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