https://doi.org/10.36290/far.2023.016

Assay of colistin A, B and colistin methanesulfonate in human plasma by LC-MS/MS and short-term plasma stability

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A simple liquid chromatography tandem mass spectrometry (LC-MS/MS) assay for the determination of colistin A and colistin B in human plasma was developed and validated. Plasma extraction was performed using Oasis HLB 1 ml cartridges, analysis was performed using Arion® Polar C18 (250 × 4,6 mm; 5 mm) column at 35 °C. Mobile phases consisted of water containing 0,1% formic acid and methanol containing 0,1% formic acid (40 : 60, v/v) delivered at a flow rate of 0,8 ml/minute. Eluent was detected in the positive ion mode using electrospray ionization at the following transitions of mass to charge (m/z): colistin A 585,55 → 101,05; colistin B 578,5 → 101,15; and IS 602,4 → 101,1; 120,15; 86,15. Short-term stability tests of colistin and CMS were performed, at room temperature and 37 °C, where the stability of both components decreases with increasing temperature. The presented paper is part of the Pharmacokinetics of Colistin in Critically Ill Patients With Extracorporeal Membrane Oxygenation (COL-ECMO2022) study in which further results will be presented.

Key words: colistin, CMS, quantification methods, clinical samples, critically ill.

Stanovení kolistinu A, B a kolistin-methanesulfonátu v lidské plazmě pomocí LC-MS/MS a jejich krátkodobá stabilita v plazmě

Byla zavedena a validována jednoduchá metoda s použitím kapalinové chromatografie a tandemové hmotnostní spektrometrie (LC-MS/MS) pro stanovení kolistinu A a kolistinu B v plazmě. Extrakce proteinů z plazmy byla provedena pomocí 1 ml kazet Oasis HLB a chromatografická separace byla provedena na koloně Arion® Polar C18 (250 × 4,6 mm; 5 mm) při 35 °C. Mobilní fáze se skládala z vody obsahující 0,1 % kyseliny mravenčí a methanolu obsahujícího 0,1 % kyseliny mravenčí v poměru 40 : 60 (v/v), při průtoku 0,8 ml/minutu. Eluent byl detekován v režimu pozitivních iontů pomocí ionizace elektrosprejem s následujícími iontovými přechody m/z: kolistin A 585,55 → 101,05; kolistin B 578,5 → 101,15; a IS 602,4 → 101,1; 120,15; 86,15. Byly provedeny testy krátkodobé stability kolistinu a CMS, a to při pokojové teplotě a 37 °C, kdy se stabilita obou složek se zvyšující se teplotou snižuje. Předložený příspěvek je součástí studie Pharmacokinetics of Colistin in Critically Ill Patients With Extracorporeal Membrane Oxygenation (COL-ECMO2022), v níž budou prezentovány další výsledky.

Klíčová slova: kolistin, CMS, kvantifikační metody, klinické vzorky, kriticky nemocní.

Introduction

Colistin (COL), also known as polymyxin E, is classified as a polypeptide antibiotic produced by *Bacillus polymyxa*. Colistin is usually used as a last-resort antibiotic for the treatment of multidrug-resistant gram-negative infections (mainly *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*), especially where new antibiotics (ceftazidime/avibactam, meropenem/vaborbactam, plasmomycin) are not available or are not preferred (1, 2, 3, 4).

As a product of fermentation, colistin is a mixture of more than thirty components that are not in a constant ratio. Therefore, its

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Cit. zkr: Klin Farmakol Farm 2023;37(3):89-92
Článek přijat redakcí: 11. 8. 2023
Článek přijat k publikaci: 24. 8. 2023

molecular weight is not precisely determined. The main components of this mixture are colistin A and colistin B, whose molecular weights are 1169,5 and 1155,4, respectively (5, 6). The base of the colistin molecule consists of a decapeptide with seven amino acid residues in a cyclic formation and a fatty acid tail attached to the tripeptide end (4, 7). Due to the toxic side effects, colistin is used as an inactive prodrug, colistin methanesulphonate (CMS). CMS is hydrolyzed spontaneously in an aqueous solution *in vivo* and *in vitro* to a series of partially methanesulfonated derivatives and colistin (1, 8).

To find the most optimal conditions for the determination of CMS and colistin, various methods have been published that differ in sample preparation, analytical conditions, and measured concentrations in patients' plasma samples (8, 9). The method described below is based on already published and established methods, which we have adapted to our instrumental conditions to follow up with a study focusing on the pharmacokinetics of colistin used in critically ill patients. Colistin plasma concentrations in critically ill patients generally range from 0,6–13 mg/L (3).

Several pitfalls can be encountered in the determination of CMS and colistin. One problem is the adsorption of colistin to a range of materials, including plastics and glass in labware. If colistin adsorbs to materials used in sample collection, processing, or storage, its concentrations may be incorrectly evaluated. Another problem is the stability of both substances, CMS and colistin. The hydrolysis of CMS to colistin and colistin stability itself depends on concentration, time, temperature, and the matrix containing the substance (7, 9, 10).

Experimental

Chemical and reagents

Colistin sulfate, Colistinmethate Sodium and Polymyxin B sulfate (internal standard, IS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). MS grade water, Formic acid ≥ 99 %, and Sulphuric acid 96 % were purchased from VWR (Radnor, PA, USA). MS grade Methanol was purchased from J. T. Baker (Avantor, Gliwice, Poland), and Sodium hydroxide was obtained from Lach-Ner (Neratovice, Czech Republic).

Chromatographic conditions

The liquid chromatography was performed using the Prominence LC–20A HPLC system (Shimadzu, Kyoto, Japan), and an analytical column Arion® Polar C18 column (250 × 4,6 mm; 5 mm) purchased from Chromservis (Prague, Czech Republic) and tempered at 35 °C. The detector was a triple quadrupole mass spectrometer LCMS-8045 (Shimadzu, Kyoto, Japan) with electrospray ionization (ESI). The mobile phases consisted of water containing 0,1% formic acid and methanol containing 0,1% formic acid (40 : 60, v/v). The flow rate was 0,8 ml/minute. Ions were generated using electrospray ionization and detected in the positive ion mode at the following transitions of mass to charge (m/z): colistin A 585,55 → 101,05; colistin B 578,5 → 101,15; and IS 602,4 → 101,1; 120,15; 86,15. The total analysis time was 3 minutes and the LabSolutions software (ver. 5,93; Shimadzu, Kyoto, Japan) was used for instrument control, data acquisition, and processing.

Sample preparation

Colistin

The volume of 140 µl of human plasma was treated with 20 µl of a solution containing 0,1 mg/ml of IS. Further preparation of all samples was performed with Oasis HLB 1 ml cartridges with 30 mg of sorbent (Waters, Prague, Czech Republic). The SPE extraction consisted of conditioning of cartridges with 1 mL of methanol, equilibration of cartridges with 1 ml of water containing 0,1 % formic acid, 160 µl of sample loading, washing away of interferences with 1 ml of water containing 0,1 % formic acid, and finally eluting of COL and IS with 0,5 ml of methanol containing 0,1 % formic acid. An amount of 10 µl of the sample obtained from the elution step was injected into HPLC.

CMS

The volume of 140 µl of human blood plasma was treated with 20 µl of a solution containing 0,1 mg/ml of IS. Acid hydrolysis was performed by adding 15 µl of 1 M sulfuric acid to a plasma sample containing CMS. After 30 minutes, 30 µl of 1M sodium hydroxide was added to stop hydrolysis. The subsequent sample treatment was the same as for the colistin samples.

Validation

Calibration curves for colistin and CMS were constructed in the range of 0,15–30 mg/L, where each calibration point of the curve was measured at least six times. All points in the calibration series were used to determine the precision and accuracy of the inter-day (intraday) measurements. Samples at 2, 10, and 30 mg/L were prepared for intraday (inter-day) precision and accuracy measurements. Plasma samples with colistin and CMS concentrations of 0,07; 0,1; 0,15; 0,5; 1; and 2 mg/L were prepared for limit of quantification (LOQ) and limit of detection (LOD) determination.

Stability of COL and CMS samples testing

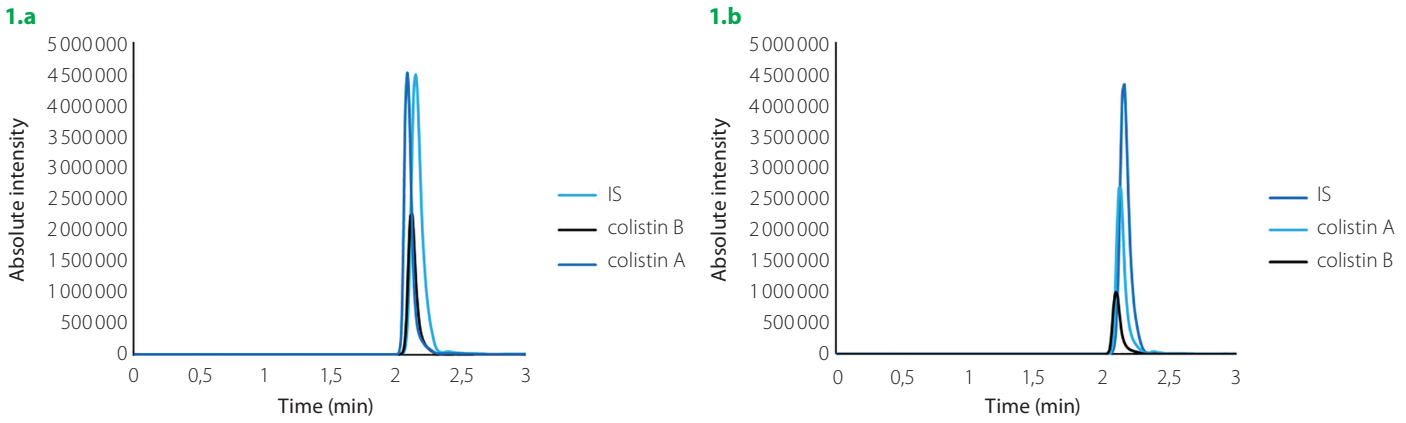
Studies for short-term, long-term, and freeze-thaw stability were also performed on the samples. The validation procedure was derived from the European Medicines Agency (EMA) recommendations. Control plasma samples from three female and three male donors obtained from the Transfusion Department of the Olomouc University Hospital were used for calibration and validation.

Results and discussion

No pure colistin A and B reference standards were available. Therefore, the pharmaceutical secondary standard of colistin sulfate was used for the method determination. The purity of the standard was determined by the manufacturer (11) to be 91 % by HPLC-UV analysis, which contained 30,46 % colistin A and 53,8 % colistin B.

As shown in Figure 1.a, the retention time of colistin A and B and IS are 2,11; 2,09, and 2,1 minutes, respectively. Calibration curves were constructed in the 0,15–30 mg/L range. Plotting the peak area versus concentration, linear calibration curves were obtained with a confidence value of $R^2 = 0,9997$ for colistin A and $R^2 = 0,9989$ for colistin B. In the case of hydrolysed CMS to COL, the confidence value

Fig. 1. *Representative chromatograms of calibration point 10 mg/L (1.a) and patient sample (1.b)*



of R^2 = 0,9995 for colistin A and R^2 = 0,9982 for colistin B. The results of the inter-day and intraday measurements for method validation are accurate and precise, with an error not exceeding 15 % with the LOQ set at 0,15 mg/L. The validation confirmed the reliability of the LC-MS method for measuring concentrations of colistin in human plasma. Summary information on the analysis parameters is given in Table 1.

The CMS concentration was measured indirectly by acid hydrolysis, for which we found it most useful to use 15 µl of 1 M sulfuric acid. After 30 minutes, the hydrolysis was stopped by adding 30 µl of 1M sodium hydroxide. As this is an indirect method, it is necessary to back-calculate the CMS concentrations from the difference:

$$CMS = COL_{total} - COL_{before\ hydrolysis}$$

where COL_{total} is the concentration after hydrolysis of CMS to colistin and $COL_{before\ hydrolysis}$ is the circulating concentration of colistin formed by endogenous transformation of the prodrug to its active form.

Long-term stability studies of colistin showed no degradation in stock solutions and patient plasma samples stored at –70 °C for at least 90 days. Also, no degradation was observed in three freeze-thaw cycles (data not shown). The short-term stability of colistin was measured for three concentration points at room temperature (RT) and 37 °C (Table 2). After 24 hours at RT, the degradation of the samples reached almost 10 %. However, the degradation of colistin was more significant at 37 °C. Already after 30 minutes, degradation reaching up to 25 % was observed, the average degradation for all samples was 11 %.

Tab. 1. *Summary information on the analysis parameters*

Chromatographic conditions		
HPLC:	Shimadzu, Prominence LC–20A	
Column:	Arion® Polar C18 (250 × 4,6 mm; 5 mm)	
Mobile phase A: Mobile phase B:	0,1% formic acid in water 0,1% formic acid in methanol	(40:60, v/v)
Flow rate:	0,8 ml/minute	
Column temperature:	35 °C	
The volume of injection:	10 µl	
Analysis time:	3 minutes	
MS/MS detection		
Mass spectrometer:	Shimadzu, LCMS-8045	
Ionization mode:	ESI positive	
Ion transition monitored:	Colistin A 585,55 → 101,05	
	Colistin B 578,5 → 101,15	
	IS 602,4 → 101,1; 120,15; 86,15	
Validation parameters		
Calibration curve range (mg/l)	0,15–30	
Limit of quantification – LOQ (mg/l)	0,2	
Limit of detection – LOD (µg/l)	4,7	
Recovery (%)	81	

Tab. 2. *Stability of colistin A and B (%) at three concentration levels (2; 10; 20 mg/L) in human plasma*

		Colistin A			Colistin B		
	Time	**2 mg/L**	**10 mg/L**	**20 mg/L**	**2 mg/L**	**10 mg/L**	**20 mg/L**
RT	0,5 h	99,28	101,28	100,18	107,01	105,14	103,41
	1 h	98,90	97,86	99,05	92,93	97,28	104,80
	2 h	96,92	97,66	96,08	92,15	95,31	99,89
	5 h	93,28	92,16	95,64	92,22	94,15	96,57
	24 h	90,57	92,71	91,37	90,15	93,93	96,46
37 °C	30 min	91,65	92,76	91,21	74,96	92,76	92,17

RT – room temperature

Tab. 3. *Stability of CMS (%) at 10 mg/L concentration level in human plasma*

	37 °C		**RT °C**	
Time	COL A	COL B	COL A	COL B
0,5 h	**103,7**	**103,1**	**100,1**	**100,6**
1 h	**108,0**	**109,2**	**102,0**	**105,6**
2 h	**114,0**	**116,3**	**108,1**	**110,3**
3 h	**117,6**	**119,9**	**111,7**	**113,8**

RT – room temperature

CMS stability in plasma samples at a selected concentration of 10mg/L was studied at room temperature and 37 °C. As expected, the conversion of CMS to colistin is more significant at 37 °C than at RT. At elevated temperatures, we observe the conversion of CMS into its colistin

components after 30 min. After 3 hours, the average conversion of CMS is about 13% at RT and 19% at 37 °C (Table 3). The stability of CMS in a matrix other than plasma was not tested. However, as there is evidence of lower stability of CMS in aqueous solutions or infusion solutions, this stability needs to be studied more closely before further measurement (7, 12).

This analytical method for determining both COL and CMS in plasma samples is applied in the ongoing phase IV clinical trial „Pharmacokinetics of Colistin in Critically Ill Patients With Extracorporeal Membrane Oxygenation (COL-ECMO2022)" (EudraCT Number 2022-000291-19; NCT05542446) (13, 14). This study is designed to assess the influence of extracorporeal membrane oxygenation on the pharmacokinetics of colistin and CMS.

Conclusion

A rapid method for measuring colistin in human plasma has been introduced and validated. The described method is sensitive and selective for the analysis of colistin in plasma and it was applied to the measurement of patient samples. The presented paper is part of the Pharmacokinetics of Colistin in Critically Ill Patients With Extracorporeal Membrane Oxygenation (COL-ECMO2022) study in which further results will be presented. The study has been approved by the Ethics Committee of St. Anne's University Hospital Brno (Number 10ML/2022-AM). EudraCT Number of the study is 2022-000291-19, registered on June 21, 2022. The study was registered at the Clinical Trials register https://clinicaltrials.gov/ct2/show/NCT05542446 on September 15, 2022.

Podpořeno ze státního rozpočtu prostřednictvím MŠMT projektem VVI CZECRIN (LM2023049) a IGA_LF_2023_004.

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