International Journal of Chemical, Materials and Biomolecular Sciences
ISSN: 2415-6620
Vol:7, No:4, 2013

High Performance Liquid Chromatographic Method for Determination of Colistin Sulfate and its Application in Medicated Premix and Animal Feed

S. Choosakoonkriang, S. Supaluknari and P. Puangkaew

***Abstract*—**The aim of the present study was to develop and validate an inexpensive and simple high performance liquid chromatographic (HPLC) method for the determination of colistin sulfate. Separation of colistin sulfate was achieved on a ZORBAX Eclipse XDB-C18 column using UV detection at λ=215 nm. The mobile phase was 30 mM sulfate buffer (pH 2.5): acetonitrile (76:24). An excellent linearity (r^2=0.998) was found in the concentration range of 25 - 400 µg/mL. Intra- day and inter-day precisions of method (%RSD, n=3) were less than 7.9%. The developed and validated method was applied to determination of the content of colistin sulfate in medicated premix and animal feed sample. The recovery of colistin from animal feed was satisfactorily ranged from 90.92 to 93.77%. The results demonstrated that the HPLC method developed in this work is appropriate for direct determination of colistin sulfate in commercial medicated premixes and animal feed.

***Keywords*—**Colistin sulfate, HPLC, medicated premix, animal feed.

I. Introduction

COLISTIN is an important member of the polymyxin group of cationic peptide antibiotics. The compound was isolated from *Bacillus polyrnyxa* subspecies *colistinus*. The amino acid components in the colistin molecule are leucine, threonine, and diaminobutyric acid. Five of the diaminobutyric acid residues are free amines which are positively charged in physiologic conditions. Colistin comprises a cyclic heptapeptide and side chain of three amino acids that acylated at the N terminus by a fatty acid (Fig.1). It is a complex mixture of at least 13 different components [1]. The two main components are colistin A (polymyxin El) and colistin B (polymyxin E2), which contain the same amino acids but different fatty acids (6-methyloctanoic acid and 6-methylheptanoic acid respectively) [2].

S. Choosakoonkriang is with the Department of Chemistry , Faculty of Science, Silpakorn University, Nakorn Pathom 73000 Thailand (e-mail: siririrat@su.ac.th).

S. Supaluknari is with the Department of Chemistry , Faculty of Science, Silpakorn University, Nakorn Pathom 73000 Thailand (e-mail: supachai@su.ac.th).

P. Puangkaew is with the Department of Chemistry , Faculty of Science, Silpakorn University, Nakorn Pathom 73000 Thailand (corresponding author to provide phone: +66-89-5515441; fax: +66-34-271356; e-mail: nuoff59@hotmail.com).

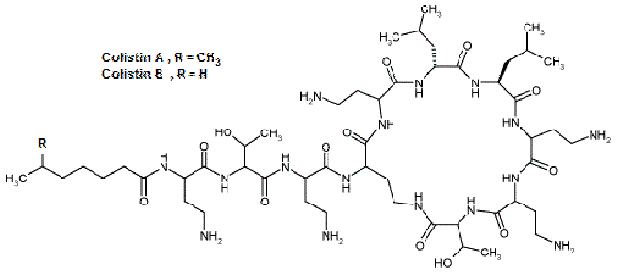

Fig. 1 Structure of colistin

This antibiotic is used in veterinary therapy as drug against certain infectious diseases caused by Gram-negative bacterias in rabbits, pigs, cattle, sheep and goat. In veterinary medicine, it is normally used as sulfate salt for treatment of intestinal infections [3]. Diseases in animals are usually treated by adding the drug (liquid or solid medicated premix) to animal feed. However, if it is used in a high concentration, colistin sulfate will be toxic to animals. The residues of colistin A and colistin B in meat product can be absorbed by human through the food chain and be harmful for human consumption. Colistin was abandoned during the last two decades because of its toxicity. There was concern regarding its nephrotoxicity. The rate of nephrotoxicity of colistin in recent studies was highly variable, ranging from 0% to 37%. To safeguard public health, national health authorities worldwide have established respective maximum residue limits (MRLs) in animal milk and tissues for regulating the use of colistin [4]. European authorities pressurize to check homogeneity, stability and storability of the antimicrobial formulations both in the medicated premixes and the medicated feed. In Thailand, the residual veterinary drugs in animal products are controlled under the notification of Department of Livestock Development (No.2/2) on 18 May, 2001. MRLs for colistin in egg and animal tissues were set at 300 µg/kg and 150–200 µg/kg, respectively [5]. Thus, this antibiotic has been measured in pharmaceutical formulations and animal feed [6]. Several techniques have been described for the quantification of colistin. The simple microbiological method has been widely used for monitoring of colistin concentration. Unfortunately, this method was low sensitivity and time consuming [7]. Different methods for colistin sulfate analysis

are paper chromatography [8], thin layer chromatography [9], isotachophoresis [10], electrophoresis [11] and liquid chromatography [1], [12]. The liquid chromatographic techniques were mainly applied for the separation of colistin components. Orwa et al. [1] could successfully separate the major components (colistin A and colistin B) and many minor components on a YMCPack Pro, C-18 column. The simple method of colistin analysis was detected by UV detection at a wavelength of 215 nm. The mobile phase used was acetonitrile - sodium sulphate (0.7% *m/v)* - phosphoric acid (6.8% *v/v*) –water (21.5:50:5:23.5) at the flow rate of 1.0 mL/min. Cancho-Grand et al. [12] reported the utilization of solid phase extraction (SPE) for cleaning up protein and amino acid contents in feed samples. A commercial medicated feed was used to evaluate the efficiency for colistin sulfate determination. The result showed that the proposed sample treatment of this method was selective because it was free of matrix interferences. Li et al. [13] developed and validated a novel HPLC method with fluorimetric detection for the assay of colistin in human plasma. Colistin was extracted from human plasma onto a SPE C18 cartridge and the compound was derivatized in the same cartridge with 9-fluoranylmethyl chloroformate (FMOC-Cl). This is the first report using FMOC-Cl as derivatizing reagent for determination of colistin. Cheng et al. [14] avoided using derivatization and SPE process. Trichloroacetic acid (TCA) was used as protein precipitating reagent. The effect of TCA concentration on plasma protein precipitation was studied. It found that the TCA sample precipitation method gave an acceptable sample recovery for colistin from plasma. This method was accurate, sensitive and precise. Sin et al. [15] developed a method for the determination of colistin in bovine milk samples using electrospray LC–MS/MS with time scheduled multiple reaction monitoring (MRM) for detection. Bovine milk samples were deproteinized and extracted by a mixture of trichloroacetic–formic acid. The results showed that LC-MS/MS provided a sensitive signal for detection of colistin in biological matrices. From these published methods, the HPLC technique was used for analysis of colistin but this compound was detecting by different methods such as UV detection, fluorimetric detection or mass spectrometric detection. The HPLC method with fluorimetric detection requires additional derivatization process with the possibility of poor recovery and reproducibility. The LC–MS/MS has higher detection sensitivity but it is expensive instrument. The HPLC-UV is simple and economical instrument for measuring drug concentration. Thus, the purpose of the present study was to develop and validate an inexpensive and simple method for determination of colistin sulfate by UV detection. Gentamicin sulfate was used as internal standard to correct for errors that may be caused by instrumental variations. Validation of the method included linearity, sensitivity, repeatability, and accuracy test. The validated method was applied to quantitative analysis of colistin sulfate in medicated premixes and animal feeds for routine analysis in veterinary pharmaceutical company. The ultrasonic-assisted extraction, protein precipitation and SPE method were used for optimized sample preparation method.

II. Materials and Methods

A. Chemicals and Materials

Colistin sulfate standard was obtained from Thai Meiji pharmaceutical Co., Ltd. (Bangkok, Thailand). Gentamicin sulfate in a commercial medicated injection form (used as an internal standard) was purchased from General drug house Co., Ltd. (Bangkok, Thailand). Acetonitrile HPLC Grade was from SIGMA-ALDRICH. The deionized (DI) water was used in sample preparation. Anhydrous sodium sulphate, phosphoric acid and all other chemicals used were of analytical grade. Solid feed premixes were purchased from a commercial veterinary retailer. Animal feed samples were supported by Thai Meiji pharmaceutical Co., Ltd. (Bangkok, Thailand). Prepacked cartridges (Sep-Pak plus 360 mg C18 chemically bonded silica) for solid phase extraction were purchased from Waters (Milford, MA, USA).

B. HPLC System and Chromatographic Conditions

Chromatographic analysis was carried out on a HPLC system equipped with Waters delta 600 controller, Waters 600E pump, Waters 2487 dual wavelength absorbance detector and Empowers software, all from Waters (Milford, MA, U.S.A.). The HPLC separation of the colistin A and colistin B was performed on a Zorbax Eclipse XDB C18 column (5 μm, 150 x 4.6 mm ID). The guard column was KJ0-4282 cartridge system (Phenomenex, USA). The isocratic elution program was used and the mobile phase was 30 mM Na_2SO_4 buffer solution (adjusted to pH 2.5 with H_3PO_4): acetonitrile (76:24 % v/v) at the flow rate of 1.0 ml/min. The mobile phase was prepared freshly every day and was filtered through a 0.45 μm membrane filter to remove any particulate matter. The UV detection was performed at a wavelength of 215 nm and the injection volume was 20 μL. Prior to injecting solutions, the column was equilibrated by flowing the mobile phase through the system at least for 15 min. All separations were performed at room temperature.

C. Preparation of Stock and Standard Solutions

The stock standard solution of colistin sulfate was prepared in acetronitrile:DI water (20:80). Working standard solutions, containing a fixed concentration of the internal standard (gentamicin sulfate), were prepared by diluting the solution with acetronitrile:DI water (20:80) to the concentrations of 25.00, 50.00, 80.00, 100.00, 150.00, 200.00 and 400.00 mg/L

D. Validation of the Method

Seven series of standard calibration solutions in the range of 25 – 400 mg/L were prepared and analyzed as described above. A calibration curve can be established by plotting the ratio of peak areas of colistin A and colistin B to the peak area of gentamicin *versus* the concentration of colistin.

The limit of detection (LOD) and quantification (LOQ) was determined by measuring the signal of colistin sulfate standard solution at a concentration of 5.00 mg/ L (n=10). The limit of

detection (LOD) was defined as the lowest analyte concentration yielding a signal-to-noise (S/N) ratio of 3. The limit of quantification (LOQ) was defined as the lowest analyte concentration yielding a signal-to-noise (S/N) ratio of 10.

The intra-day and inter-day accuracy and precision of the method were evaluated using colistin sulfate synthetic solution at concentration of 25, 150 and 400 mg/L. The colistin sulfate synthetic solutions were prepared by appropriately adding the standard solution of colistin sulfate to blank solution (acetronitrile: DI water, 20:80).

E. Application of the Method in Medicated Premix and Animal Feed

Representative portions of about 0.1-0.5 g feed premix sample were added with 30 mL of acetronitrile:DI water (20:80). The mixtures were sonicated in an ultrasonic bath for 30 min and stirred for 30 min at room temperature. Then the homogenates were made up to 50 mL with acetronitrile:DI water (20:80). The obtained solution was added with gentamicin sulfate (internal standard). The final solutions were filtered through a 0.45 μm nylon membrane filter before HPLC analysis.

The animal feed samples were homogenized by using a blender. In order to extract the colistin sulfate from animal feed samples, representative portions of about 2 g animal feed sample were added with 10 mL of 0.1M hydrochloric acid. The mixtures were sonicated in an ultrasonic bath for 60 min and shaken for 30 min at room temperature. The homogenates were centrifuged at 3300 rpm, 4 ºC for 20 min. The extraction was repeated two times. All the supernatants were combined. Clear supernatants were not directly injected to HPLC due to interference caused by the high level of proteins in the animal feed samples. Clear supernatants were added with 5 ml of acidic acetronitrile, acetronitrile:0.1 M HCl (90:10). Then, the mixtures were centrifuged at 3300 rpm, 4 ºC for 10 min. The obtained supernatants were separated for the clean-up by solid phase extraction (SPE). Clean-up and preconcentration process were performed using C18 Sep-Pak cartridges. The SPE cartridges were conditioned by washing 5 mL of methanol and followed by 5 mL of DI water. The final supernatants were applied to the cartridges. Then, the columns were washed with 3 mL of 0.1 M HCl. The compounds of interest were eluted with 2 mL methanol. The eluates were evaporated under a gentle stream of nitrogen for 10 min and the concentrates were made up with 0.5 mL of gentamicin sulfate solution (internal standard) at a final concentration of 80.00 mg/L. Then, the obtained sample solutions were filtered through a 0.45 μm nylon membrane filter before HPLC analysis.

III. Results and Discussion

A. The Chromatographic Separation

The separation mechanism in reversed phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the stationary phase. Colistin are basic, hydrophilic molecules containing numerous amino groups with p*K*a values ranging between 7.0 and 10.0 leading to the possible use of reversed phase with mobile phase at low pH. In this work, the different mixtures of 30 mM Na_2SO_4 buffer solution (pH 2.5)/ acetonitrile were evaluated. The optimum elution performance for resolution of the two colistin peaks was obtained under the proposed isocratic conditions. Gradient elution was tested, but did not improve sensitivity, peak separation and run time.

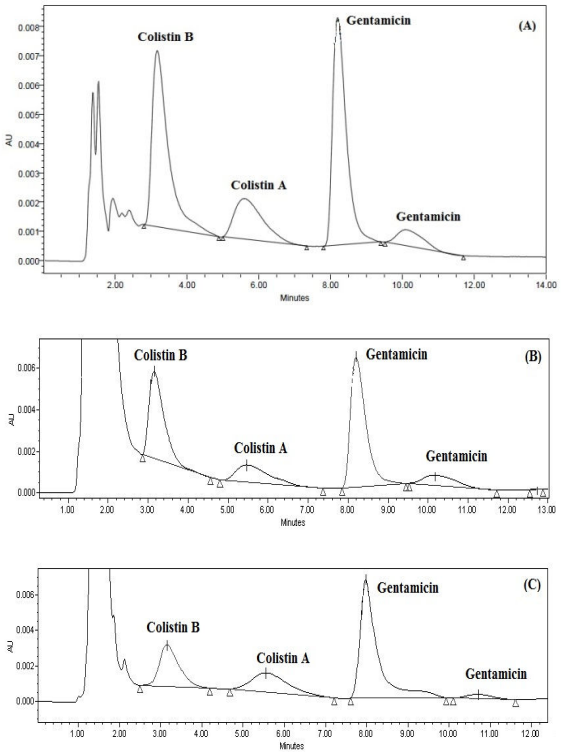

Fig. 2 HPLC chromatograms of colistin standard at concentration of 100 mg/L (A), commercial medicated premix sample (B) and animal feed sample spiked with 10 mg of colistin sulfate in100mg of feed sample (C). HPLC operating conditions: a Zorbax Eclipse XDB C18 column (150 x 4.6 mm ID) eluted with 30 mM Na_2SO_4 buffer solution (pH 2.5): acetonitrile (76: 24 % v/v) at a flow rate of 1.0 mL/min and UV detection at a wavelength of 215 nm. Colistin A, t_r = 5.5 min; colistin B, t_r = 3.1 min; gentamicin, t_r = 8.1 and 10.0 min

Chromatograms of a colistin standard, a commercial medicated premix and a sample of animal feed were presented in Fig. 2. The chromatograms exhibited well resolved peaks of colistin and gentamicin. The peaks of colistin B and colistin A appeared at the retention times of 3.1 min and 5.5 min. The colistin B was eluted first since it had less hydrophobic moiety of fatty acids than the colistin A. The internal standard, gentamicin was detected as two components at the retention times of 8.1 min and 10.0 min.

B. Validation of the Method

The chromatographic responses were found to be linear over an analytical range of 25-400 mg/L. A good linearity (r^2=0.998) of the regression line was obtained for the graph of seven points with the equation: y = 0.015x-0.166. The limit of detection and limit of quantification were 9.46 and 13.93 mg/L respectively. The accuracy (%recovery) and precision (%RSD) of the method used were studied and the results were reported in Table I.

TABLE I
ACCURACY AND PRECISION OF THE METHOD

intra-day(n=3)			
Colistin sulfate solutions (mg/L)	Found (Mean±SD) (mg/L)	%RSD	% recovery
25	23.98±1.89	7.90	95.92
150	146.06±5.77	3.95	97.37
400	461.01±4.71	1.02	115.25
inter-day(n=9)			
Colistin sulfate solutions (mg/L)	Found (Mean±SD) (mg/L)	%RSD	% recovery
25	23.25±1.34	5.77	92.98
150	153.81±9.20	5.98	102.54
400	447.95±6.98	1.56	111.99

C. Analysis of Commercial Medicated Premix Samples and Animal Feed

For the successful determination of colistin sulfate in animal feed, sample pretreatment prior to HPLC analysis is generally required. It is well known that animal feed samples have matrix interferences for the chromatographic separation of analyte. In this study, ultrasonic solvent extraction and protein precipitation combining with SPE clean-up was used for sample pretreatment process. The chromatograms (Fig. 2(B) and 2(C)) obtained for medicated premix and animal feed analysis indicated that the proposed sample pretreatment method was selective because it was free of matrix interferences.

The method was then applied to the determination of colistin in the commercial medicated premix and animal feed samples. Triplicate samples were analysed by the procedures described above. Each measurement was performed in triplicate injection. Recovery was performed to verify the effectiveness of the sample pretreatment process and the accuracy of the proposed method. The recovery of colistin sulfate from animal feed samples was satisfactorily ranged from 90.92 to 93.77%. RSD were less than 7.1% at all the concentration levels. The results were displayed in Table II. The recovery of colistin sulfate from commercial medicated premix samples was satisfactorily ranged from 94.50 to 109.90%. RSD were less than 5.0% at all samples, as reported in Table III.

TABLE II
AMOUNTS OF COLISTIN
IN ANIMAL FEED SAMPLES (n=3).

sample	Labeled amount (%w/w)	Colistin found (%w/w)	%REC	%RSD	t_{cal}
1	0.0080	0.0074	92.50	7.06	1.99
2	0.0150	0.0141	93.77	5.53	2.00
3	0.0200	0.0182	90.92	2.35	3.76
4	10	9.47	93.68	2.48	3.91
5	40	36.60	91.51	4.27	3.77

%REC = %recovery

TABLE III
AMOUNTS OF COLISTIN
IN COMMERCIAL MEDICATED PREMIX (n=3).

Sample	Labeled amount (%w/w)	Colistin found (%w/w)	%REC	%RSD	t_{cal}
Nulistin - SP100	10	10.56	109.90	2.15	4.27
Octamix A.C.	2	1.89	94.50	3.53	2.86
Yenlistin 40%	40	41.33	107.82	1.46	3.82
Moxlistin	5	4.75	95.00	4.68	1.95

%REC = %recovery

The amounts of colistin found in the animal feed and commercial medicated premix samples were comparable to those labeled on the products by using a t-test. The test statistics calculated from the experimental values (t_{cal}) were reported in Table II and III. The critical t value at a significance level, α of 0.05 and 2 degree of freedom, $t_{0.975,2}$ = 4.30. Since the value of the test statistics is smaller than the critical t value, the test thus suggests that at 95% confidence level, no significant difference between the results of assay and the labeled amount.

IV. CONCLUSIONS

The results from this work demonstrated that the HPLC method developed is appropriate to the direct and rapid determination of colistin sulfate in commercial medicated premixes and animal feeds. The method used is simple, convenient and inexpensive. The repeatability and accuracy of method have been evaluated and found to be in acceptable ranges. This method can be applied to the determination of colistin sulfate in quality control laboratory of veterinary pharmaceutical company.

ACKNOWLEDGMENT

We thank Thai Meiji pharmaceutical Co., Ltd. for colistin sulfate standard and feed samples. We thank the Department of Chemistry, Faculty of Science, Silpakorn University and Development and Promotion of Science and Technology talents project (DPST) for financial support.

REFERENCES

[1] J. Orwa, A. Van Gerven, E. Roets, and J. Hoogmartens, "Development and validation of a liquid chromatography method for analysis of colistin sulphate," Chromatographia, vol 51, pp. 433-436, 2000.
[2] Y. Ikai, H. Oka, J. Hayakawa, N. Kawamura, K Harada, M.Suzuki, H.Nakazawa and Y. Ito, Journal of Liquid Chromatography and Related Technologies, vol 21, pp.143, 1998
[3] A.R. Martin, J.L. Delgado, and W.A. Remers (Eds.), Wilson and Gisvold's Textbook of Organic Medicinal and Pharmaceutical Chemistry, 10th ed., 1998.
[4] Y. Xu, X.i Tian, C. Ren, H. Huang, X. Zhang, X. Gong, H. Liu, Z. Yu, and L. Zhang, "Analysis of colistin A and B in fishery products by ultra performance liquid chromatography with positive electrospray ionization tandem mass spectrometry," Journal of Chromatography B, vol 899,pp. 14-20, 2012.
[5] The notification of the Department of Livestock Development (No.2/2) , " the maximum residue limits of animal product", Department of Livestock Development, Bangkok, Thailand, May, 2001.
[6] S. Morales-Munoz and M. D. Luque de Castro, "Dynamic ultrasound-assisted extraction of colistin from feeds with on-line pre-column derivatization and liquid chromatography-fluorimetric detection," Journal of Chromatography A, vol 1066, pp. 1-7, 2005.
[7] P. Leroy, D. Decolin, S. Nicolas, P. Archimbault and A. Nicolas, "Residue determination of two co-administered antibacterial agents cephalexin and colistin in calf tissues using high-performance liquid chromatography and microbiological methods.," Journal of Pharmaceutical and Biomedical Analysis, vol 7, pp.1837-1846, 1989.
[8] T. Suzuki, H. Inouye, K. Fujikawa and Y. Suketa, "Studies on the Chemical Structure of Colistin: I. Fractionation, Molecular Weight Determination, Amino Acid and Fatty Acid Composition," Journal of Biochemistry, vol 54, pp.25-33 ,1963.
[9] A. H. Thomas and L. Holloway, "Thin-layer chromatographic method for the identification of the polymyxins," Journal of Chromatography A, vol 161, pp.417-420, 1978.
[10] W. Holska and E. Gwozdz.,"Determination of colistin by isotachophoresis," Journal of Liquid Chromatography, vol 12, pp. 2761-67, 1989.
[11] H. K. Kristensen, and S. H. Hansen, "Separation of Polymyxins by Micellar Electrokinetic Capillary Chromatography," Journal of Chromatography, vol. 628, pp. 309-315, 1993.
[12] B. Cancho Grande, M. García Falcón, C. Pérez-Lamela, M. Rodríguez Comesaña, and J. Simal Gándara, "Quantitative analysis of colistin and tiamulin in liquid and solid medicated premixes by HPLC with diode-array detection," Chromatographia, vol 53, pp. 460-463, 2001.
[13] J. Li, R. W. Milne, R. L. Nation, J.D. Turnidge, K. Coulthard, and D. W. Johnson, "A simple method for the assay of colistin in human plasma, using pre-column derivatization with 9-fluorenylmethyl chloroformate in solid-phase extraction cartridges and reversed-phase high-performance liquid chromatography," Journal of Chromatography B: Biomedical Sciences and Applications, vol 761, pp.167-175, 2001.
[14] C. Cheng, S. Liu, D. Xiao, J. Hollembaek, L. Yao, J. Lin, and S. Hansel, "LC-MS/MS method development and validation for the determination of polymyxins and vancomycin in rat plasma," Journal of Chromatography B, vol 878, pp.2831-2838, 2010.
[15] D. W. Sin, E. C. Wan, C. Ho, and Y. C. Wong., "Detection of residual bacitracin A, colistin A, and colistin B in milk and animal tissues by liquid chromatography tandem mass spectrometry," Analytical and Bioanalytical Chemistry , vol 385, pp.181-188, 2006.