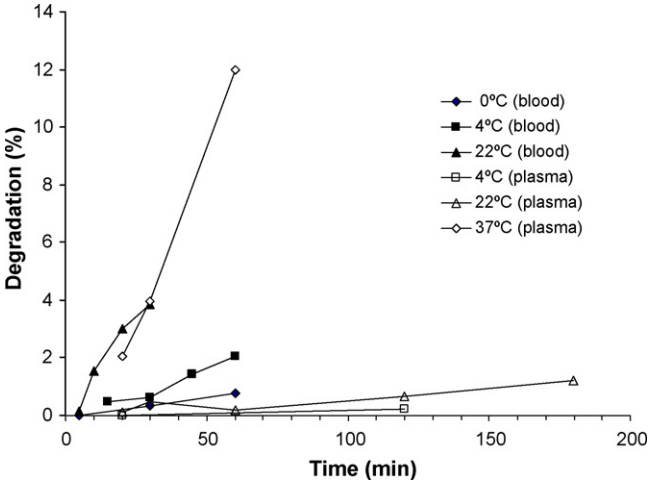

Fig. 4. Degradation of CMS in blood and plasma after storage at different temperatures.

The freeze–thaw experiment for CMS showed that the formed colistin was below LLOQ for all cycles. Colistin was also stable for at least three freeze–thaw cycles (Fig. 5).

The hydrolysis of CMS to colistin increases at low pH [11], therefore the precipitation step must be quick. After dilution with 0.03% TFA the pH is still low and to avoid degradation of CMS, the sam-

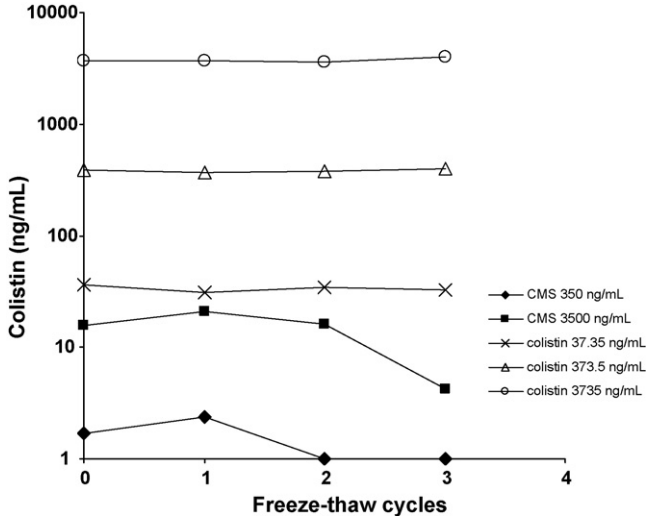

Fig. 5. Measured concentration of colistin after three freeze–thaw cycles of plasma spiked with CMS or colistin.

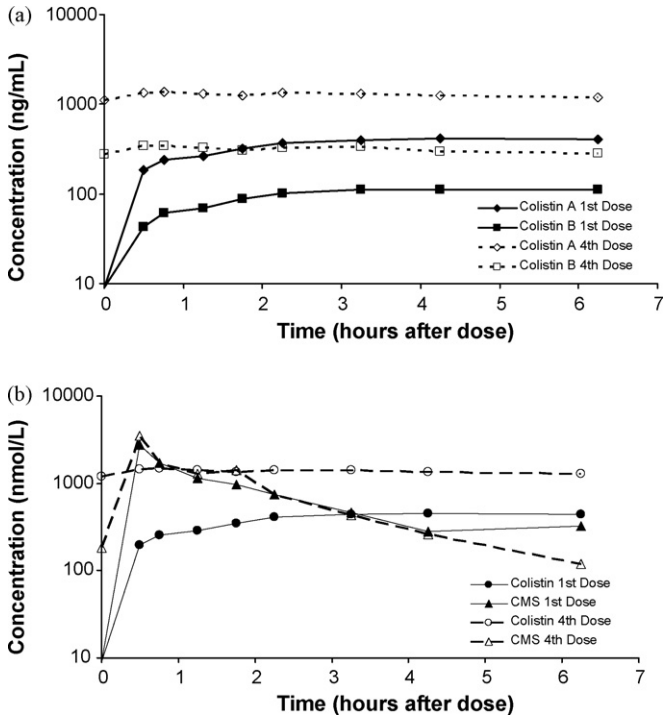

Fig. 6. Measured colistin A and colistin B concentrations versus time after the 1st and 4th dose (a) and measured total colistin and total CMS after the 1st and 4th dose (b) for a typical patient.

ples were stored at 4 °C in the refrigerator or the autosampler. At these conditions the worked-up samples were stable for 5 h (data not shown).

3.4. Study samples

The method described was used to analyze samples from clinical studies. Typical concentration versus time curves for colistin A and B after the first CMS dose of 3 million Units is presented in Fig. 6(a). Measured total colistin and CMS concentrations for one patient after the first and fourth dose are shown in Fig. 6(b).

4. Conclusions

The described method is sensitive and selective for the analysis of colistin in plasma as well as for culture medium. Only 100 μL plasma is required and the simple and rapid sample preparation method makes it possible to analyze samples from clinical studies without getting falsely high colistin from hydrolysis of CMS. The inter-day variability of the interspersed QC samples for plasma was low, which shows that the method is reliable and robust. For the culture medium, only 50 μL sample is needed for the assay. The fact that colistin is stable for at least 3 h at 37 °C allows for *in vitro* studies at physiological temperature. Important factors to take into account are the acid and thermo instability of CMS and the adsorption of colistin in water solutions to different materials used in the laboratory.

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