The validation of the Analytical method (HPLC), use for identification and assay of the pharmaceutical active ingredient, colistine sulphate and the finished product Colidem 50 – hydrosoluble powder, in SC DELOS impex '96 SRL.

Validarea metodei analitice (HPLC), utilizata pentru identificarea si dozarea ingredientului farmaceutic activ, colistine sulfat si a produsului finit Colidem 50 – puldere hidrosolubila, in cadrul SC DELOS Impex '96 SRL

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Abstract

In **SC DELOS IMPEX '96 SRL** the quality of the active pharmaceutical ingredient (API) for the finished product **Colidem 50** - hydrosoluble powder is make according to ***European Pharmacopoeia***, curent edition. The method for analysis use in this purpose is the compendial method ***„Colistine sulphate"*** in E.P. in current edition and represent a optimized variant, developed and validated „in house".The parameters which was included in the methodology validation for chromatographic method are the follow: Selectivity/Specificity, Linearity, Range of Linearity, Limit of Detection and Limit of Quantification , Precision (Repeatability - intra day, inter-Day Reproducibility), Accuracy, Robustness, Stability Solutions and System Suitability.

Key words: Colistine sulphate, Analytical method validation (HPLC), Identification, Assay, Related substances.

Rezumat

In cadrul **SC DELOS IMPEX '96 SRL** calitatea ingredientul farmaceutic activ (API) si a produsului finit **Colidem 50** – pulbere hidrosolubila, se face in conformitate cu **Farmacopeea Europeana** editia in vigoare. Metoda de analiza folosita (HPLC) in acest scop este metoda compendială ***„Colistine Sulphate"*** din ediţia curentă a ***E.P.*** şi reprezintă o variantă optimizata, dezvoltată si validată *„in house".* Parametrii inclusi in metodologia de validare a metodei cromatografice sunt urmatorii: Selectivitatea/Specificitate, Liniaritatea. Domeniul de liniaritate, Limita de detectie, Limita de cuantificare, Precizia, Exactitatea, Robustetea, Stabilitatea solutiilor.

Cuvinte cheie: colistine sulfat, validare metoda analitica HPLC, identificare, dozare, impuritati inrudite chimic.

The high pressure liquid chromatography (HPLC) is a physico – chemical method of chromatographic separation, in which the mobile phase is a liquid and, the stationary phase, contained in a column, is a solid with fine granulation, a solid impregnated with liquid or an solid that are grafted organic groups (-CN, NH_2, Diol, Phenyl). It is the most used at current time for identification, the active pharmaceutical ingredient (API) assay and related substances.

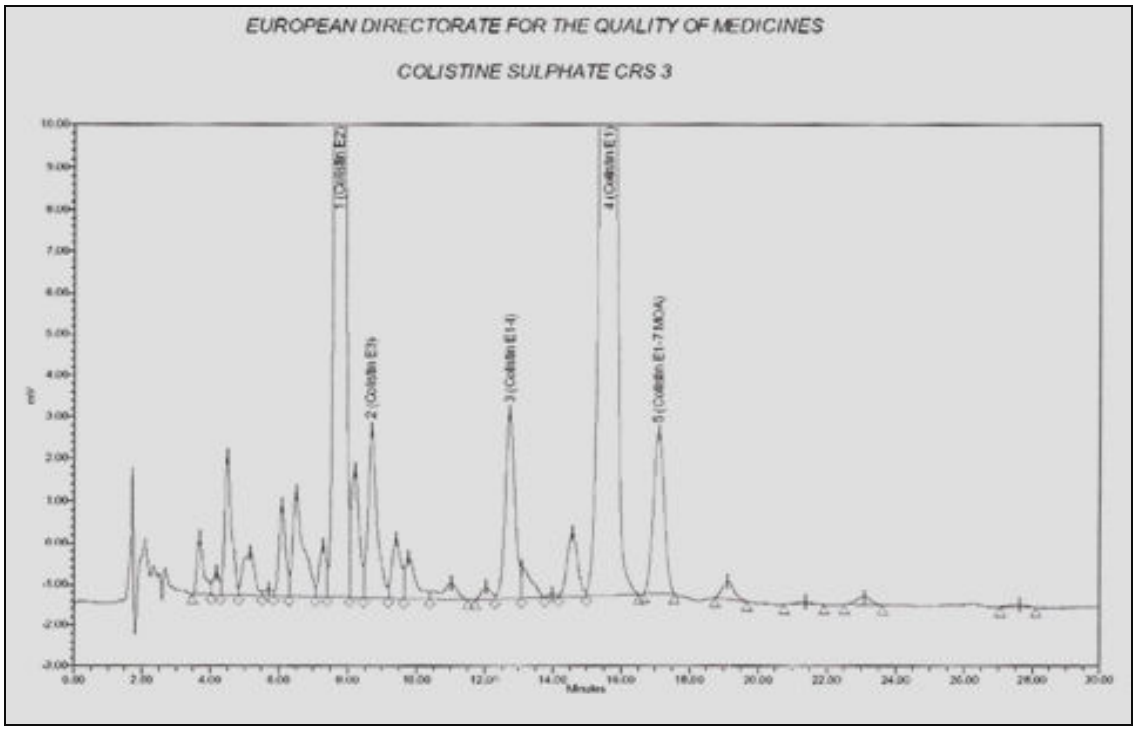
The colistine is commercial available under two forms:

- colistine sulphate and
- sodium colistimethate (colistine methanesulponate sodium and colistine sulphomethate sodium).

Colistine sulphate is a mixture of Polymyxins E1, E2, E3, E1-I, E1-7MOA. The colistine sulphate procent of API is calculate as sum of the five compounds. Unlike the microbiological assay, the HPLC method has possibility to identify and assay each of the five compounds of colistine sulphate.

European Pharmacopoeia requires a maximum limit of 10% of Polimixines E1-I, Polimixines E1-7MOA and Polimixines E3. This is the reason for which microbiological assay is not mentioned in European Pharmacopoeia, as assay method for colistine sulphate. In colistine sulphate monography from E.Ph. Ed. 6.0. is present a HPLC method for identification, related substances determination and assay for API. The time of the chromatogram registration conform with method presented, as see in the follow chromatogram (fig.1) of 30 minute. The time necessary for one analysis at one lot of API, respective of finished product ***COLIDEM 50***, being of 270 minutes.

Fig. 1. The afferent chromatogram of colistine sulphate, reference standard , lot 3, European Pharmacopoeia:

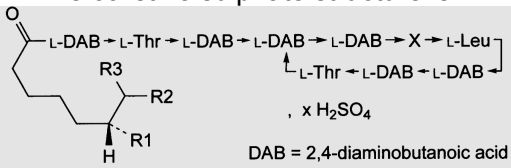


The colistinee sulphate is a mixture of the sulphates of polypeptides produced by certain strains of *Bacillus polymyxa* var. *colistineus* or obtained by any other means.

The colistine sulphate is a mixture of the polymyxins E1, E2, E3, E1-I, E1-7MOA, and according to European Pharmacopoeia current edition:

- sum of polymyxins E1, E2, E3, E1-I and E1-7MOA min. 77.0% (dried substance)
- polymyxins E1-I: maximum 10.0 per cent (dried substance),
- polymyxins E1-7MOA: maximum 10.0 per cent (dried substance),
- polymyxins E3: maximum 10.0 per cent (dried substance).

The colistine sulphate structure is:



colistin	X	R1	R2	R3	Mol. Formula	M_r
E1	D-Leu	CH_3	CH_3	H	$C_{53}H_{100}N_{16}O_{13}$	1170
E2	D-Leu	CH_3	H	H	$C_{52}H_{98}N_{16}O_{13}$	1155
E3	D-Leu	H	CH_3	H	$C_{52}H_{98}N_{16}O_{13}$	1155
E1-I	D-Ile	CH_3	CH_3	H	$C_{53}H_{100}N_{16}O_{13}$	1170
E1-7MOA	D-Leu	H	CH_3	CH_3	$C_{53}H_{100}N_{16}O_{13}$	1170

The integration report is:

Signal 1: VWD1 A, Wavelength=215 nm

RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
4.976	-	1653.83325	150.93642	1.03	0.1666	4940	-	-
5.614	-	104.85455	7.94073	0.88	0.1917	4752	2.09	1.13
8.459	-	7.78439	4.46000e-1	0.90	0.2718	5367	7.21	1.51
10.467	-	589.61023	29.46828	1.04	0.3033	6597	4.10	1.24
11.553	-	91.10169	4.30229	0.99	0.3252	6993	2.03	1.10

The chromatographic separation quality, through change initial method is not influenced, as it is can observe of the integration report of above, being met imposed for resolution condition of European Pharmacopoeia Ed 6.0.

Conclusions:

The colistine sulphate assay through HPLC optimization method is cheaper and faster than the standardized method of European Pharmacopoeia 6.0, the result obtained through both methods being comparable. The time necessary for one analysis, in case of optimization method is 2,40 hours comparative with 4,5 hours in case of standardized method.

Simultaneously with the assay, through HPLC method (European Pharmacopoeia

6.0 and the optimized) can make the identification the five compounds, Polymyxin E1, Polymyxin E2, Polymyxin E1-I, Polymyxin E1-7MOA and Polymyxin E3, and related substances assay.

In case of " microbiological assay" is not possible the separation and Polymyxins E3, E1-I, E1-7MOA identification, therefore is not possible to calculate their concentration, the accepted maximum, according to European Pharmacopoeia 6.0., for each of three compounds, being of 10%.

Also, it is not possible to determine the related substances. Of this considerations, in the colistine sulphate case, in the specialized literature, is not mentioned the microbiological method as assay method, evaluation have a guidance character. Through mobile phase ratio modification, chromatographic column temperature and mobile phase flow, from the proposed European Pharmacopoeia ed. 6.0 method is obtained a chromatogram of maximum 16 minute. The complete analysis duration, API identification, assay and related substances through optimization method is 144 minute.

In case of "microbiological assay" the time necessary for analysis is of about 24 hours, if is use the test microorganisms in vegetative form and is maximum 10 days if is use microorganism test in sporulated form. Below is show the chromatogram obtained after the method presented in E.P. 6.0 was modified. For obtained the retention time less, for each compounds of colistine sulphate was modified follow parameters:

- Mobile phase solvent ratio;
- Mobile phase flow;
- Chromatographic column temperature;

The chromatographic column used, was octadecilsilil silica gel (5µm) – Agilent with length of 150 cm and Ø=4,6mm;

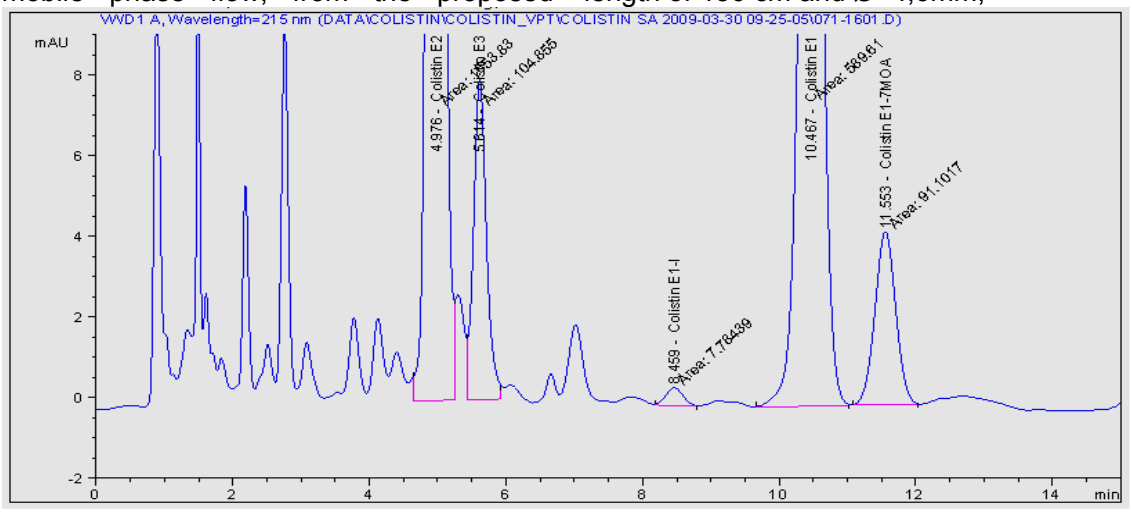


Fig.2: Chromatogram obtained after the analysis method is optimization.

The integration report is the follow:

Signal 1: VWD1 A, Wavelength=215 nm

RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
4.976	-	1653.83325	150.93642	1.03	0.1666	4940	-	-
5.614	-	104.85455	7.94073	0.88	0.1917	4752	2.09	1.13
8.459	-	7.78439	4.46000e-1	0.90	0.2718	5367	7.21	1.51
10.467	-	589.61023	29.46828	1.04	0.3033	6597	4.10	1.24
11.553	-	91.10169	4.30229	0.99	0.3252	6993	2.03	1.10

The chromatographic quality separation through initial method modification is not influenced, as is possible to see in the above integration report, being accomplished the European Pharmacopoeia 6.0 imposed conditions for resolution.

Conclusions:

The colistine sulphate assay, through HPLC optimization method is cheaper and faster than the standardization method of E.P. 6.0., the obtained result obtained through both methods being comparable.

The time necessary for one analysis in the optimization method case is 2,40 hours from the 4,5 hours in the standardization method. Simultaneously with assay through HPLC method (European Pharmacopoeia Ed.6.0 and optimized) is can do the five compounds identification (Polymysin E1, Polymyxin E2, Polymyxin E3, Polymyxin E1-I, Polymyxin E1-7MOA and the related

substances assay. In case of "microbiological assay" is not possible the separation and the Polymyxins E1-I, E1-7MOA and E3 identification.

Therefore is not possible to calculate the concentration for them, accepted maximum, according to European Pharmacopoeia, for each of compounds being 10%. Also, is not possible to determinate the related substances. Of this consideration, in colistine sulphate case, in specialized literature, is not mentioned the microbiological method as assay method, the evaluation has a guidance character. The Validation method for quantitative and qualitative determination is presented below:

Aim and methodology

1. The aim of this study is to validate the analytical method used in the determination of the levels of Colistine sulphate (polymyxin E1, E2, E3, E1-I, E1-7MOA) and related substances for this drug product ***Colistine sulphate*** – *hydrosoluble powder*. The finished product *COLIDEM 50* is processed by *SC DELOS Impex '96 SRL*, Romania.

2. The analytical performances are evaluated for the method used for identification and assay of active substance and determination of related substances.

3. The start method is presented in ***European Pharmacopoeia***, current edition, but it is developed and optimized "in house" and the following parameters have been validated for: Specificity (Peak identification and Interference Study), Precision (Repeatability and Intermediate Precision), LOQ/LOD, Linearity and Range, Accuracy, Robustness, Stability of Standard and Sample Solution.

Responsibilities

1. Research and Development Department has the responsibility to perform validation study of the method, and to record all observations in the numbered laboratory book.

2. Research and Development, Quality Assurance and Regulatory Affair Department have the responsibility to review the method validation data and to sign the approval section, if complies and it is applicable.

3. Based on the results, Research and Development Department has the responsibility to update/prepare the analytical method for routine use, if necessary.

Method's rationale

1 The method rationale is to introduce a single chromatographic procedure HPLC-UV to quantify and monitor related compounds and active substance level for the following:

- API Identification
- API Assay
- API Quantization of Related Substances
- Identification/Quantization of API and Related Compounds in bulk product, Finished Product and Stability Samples.

Associated documents:

ICH –Q2A – Text on Validation of Analytical Procedure

ICH-Q2B – Validation of analytical Procedure: Methodology

CPMP/ICH/381/-ICH Q(R1)

Validation terms

Refer to the Associated Documents for definition of all terms used in this study

Content

1. General Aspects
2. Operational Parameters of the Chromatographic Method
3. Substances and reference materials
4. Reagents. Solvents. Solutions
5. Equipments
6. Description of the Method

Validation report and experimental results

7. Selectivity
8. Linearity. Range of Linearity. Limit of Detection and Limit of Quantification
9. Precision
- Repeatability (intra-day)
- Reproducibility (inter-day)

10. – Accuracy Study for active compound in ***Reference solution (a)*** – reference for assay and ***Reference solution (b)*** – reference for related substances

11. Robustness
12. Stability Solutions

General aspects

This validation is composed of two parts: the validation report and the experimental results. In the validation report part are presented the parameters of the suggested method, the purpose and procedures, the working procedure, the calculation formulas, calculations and the acceptance conditions. In the experimental results part these are presented and explained the analytical results. To minimize

errors affecting the analytical process should be considered the following:

- The quantities of reference substances (CRS or SRS), secondary reference substances and samples below were weighed with a precision of 0.01 mg. Analytical balance has to be verified on a daily basis regarding the accuracy by using at least three certified weights E2, and should have a valid metrologic certificate at the date of use;

- The volumetric glassware should be quality A class (with batch certificate), gravimetrically tested at reception.

- The volumes of liquids should be measured through difference between marks and not by completely emptying the pipette;

- When the ultrasonic bath is used in the dissolution process of the samples, a proper cooling should be provided in order to avoid local overheating;

- the chromatographic system should be valid when operating and performance qualification certificates at the date of analysis;

- The concentrations of the solutions referred in the text are expressed as ppm (µg/mL).

Operational parameters for chromatographic method

Chromatografic column:	octadecylsilyl silica gel for chromatography R, 150 mm L x 4,6 mm i.d. x 5 µm d.p.
ColumnTemperature:	30 oC
Injection volume:	20 µL
Elution:	Isocratic
Compozition for mobile phase:	Solvent A: Dissolve 4.46 g of anhydrous sodium sulphate R in 900 ml of water R, adjust to pH 2.4 with dilute phosphoric acid R and dilute to 1000 ml with water R. Solvent B: Acetonitrile R
Mobile phase:	Composition: % Solvent A 78 % Solvent B 22
Flow rate:	1 mL/min
Detection	UV
Detection at:	215 nm

Reference substances

Colistine sulphate CRS European Pharmacopoeia lot 3 or working standard;

Reagents. Solvents. Solutions

Anhydrous sodium sulphate R;

Solvent for samples – Water: Acetonitrile 80:20;

Water for chromatographic use – (18,2 MΩ, TOC maximum 30 ppb (ultra purified water).

Stock Test solution – In volumetric flask, quality A class, 50 ml are transferred 100mg of Colidem 50, finished product, added to volume with sample solvent. It is sonicate 1 minute for homogenization.

Test solution – In volumetric flask, quality A class, 10 mL are transfered 5 ml of Stock Test solution, added to volume with sample solvent. It is sonicate 1 minute for homogenization.

Stock Reference Solution (a) – In volumetric flask, quality A class, 50mL are transferred 50 mg of Colistine sulphate CRS lot 3 European Pharmacopoeia, added to volume with sample solvent. It is sonic ate 1 minute for homogenization.

Reference Solution (a) – In volumetric flask, quality A class, 10 mL are transferred 5 ml of Stock Reference Solution (a) added to volume with sample solvent. It is sonic ate 1 minute for homogenization

Reference Solution (b) – Dilute 1.0 ml of Reference Solution (a) to 100 ml with sample solvent. It is sonic ate 1 minute for homogenization.

Equipment: Chromatographic system (common to all determinations)

Agilent 1200 With following modulus:	Solvents cabinet; Quaternary pump high pressure G 1354A with degassing G 1379B, series 1200; Thermostat for column G 1316A, series 1200; Spectrometric Detector (VWD) G 1314B, series 1200, or another similarly; Auto sampler G 1329A; Thermostat for auto sampler G1330B, series 1200;

Additional Equipment:

- sonicate bath;
- analytical balance (precision 0,01mg);

Instruments for laboratory:

- Graduated pipette;
- A class volumetric flask;
- Graduated cilinder;
- Vials for automatic injector of HPLC.

Method Description

The assay of the active pharmaceutical ingredient in the drug product ***Colidem 50 –*** *hydrosoluble powder* is performed using a HPLC method, validated by the drug product manufacturer.

- The identification of active substance from pharmaceutical product ***Colidem 50 -*** *hydrosoluble powder* is based on:

- Comparing the retention time which corresponds for ***colistine sulphate*** peaks (polymyxins E1, E2, E3, E1-I, E1-7MOA) in chromatogram recorded for the ***Test Solution –*** *(sample of assay and related substances),* with the retention time for ***colistine sulphate*** peaks (polymyxins E1, E2, E3, E1-I, E1-7MOA from ***Reference Solution (a).***

Samples concentration which are used are presented in the next table:

Type of determination	Sample type	Sample concentration (ppm)
Active substance identification	Reference solution – Reference solution (a)	Colistine sulphate CRS - 500
	Sample solution – Test solution	Colistine sulphate - 500
Active substance assay	Reference solution – reference solution (a)	Colistine sulphate CRS - 500
	Sample solution – Test solution	Colistine sulphate - 500
Related substances	Reference Solution – reference solution (b)	Colistine sulphate CRS - 5
	Sample solution – Test solution	Colistine sulphate - 500

The same solutions is used for active substance identification and for assay.

Selectivity

Aim of procedure:

The aim is to demonstrate that the chromatographic method which is proposed has the capacity of separation of:

1) Active substance against the related substances;

2) Active substance against the related substances and against the excipients; eventual coextracts in the process of samples preparation;

3) Active substance against the related substances which are resulted through its degradation;

In order to evaluate the ability of the method to separate the active substance from the degradation products there were applied physical and chemical processes which generate the degradation of the API. The degradation processes which were chosen are: oxidative exposure, alkaline exposure, acid exposure, heat exposure, UV and natural light exposure.

Sample preparation:

Stock Test solution – In volumetric flask, quality A class, 50 ml are transfered 100 mg of ***Colidem 50***, finished product, added to volume with sample solvent. It is sonicate 1 minute for homogenization.

Placebo Solution – the solution containes the reconstituted mix of excipients used in pharmaceutical formulation of the drug product ***Colidem 50*** – *hydrosoluble powder.*

In a 50 mL A class, volumetric flask add the folowing excipients: 25mg Lactose monohydrate, complete to volume with sample solvent and sonicate for homogenisation.

Stress solution 1

Pipette 5 ml ***Stock Test solution***, in a 10 ml A class, volumetric flask, complete to volume with ***Sample Solvent***.

Stress solution 2

Pipette 5 ml ***Stock Test solution***, in a 10 ml class A, volumetric flask, add 1 ml 0,1 M NaOH solution and complete to volume with ***Sample Solvent.***

Stress solution 3

Pipette 5 ml ***Stock Test solution***, in a10 ml A class, volumetric flask, add 2 ml 0.1 M NaOH solution and complete to volume with ***Sample Solvent.***

Stress solution 4

Pipette 5 ml ***Stock Test solution***, n a 10 ml, A class volumetric flask, add 1 ml 0.1 M HCl solution and complete to volume with ***Sample Solvent.***

Stress solution 5

Pipette 5 ml ***Stock Test solution***, in a 10 ml, A class volumetric flask, add 2 ml 0.1 M HCl solution and complete to volume with ***Sample Solvent.***

Stress solution 6

Pipette 5 ml ***Stock Test solution***, in a 10 ml, A class volumetric flask, add 0.5 ml H_2O_2 3% solution and complete to volume with ***Sample Solvent.***

Stress solution 7

Pipette 5 ml of ***Stock Test solution***, in a 10 ml, A class volumetric flask, add 1 ml H_2O_2 3% solution and complete to volume with ***Sample Solvent***.

Stress solution 8

Pipette 5 ml ***Stock Test solution***, in a 10 ml, A class volumetric flask, complete to

volume with ***Sample Solvent.*** Keep the solution in drying for 5 hours at 40˚C**.**

Stress solution 9

Pipette 5 ml ***Stock Test solution***, in a 10 ml, A class volumetric flask, complete to volume with Sample *Solvent*. Expose the obtained solution to UV irradiation for 24 h.

Stress solution 10

Pipette 5 ml of ***Stock Test solution***, in a 10 ml, A class volumetric flask, complete to volume with Sample *Solvent.* Expose the obtained solution to irradiation with fluorescent light for 72 hours.

Stress solution 11

Pipette 5 ml ***Stock Test solution*****,** in a 10 ml, A class volumetric flask, add 1 ml $HClO_4$, and complete to volume with Sample *Solvent.*

Stress solution 12

Pipette 5 ml ***Stock Test solution***, in a 10 ml, A class volumetric flask, add 0,5 mL 1M NaOH solution and 0,5 ml H_2O_2, 3% solution, complete to volume with Sample Solvent.

Working method: The prepared ***Stress solutions*** are injected in chromathographic system. The obtained chromatograms are presented below.

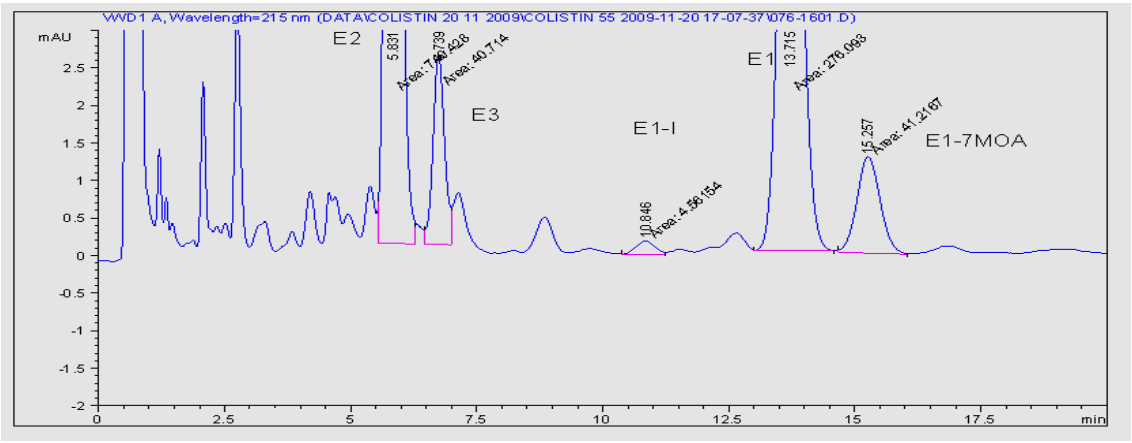


Figure 1. The chromatogram recorded for Stress solution 1

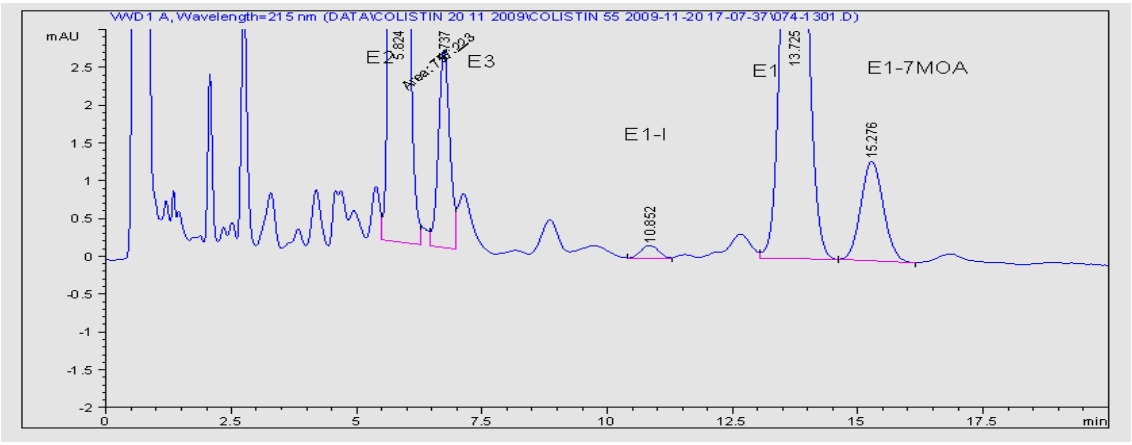


Figure 2. The chromatogram recorded for Stress solution 2

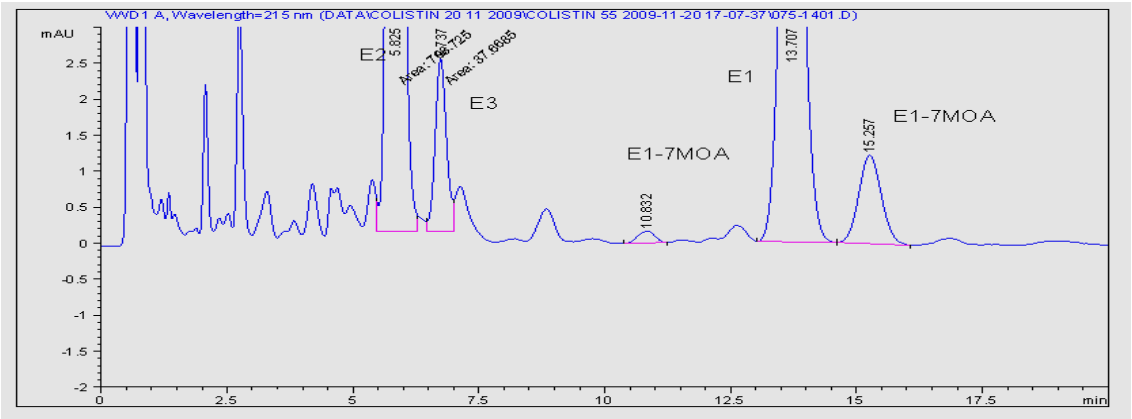


Figure 3. The chromatogram recorded for Stress solution 3

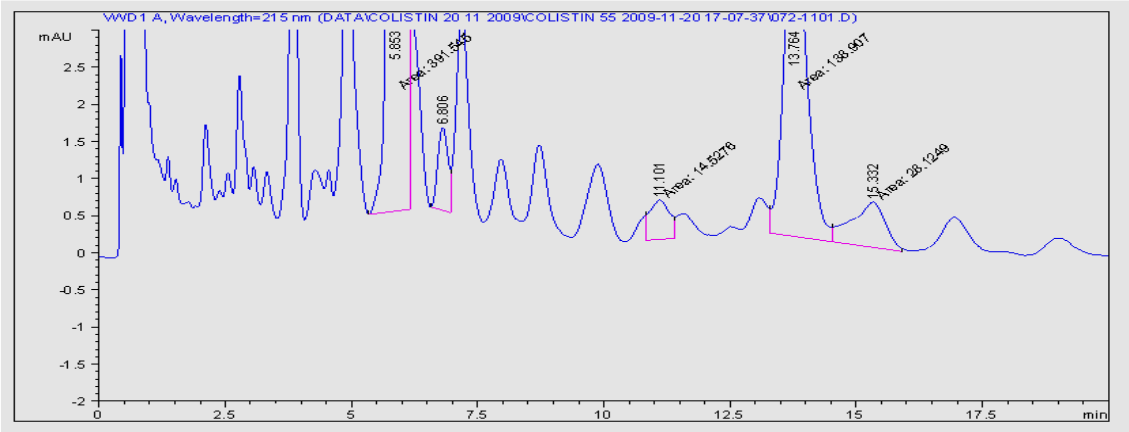


Figure 4. The chromatogram recorded for Stress solution 4

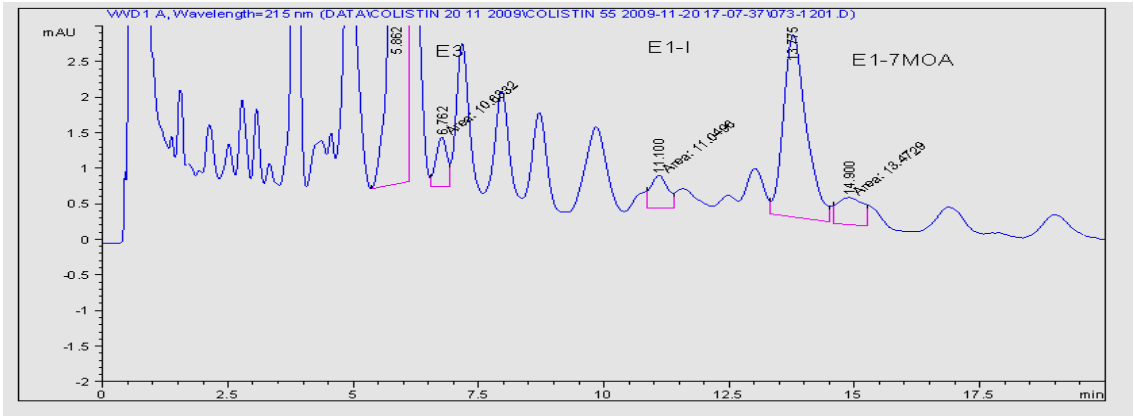


Figure 5. The chromatogram recorded for Stress solution 5

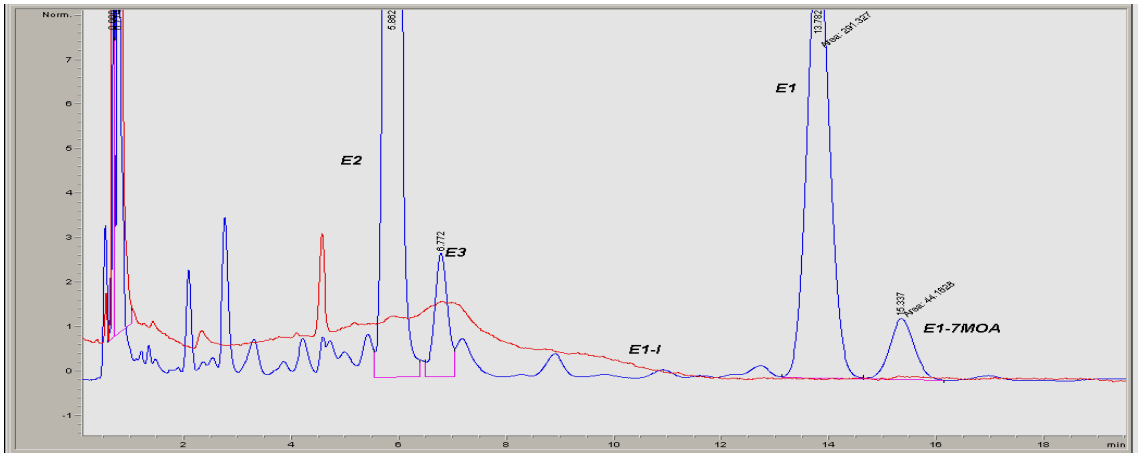


Figure 6. The overlapped chromatograms recorded for Stress solution 1 and Placebo

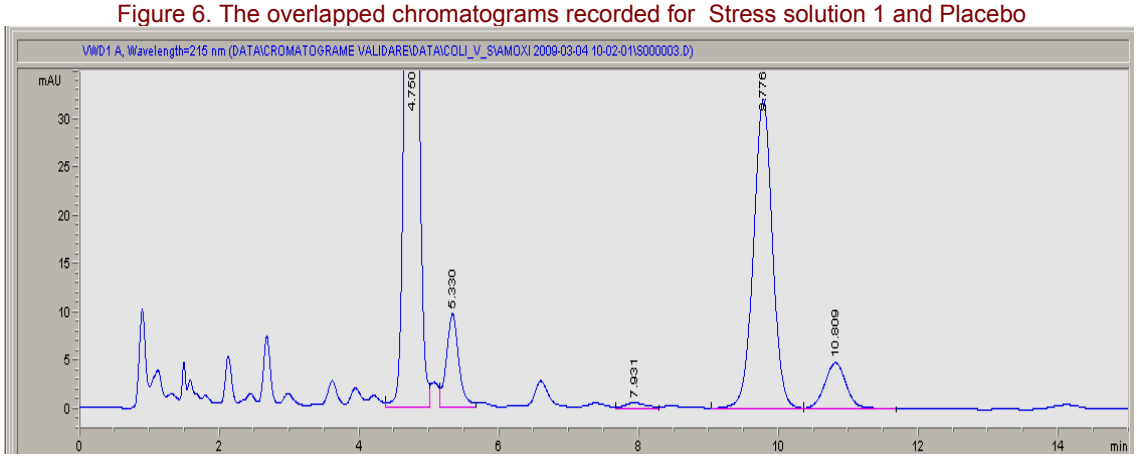


Figure 7. The chromatogram recorded for Stress solution 8:

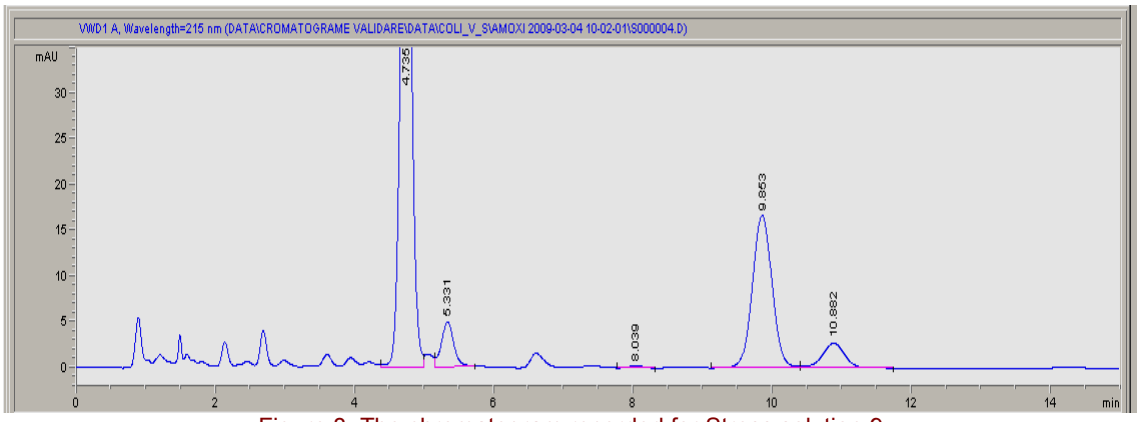


Figure 8. The chromatogram recorded for Stress solution 9

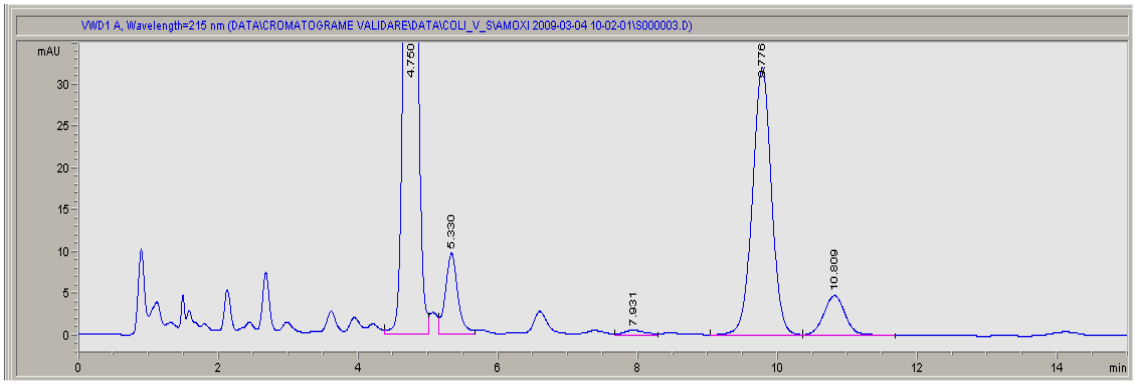


Figure 9. The chromatogram recorded for Stress solution 10

The difference between the retention time in the chromatograms presented above was due to the chromatographic columns which was different usage degree and another similar equipment.

Acceptance criteria:

The active substance must be separated against the related substances resulted after exposure to stress conditions against the eventually co extracted excipients during the samples preparation, against the related substances resulted through degradation.

Conclusions

The proposed method is selective because it has the capacity of separating the corresponding peaks of colistine sulphate against the peaks corresponding to the related substances. The excipients from the pharmaceutical formulation are not interfering with the peaks corresponding to colistine sulphate (in elution order - polymyxin E2, E3, E1-I, E1, E1-7MOA). The method has also the ability to separate the peaks corresponding to colistine sulphate the peaks corresponding to degradation products of Colistine sulphate obtained after physical and chemical stresses.

Linearity. Range of linearity. Limit of detection and quantification.

Procedure purpose:

The aim is to prove that there is a linear relationship between concentrations of the target analytes solutions which are injected in chromatographic column and the peaks areas resulted in the corresponding chromatograms. The purpose is to establish the concentration range for which the linear relationship mentioned above is valid. The lowest amount of analytes which can be quantitatively determined with suitable precision and accuracy (limit of quantification or LOQ) and the lowest amount of analytes in a sample which can be detected but not necessarily quantitated as an exact value are determined.

Linearity. Range of linearity. Limit of detection. Limit of quantification (for Colistine sulphate in sample solution – assay and related substance sample)

Samples preparation:

Stock Test solution, 1000 ppm – In volumetric flask, quality A class, 50 mL are transferred 100 mg of Colidem 50, finished product, added to volume with sample solvent. It is sonicated 1 minute for homogenization.

Linearity solution 1 - 400 ppm:

Pipette 4 ml Stock Test solution in a 10 ml A class volumetric flask, and complete to volume with sample solvent. Sonicated 1 minute for homogenization.

Linearity solution 2 - 450 ppm:

Pipette 4,5 ml Stock Test solution, in a 10 ml A class volumetric flask, and complete to volume with Sample solvent. Sonicated 1 minute for homogenization.

Linearity solution 3 - 500 ppm:

Pipette 5 ml Stock Test solution, in a 10 ml, A class volumetric flask, complete to volume with Sample solvent. Sonicate 1 minute for homogenization.

Linearity solution 4 - 550 ppm:

Pipette 5,5 ml Stock Test solution in a 10 ml, A class volumetric flask, complete to volume with Sample solution. Sonicated 1 minute for homogenization.

Linearity solution 5 - 600 ppm:

Pipette 6 ml Stock Test solution in a 10 ml, A class volumetric flask, and complete to volume with Sample solvent. Sonicated 1 minute for homogenization.

Working method:

Inject three times, each of Linearity solution (1-5), above presented, in the order of increasing of concentrations .

In the chromatograms obtained for each analyzed substance, integrate the peaks corresponding o Colistine sulphate (polimyxyns E1, E2, E3, E1-I, E1-7MOA) and calculate the average value for peak's areas, standard deviation and relative standard deviation (%), using the following formulas for calculation:

$\overline{A_p} = \frac{\sum_{i=1}^{n} \overline{A_p^i}}{n}$; Average

$s = \sqrt{\frac{\sum_{i=1}^{n} (A_p^i - \overline{A_p})2}{(n-1)}}$; Standard deviation

RSD % = s*100/$\overline{A_p}$;

Relative standard deviation

The graphic disposal of the nominal concentrations of the analytes (on the abscissa, expressed in µg/ml or ppm) as function of the mean peak area value (on ordinate, expressed as mAU*s) is presented. Using the formulae from below, the linear regressions, correlation coefficient, normal variation ranges for the slope and the intercept and also the limit of quantification (for a 95% level of certitude and n - 2 freedom degrees are calculated for the analytes of interest.

The statistical formulae involved in the data processing for linear regression are:

Covariance:

$Sxy = \frac{\sum xy - \frac{\sum x - \sum y}{n}}{n-1}$;

Standard deviation for x value population:

$Sx = \sqrt{\frac{\sum x^2 - \frac{(\sum [x])^2}{n}}{n-1}}$;

Standard deviation for y value population:

$Sy = \sqrt{\frac{\sum y^2 - \frac{(\sum [y])^2}{n}}{n-1}}$;

Correlation coefficient:

$rxy = \frac{S_{xy}}{S_x \, S_y}$;

Slope:

$B = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sum x^2 - \frac{(\sum [x])^2}{n}}$;

Intercept:

$A = \frac{\sum y}{n} - B \frac{\sum x}{n}$;

Standard deviation for y value population :

$S0 = \sqrt{\frac{\sum y^2 - A \sum y - B \sum xy}{n-2}}$

Standard deviation associated with slope:

$Sb = \sqrt{\frac{nS_0^2}{n \sum x^2 - (\sum x)^2}}$

Standard deviation associated with intercept:

$S\sigma = \sqrt{\frac{S_b^2}{n} \sum x^2}$;

Variation range for A: A±t*Sa;

Variation range for B: B±t*Sb;

Identification limit:: Xi = $\frac{2t \left(S_a + \frac{\sum x}{n} * S_b\right)}{b + 2t * S_b}$;

where:

x = The nominal concentration value of the target analyte µg/mL or ppm;

y = The mean peak area value for target analytes, integrated in the recorded chromatograms of consecutively injections of each Linearity solutions (1-5).

t = The "Student" coefficient (for a known certainty level P% and for a *number v* of freedom degrees),

v = the number of freedom degrees (v = n-2),

n = the number of sets pairs of experimental data (concentration – mean area).

Acceptance criteria:

The accepted relative standard deviation for the peak areas must not exceed 0,62% (in accordance with E.Ph, current edition) when injecting the same solution three times consecutively.

The correlation coefficient which characterizes the linear regression has not be equal or higher than 0.9990.

Results and conclusions

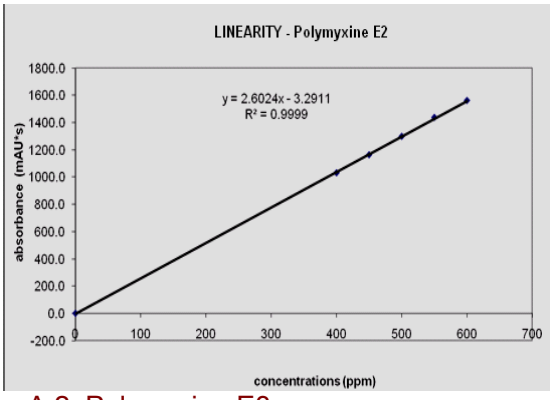
In next table are shown the standard deviation and relative standard deviation (%) for three injection of the Linearity solution (1-5), for Colistine sulphate (polymyxins E1, E2, E3, E1-I, E1-7MOA). All results for linearity are show below (in elution order).

A.1. Polymyxine E2:

Solutions concentration (ppm) for linearity	Mean areas for three injections (mAU) Colistine E2
0	0.0
400,0	1030.1
450,0	1162.3
500,0	1297.5
550,0	1436.0
600,0	1560.3

B=	2,602	Slope
A=	-3,2896	Intercept
rxy	0,999947	Correlation coeficient
Sxy	121443,80	
Sx	216,02	
Sy	562,21	
So^2	41,6	Dispersion of y value population
So	6,4	Standard deviation for y value population
Sb^2	0,0	Slope dispersion of linear regression
Sb	0,0	Standard deviation associated with the slope
tsb	0,0	Variation domain of B
Sa^2	37,9	Dispersion of origin of the ordinate
Sa	6,1526	Standard deviation associated with the intercept
tsa	13,12	Variation domain of A
Xi	18,7823	Limit of quantification LOQ
	5,6347	Limit of detection LOD

The dependence between peaks area (mAU*s) of Polymyxin E2 and the concentration of the active substance in the sample (ppm) is presented in the next plot

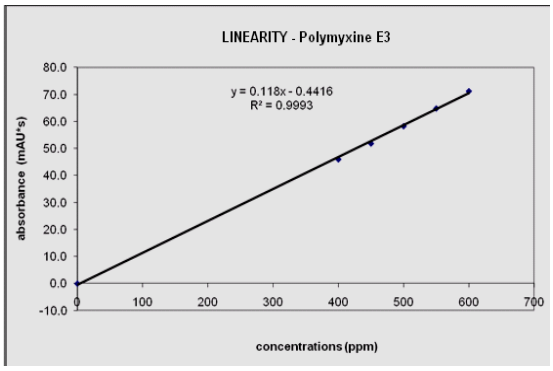


A.2. Polymyxine E3:

Solutions concentration (ppm) for linearity	Mean areas for three injections (mAU) Colistine E3
0	0
400,0	46.02
450,0	51.91
500,0	58.32
550,0	64.87
600,0	71.25

B=	0,118	Slope
A=	-0,4416	Intercept
rxy	0,999643	Correlation coeficient
Sxy	5507,03	
Sx	216,02	
Sy	25,50	
So^2	0,6	Dispersion of y value population
So	0,8	Standard deviation for y value population
Sb^2	0,0	Slope dispersion of linear regression
Sb	0,0	Standard deviation associated with the slope
tsb	0,0	Variation domain of B
Sa^2	0,5	Dispersion of origin of the ordinate
Sa	0,7270	Standard deviation associated with the intercept
tsa	1,55	Variation domain of A
Xi	47,3171	Limit of quantification LOQ
	14,1951	Limit of detection LOD

The dependence between peaks area (mAU*s) of Polymyxin E3 and the concentration of the active substance in the sample (ppm) is presented in the next plot.



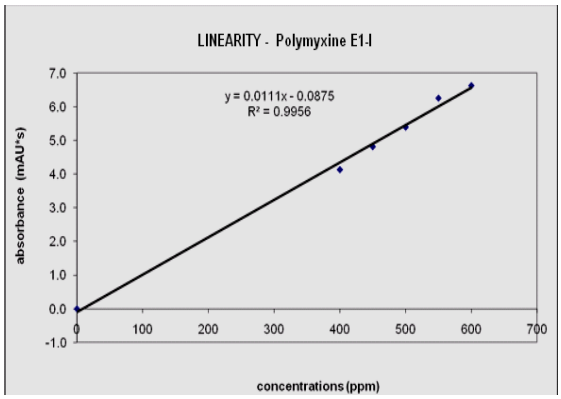
A.3. Polymyxine E1-I:

Solutions concentration (ppm) for linearity	Mean areas for three injections (mAU) Colistine E3
0	0
400,0	4.13
450,0	4.81
500,0	5.39
550,0	6.25
600,0	6.62

B=	0,011	Slope
A=	-0,0875	Intercept
rxy	0,997775	Correlation coeficient
Sxy	517,53	
Sx	216,02	
Sy	2,40	
So^2	0,0	Dispersion of y value population
So	0,2	Standard deviation for y value population
Sb^2	0,0	Slope dispersion of linear regression
Sb	0,0	Standard deviation

		associated with the slope
tsb	0,0	Variation domain of B
Sa^2	0,0	Dispersion of origin of the ordinate
Sa	0,1708	Standard deviation associated with the intercept
tsa	0,36	Variation domain of A
Xi	109,4310	Limit of quantification LOQ
	32,8293	Limit of detection LOD

The dependence between peaks area (mAU*s) of Polymyxin E1-I and the concentration of the active substance in the sample (ppm) is presented in the next plot.

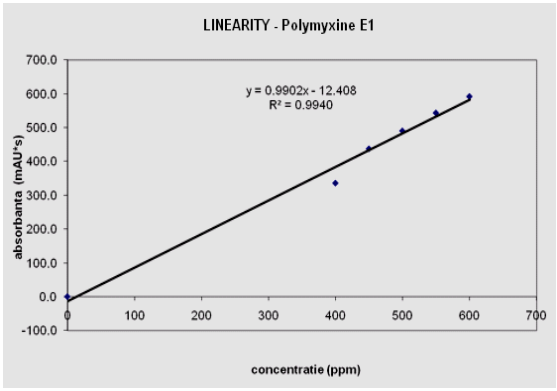


A.4. Polymyxine E1:

Solutions concentration (ppm) for linearity	Mean areas for three injections (mAU) Colistine E3
0	0
400.0	386.11
450.0	437.68
500.0	490.77
550.0	543.88
600.0	592.6

B=	0.990	Slope
A=	-12.4082	Intercept
rxy	0.994030	Correlation coeficient
Sxy	46209.13	
Sx	216.02	
Sy	215.19	
So^2	689.1	Dispersion of y value population
So	26.3	Standard deviation for y value population
Sb^2	0.0	Slope dispersion of linear regression
Sb	0.1	Standard deviation associated with the slope
tsb	0.1	Variation domain of B
Sa^2	627.6	Dispersion of origin of the ordinate
Sa	25.0519	Standard deviation associated with the intercept
tsa	53.41	Variation domain of A
Xi	166.4382	Limit of quantification LOQ
	49.9315	Limit of detection LOD

The dependence between peaks area (mAU*s) of ***Polymyxine E1*** and the concentration of the active substance in the sample (ppm) is presented in the next plot.

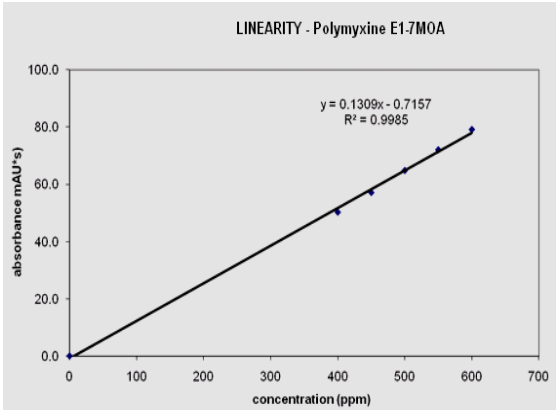


A.5. Polymyxine E1-7MOA:

Solutions concentration (ppm) for linearity	Mean areas for three injections (mAU) Colistine E3
0	0
400.0	50.20
450.0	57.05
500.0	64.75
550.0	71.97
600.0	79.05

B=	0,131	Slope
A=	-0,7157	Intercept
rxy	0,999266	Correlation coeficient
Sxy	6109,87	
Sx	216,02	
Sy	28,30	
So^2	1,5	Dispersion of y value population
So	1,2	Standard deviation for y value population
Sb^2	0,0	Slope dispersion of linear regression
Sb	0,0	Standard deviation associated with the slope
tsb	0,0	Variation domain of B
Sa^2	1,3	Dispersion of origin of the ordinate
Sa	1,1566	Standard deviation associated with the intercept
tsa	2,47	Variation domain of A
Xi	66,2959	Limit of quantification LOQ
	19,8888	Limit of detection LOD

The dependence between peaks area (mAU*s) of Polymyxin E1-7MOA and the concentration of the active substance in the sample (ppm) is presented in the next plot.



Conclusions:

The relationship between the concentration of the solutions of the active pharmaceutical ingredient injected in the chromatographic column and the resulted peak areas in the resulting chromatograms is linear within the range 400-600 ppm (80-120%). From the tables presented at Accuracy section (Repeatability intra-day, inter-day Reproducibility) – the solutions for accuracy are the same with the linearity solutions, it can be observed that the relative standard deviations calculated for three consecutive injections are below the imposed acceptance criteria of not more than 0.62%.

The obtained correlation coefficient which characterizes the linear regression is higher than 0.9900.

The limit of quantification and the limit of detection determined by statistical means are presented in the table below:

Compound	Limit of quantification (LOQ)* ppm	Limit of detection (LOD)* ppm
Polymyxin E1	166.4	49.9
Polymyxin E2	18.8	5.63
Polymyxin E3	47.3	14.2
Polymyxin E1-I	109.4	32.8
Polymyxin E1-7MOA	66.3	19.9

*Because the peaks areas have more variation function of the raw material, LOD, LOQ, in SC DELOS IMPEX '96 SRL, always, the calculations are function by the signal/ noise in this way:

- According to E.Ph. LOD – S/N≥3, LOQ≥10;
- According to ICH guide book LOD – S/N≥ 2 - 3, LOQ≥10;

Precision

Procedure purpose:

The aim is to prove that, by repeatedly applying the method to the same sample, similar results will be consistently obtained.

As the sample is analyzed within the same experimental session, the procedure is also known as Repeatability.

Another aim is to prove that, by repeatedly applying the method at time intervals of at least one day between two consecutive tests, upon similar samples, similar results will be consistently obtained.

It is recommended that the determinations should be performed by different analysts and - if possible – with different equipment.

The entire procedure is performed during different experimental sessions and is known as Intermediate Reproducibility.

Sample preparation:

Test solution, 500 ppm – In volumetric flask, quality A class, 50ml are transferred 50 mg of Colidem 50, finished product, added to volume with sample solvent. It is sonicate 1 minute for homogenization.

Working method:

Injected the Test Solution for six consecutively times. Integrate the corresponding peaks for Colistine sulphate (sum of Polymyxins E1, E2, E3, E1-I, E1-7MOA) in the recorded chromatograms.

Calculate the standard deviation and the relative standard deviation (%) for the obtained peaks areas.

Acceptance criteria:

The relative standard deviation calculated for peaks areas of the target analytes must not exceed 2%.

A. Repetability

A.1. The peak areas for Colistine sulphate (Polymyxins E1, E2, E3, E1-I, E1-7MOA) obtained by injecting for six times consecutively the Test solution, the standard deviation and the relative standard deviations are presented in the tables, below.

Sample	Compound (in elution order)	Conc. (ppm)	Retention time (min.)	Peak area (mAU*s)
1	Polymyxin E2	500	4.78	1349.59
	Polymyxin E3		5.39	118.51
	Polymyxin E1-I		8.15	13.66
	Polymyxin E1		10.03	629.01
	Polymyxin E1-7MOA		11.07	117.01
2	Polymyxin E2	500	4.79	1346.36
	Polymyxin E3		5.42	119.29
	Polymyxin E1-I		8.16	13.52
	Polymyxin E1		10.04	628.67
	Polymyxin E1-7MOA		11.09	122.15
3	Polymyxin E2	500	4.79	1347.98
	Polymyxin E3		5.41	119.03
	Polymyxin E1-I		8.21	12.42
	Polymyxin E1		10.11	629.43
	Polymyxin E1-7MOA		11.16	121.81
4	Polymyxin E2	500	4.80	1346.74
	Polymyxin E3		5.43	123.94
	Polymyxin E1-I		8.21	12.62
	Polymyxin		10.12	627.87

	E1			
	Polymyxin E1-7MOA		11.17	121.81
	Polymyxin E2		4.82	1347.13
	Polymyxin E3		5,45	123,49
5	Polymyxin E1-I	500	8.21	11.95
	Polymyxin E1		10.13	626.75
	Polymyxin E1-7MOA		11.18	119.92
	Polymyxin E2		4.80	1347.7
	Polymyxin E3		5.42	119.88
	Polymyxin E1-I		8.22	12.59
	Polymyxin E1		10.12	628.12
6	Polymyxin E1-7MOA	500	11.18	121.90
	Polymyxin E2		8.26	12.18
	Polymyxin E3		10.17	627.02
	Polymyxin E1-I		11.25	118.60

Compound	Injection no.	Retention time	Compound area
Polymyxin E2	1	4.78	1349.59
	2	4.79	1346.36
	3	4.79	1347.98
	4	4.80	1346.74
	5	4.82	1347.13
	6	4.80	1347.70
	Average	4.81	1347.44
	RSD	0.40	0.07
Polymyxin E	1	5.39	118.51
	2	5.42	119.29
	3	5.41	119.03
	4	5.43	123.94
	5	5.45	123.49
	6	5.42	119.88
	Average	5.43	119.44
	RSD	0.46	2.10
Polymyxin E1	1	8.15	13.66
	2	8.16	13.52
	3	8.21	12.42
	4	8.21	12.62
	5	8.21	11.95
	6	8.22	12.59
	Average	8.21	12.53
	RSD	0.47	4.87
Polymyxin E1	1	10.03	629.01
	2	10.04	628.67
	3	10.11	629.43
	4	10.12	627.87
	5	10.13	626.75
	6	10.12	628.12
	Average	10.11	628.20
	RSD	0.49	0.14
Polymyxin E1 7MOA	1	11.07	117.01
	2	11.09	122.15
	3	11.16	121.81
	4	11.17	121.81
	5	11.18	119.92
	6	11.18	121.9
	Average	11.17	120.8
	RSD	0.49	1.66

From the experimental data listed above, result that the method is ***Reproductible***.

B. Intermediate reproducibility

Intermediate reproducibility was determined by injecting the ***Test solution (500 ppm)*** in two experimental sessions.

In the obtained chromatograms peaks coresponding to ***Colistine sulphate (Polymyxins E1, E2, E3, E1-I, E1-7MOA)*** are integrated. The standard deviation and the relative standard deviation (%). There are calculated the standard deviation and the relative standard deviation (%) for the peaks areas of to ***Colistine sulphate (Polymyxins E1, E2, E3, E1-I, E1-7MOA),*** are calculated.

B.1. In the next table there are presented the obtained peaks areas by injecting ***Test Solution,*** standard deviation and relative standard deviation (%) for those six injections.

Sample	Compound (in elution order)	Conc. (ppm)	Retention time (min.)	Peak area (mAU*s)
1	Polymyxin E2	500	5.85	776.41
	Polymyxin E3		6.77	43.15
	Polymyxin E1-I		10.91	5.07
	Polymyxin E1		13.79	290.6
	Polymyxin E1-7MOA		15.33	42.54
2	Polymyxin E2	500	5.86	774.97
	Polymyxin E3		6.78	42.94
	Polymyxin E1-I		10.91	5.1
	Polymyxin E1		13.8	290.7
	Polymyxin E1-7MOA		15.36	42.52
3	Polymyxin E2	500	5.86	775.78
	Polymyxin E3		6.77	43.18
	Polymyxin E1-I		10.9	5.09
	Polymyxin E1		13.79	290.92
	Polymyxin E1-7MOA		15.34	42.50
4	Polymyxin E2	500	5.86	776.33
	Polymyxin E3		6.77	43.17
	Polymyxin E1-I		10.88	5.12
	Polymyxin E1		13.78	290.1
	Polymyxin E1-7MOA		15.34	42.56
5	Polymyxin E2	500	5.85	776.53
	Polymyxin E3		6.76	43.26
	Polymyxin E1-I		10.87	5.03

	Polymyxin E1		13.77	290.64
	Polymyxin E1-7MOA		15.32	42.52
	Polymyxin E2		5.86	776.79
	Polymyxin E3		6.77	43.18
6	Polymyxin E1-I	500	10.89	5.09
	Polymyxin E1		13.78	291.07
	Polymyxin E1-7MOA		15.34	42.48

Compound	Injection no.	Retention time	Compound area
Polymyxin E2	1	5.85	776.41
	2	5.86	774.97
	3	5.86	775.78
	4	5.86	776.33
	5	5.85	776.53
	6	5.86	776.79
	Average	5.86	776.14
	STD	0.01	0.66
	RSD	0.09	0.09
Polymyxin E3	1	6.77	43.15
	2	6.78	42.94
	4	6.77	43.17
	5	6.76	43.26
	6	6.77	43.18
	Average	6.77	43.15
	STD	0.01	0.11
	RSD	0.09	0.25
Polymyxin E1-I	1	10.91	5.1
	2	10.91	5.1
	3	10.9	5.09
	4	10.88	5.12
	5	10.87	5.03
	6	10.89	5.09
	Average	10.89	5.0883
	STD	0.02	0.03
	RSD	0.15	0.60
Polymyxin E1	1	13.79	290.6
	2	13.8	290.7
	3	13.79	290.92
	4	13.78	290.1
	5	13.77	290.64
	6	13.78	291.07
	Average	13,785	290,672
	STD	0,0105	0,33289
	RSD	0,0761	0,11453
Polymyxin E1-7MOA	1	15.33	42.54
	2	15.36	42.52
	3	15.34	42.50
	4	15.34	42.56
	5	15.32	42.52
	6	15.34	42.48
	Average	15.33	42.52
	STD	0.01	0.03
	RSD	0.09	0.07

Accuracy

Procedure purpose:

The aim is to prove that the analytical method (sample preparation and chromatographic analysis generates results which are closest to those considered as being true. The study of accuracy is applied to synthetic mixtures of the drug product components to which known quantities of the analytes have been added.

Samples preparation:

Stock Test solution, 1000 ppm – In volumetric flask, quality A class, 50 mL are transfered 100 mg of ***Colidem 50***, finished product, added to volume with sample solvent. It is sonicate 1 minute for homogenization.

Accuracy solution 1 - 400 ppm:

Pipette 4 ml ***Stock Test solution*** in a 10ml A class volumetric flask, and complete to volume with ***Sample solvent***. Sonicated 1 minute or homogenisation.

Accuracy solution 2 - 450 ppm:

Pipette 4,5 ml ***Stock Test solution,*** in a 10 ml A class volumetric flask, and complete to volume with ***Sample solvent***. Sonicated 1 minute for homogenisation.

Accuracy solution 3 - 500 ppm:

Pipette 5 ml ***Stock Test solution,*** in a 10 ml A class volumetric flask, complete to volume with ***Sample solvent***. Sonicate 1 minute for homogenisation.

Accuracy solution 4 - 550 ppm:

Pipette 5,5 ml ***Stock Test solution*** in a 10 ml A class volumetric flask, complete to volume with ***Sample solution***. Sonicate 1 minute for homogenisation.

Accuracy solution 5 - 600 ppm:

Pipette 6 ml ***Stock Test solution*** in a 10 ml, A class volumetric flask, and complete to volume with ***Sample solvent***. Sonicate 1 minute for homogenisation.

Working method

Each ***accuracy solutions (1-5)*** is injected for three times and the obtained peaks of ***Colistine sulphate (Polymyxins E1, E2, E3, E1-I, E1-7MOA),*** are integrated. The mean peak area, standard deviation and relative standard deviation (%) for each peak of the polymyxins are calculated.

The ***Recovery,*** R, for each accuracy solution is calculated using the relation: solution using the relation:

R(%) = Cexp/Cteor*100

It is expressed the percentage ratio between the analytes quantity experimental determined $C_{exp.}$ and nominal value $C_{teor.}$ (theoretical).

Acceptance criteria:

The ***Recovery value*** for ***Colistine sulphate (Polymyxins E1, E2, E3, E1-I, E1-7MOA),*** should be in range 95.0-105.0%

Conclusions and results

In the next table was presented the theoretical values and the experimental values for each accuracy solution and the recovery for these.

Recovery calculation for four accuracy solutions				
400 ppm – Theoretical values				
Polymyxin E2	**Polymyxin E3**	**Polymyxin E1-I**	**Polymyxin E1**	**Polymyxin E1-7MOA**
1037.99	46.66	4.31	392.6	51.8
400 ppm – Experimental values				
Polymyxin E2	**Polymyxin E3**	**Polymyxin E1-I**	**Polymyxin E1**	**Polymyxin E1-7MOA**
1030.1	46.02	4.13	386.11	50.2
Recovery -400 ppm				
Polymyxin E2	**Polymyxin E3**	**Polymyxin E1-I**	**Polymyxin E1**	**Polymyxin E1-7MOA**
99.23988 %	98.62838 %	95.82367 %	98.34692 %	96.9112 %
450 ppm - Theoretical values				
Polymyxin E2	**Polymyxin E3**	**Polymyxin E1-I**	**Polymyxin E1**	**Polymyxin E1-7MOA**
1167.74	52.49	4.85	441.7	58.28
450 ppm - Experimental values				
Polymyxin E2	**Polymyxin E3**	**Polymyxin E1-I**	**Polymyxin E1**	**Polymyxin E1-7MOA**
1162.3	51.91	4.81	437.68	57.05
Recovery - 450 ppm				
Polymyxin E2	**Polymyxin E3**	**Polymyxin E1-I**	**Polymyxin E1**	**Polymyxin E1-7MOA**
99.53414 %	98.89503 %	99.17526 %	99.08988 %	97.8895 %
550 ppm - Theoretical values				
Polymyxin E2	**Polymyxin E3**	**Polymyxin E1-I**	**Polymyxin E1**	**Polymyxin E1-7MOA**
1427.24	64.15	5.93	539.85	71.23
550 ppm - Experimental values				
Polymyxin E2	**Polymyxin E3**	**Polymyxin E1-I**	**Polymyxin E1**	**Polymyxin E1-7MOA**
1435.98	64.87	6.25	543.88	71.97
Recovery - 550 ppm				
Polymyxin E2	**Polymyxin E3**	**Polymyxin E1-I**	**Polymyxin E1**	**Polymyxin E1-7MOA**
100.6124 %	101.1224 %	104.5531 %	100.7465 %	101.0389 %
600 ppm - Theoretical values				
Polymyxin E2	**Polymyxin E3**	**Polymyxin E1-I**	**Polymyxin E1**	**Polymyxin E1-7MOA**
1556.99	69.99	6.47	588.93	77.7
600 ppm - Experimental values				
Polymyxin E2	**Polymyxin E3**	**Polymyxin E1-I**	**Polymyxin E1**	**Polymyxin E1-7MOA**
1560.23	71.25	6.62	592.6	79.05
Recovery - 600 ppm				
Polymyxin E2	**Polymyxin E3**	**Polymyxin E1-I**	**Polymyxin E1**	**Polymyxin E1-7MOA**
100.2081 %	101.8003 %	102.3184 %	100.6232 %	101.7375 %

From experimental data which were presented above results that the chromatographic method is accuracy and retrieval efficiency is situated in range of 95 ÷105%.

Robustness

Procedure purpose:

The procedure is designed to demonstrate that the results of the analytical method are not influenced by small variations of the experimental parameters.

The investigated operational parameters for the proposed chromatographic method are the following:

A: The length of chromatographic column influence;

B: pH of the Solvent A (aqueous solvent), in mobile phase, influence;

C: The mobile phase composition influence;

D: Modify the ionic force of the Solvent A (aqueous solvent);

E: The temperature of the chromatographic column influence;

F: The flow of mobile phase influence;

G: The injection volume influence about the chromatographic separation;

Sample preparation:

A1 variant:

Test solution, 500 ppm – In volumetric flask, quality A class, 50 ml are transferred 50 mg of Colidem 50, finished product, added to volume with sample solvent. It is sonicate 1 minute for homogenization.

A: The length of chromatographic column:

The following chromatographic column have been used in order to study the influence of the length of the chromatographic columns:

A1- Kromasil 100 end – capped octadecylsilyl silica gel for chromatography R (5 µm) with 150 mm length.

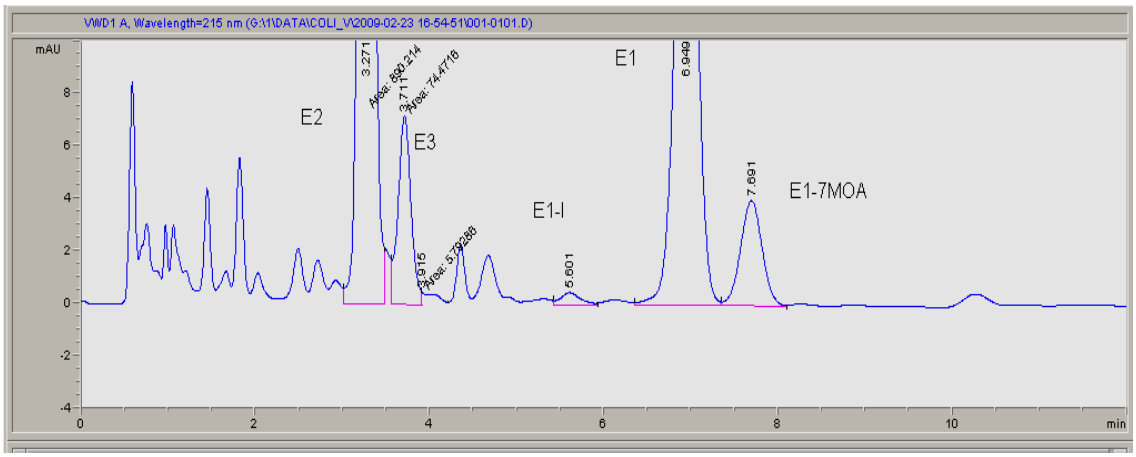
A2- Kromasil 100 end – capped octadecylsilyl silica gel for chromatography R (5 µm) with 250 mm length.

Working method:

Apply the operational parameters described in the proposed method, using, first, the column of variant ***A1*** and after that, the column of variant 2. Allow the chromatographic system to equilibrate for each of these columns.

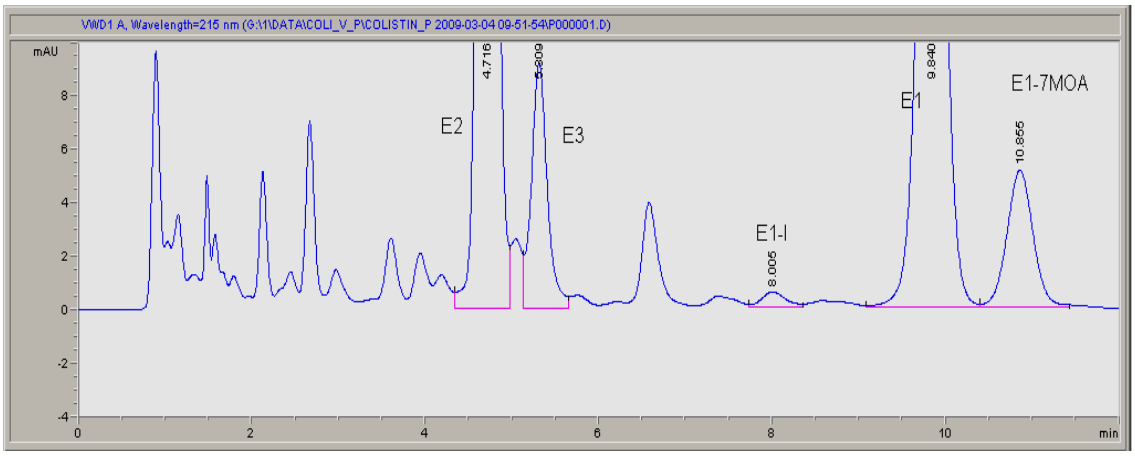
For each case, injected ***Test solution*** and record the chromatograms.

Conclusions and results:



#	Time	Area	Height	Width	Area%	Symmetry
1	3.271	890.2	102.8	0.1444	60.868	0.969
2	3.711	74.5	7.2	0.172	5.092	0.948
3	3.915	5.8	4.7E-1	0.1795	0.396	0
4	5.601	8.4	5E-1	0.2425	0.571	0.667
5	6.949	413.6	25.6	0.2498	28.278	1.068
6	7.691	70.1	4	0.2695	4.794	0.997

A2 variant:



#	Time	Area	Height	Width	Area%	Symmetry
1	4.716	1365.5	128.9	0.1643	60.534	1.004
2	5.309	117.5	9.2	0.1912	5.210	0.851
3	8.005	12.8	6.2E-1	0.3094	0.569	0.786
4	9.84	638.1	32.6	0.3038	28.288	1.024
5	10.855	108.3	5.1	0.3244	4.803	0.953
6	14.237	13.4	5.6E-1	0.3831	0.596	0.911

As expected, the use of chromatographic column of 250 mm length , Kromasil 100 end – capped octadecylsilyl silica gel for chromatography R (3,5µm) instead of the chromatographic column of 150 mm, Kromasil 100 end – capped octadecylsilyl silica gel for chromatography R (5 µm), leads to an increase of retention times of the compounds.

The obtained results are presented in the above in a integration report:

Kromasil – C18, (5µm), 150 mm length					Kromasil – C18, (5µm), 250 mm length				
Retention time					Retention time				
E2	E3	E1-I	E1	E1-7MOA	E2	E3	E1-I	E1	E1-7MOA
3.3	3.7	5.6	7	7.7	4.7	5.3	8.0	9.8	10.9
Resolution					Resolution				
E2	E3	E1-I	E1	E1-7MOA	E2	E3	E1-I	E1	E1-7MOA
-	1.81	5.54	3.22	1.71	-	2.02	6.43	3.57	1.95

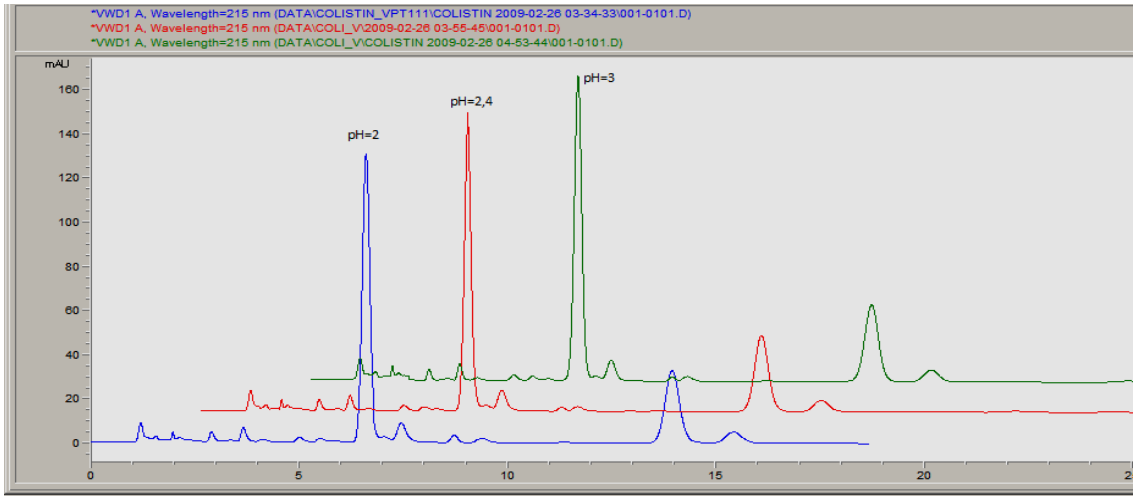
A. pH of the Solvent A, in mobile phase, influence;

Working method:

For evaluated the pH influence of the Solvent A, in mobile phase, have been prepared the Solvent A, in following mode:

- dissolve 4.46 g of *anhydrous sodium sulphate R* in 900 ml of *water R*,
- adjust to pH 2.4 with *dilute phosphoric acid R* and
- dilute to 1000 ml with *water R*. For each case (the **Solvent A** had pH = 2, pH = 2.4, pH =3, injected ***Test solution*** and
- record the chromatograms.

Thus as is observing on overlapped chromatograms used the ***Solvent A*** with pH 2.0, 2.4 and 3.0, the retention times and the resolution, corresponding for colistine sulphate peaks, was insignificant modified, was not modify nor chromatographic separation (see the below integration report).



1. Integration report for Solvent A with pH = 3

RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
6.393	1.56	1757.45251	135.08139	0.99	0.1966	5861	-	-
7.217	1.90	140.06483	9.41997	0.83	0.2184	6049	2.33	1.21
9.015	2.62	37.44241	2.10445	0.72	0.2645	6435	4.37	1.38
13.434	4.39	817.08990	34.53968	0.97	0.3616	7648	8.29	1.68
14.849	4.96	136.93440	5.24503	0.88	0.3980	7713	2.19	1.13

2. Integration report for Solvent A with pH = 2.4

RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
6.392	1.56	1766.91101	137.74971	1.03	0.1941	6006	-	-
7.209	1.89	135.42654	9.42723	0.90	0.2135	6314	2.35	1.21
9.019	2.62	39.42259	2.31080	0.83	0.2621	6560	4.47	1.38
13.445	4.39	823.60114	34.80005	1.03	0.3616	7661	8.34	1.68
14.864	4.96	136.04973	5.34735	0.98	0.3883	8119	2.22	1.13

3. Integration report for Solvent A with pH = 2

RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
6.199	3.14	1787.43555	149.15390	0.91	0.1796	6602	-	-
6.974	3.66	138.98813	10.30169	0.91	0.1974	6916	2.41	1.16
8.414	4.63	43.69227	2.61457	0.74	0.2378	6935	3.89	1.26
12.652	7.46	823.54926	39.02324	0.97	0.3227	8513	8.88	1.61
13.956	8.33	146.08450	5.90403	0.92	0.3640	8143	2.23	1.12

System suitability: minimum 8.0 between the peaks due to polymyxin E2 and polymyxin E1, minimum 6.0 between the peaks due to polymyxin E2 and polymyxin E1-I, minimum 2.5 between the peaks due to polymyxin E1-I and polymyxin E1, minimum 1.5 between the peaks due to polymyxin E1 and polymyxin E1-7MOA;

C: Influnce of the variation of mobile phase composition. Working method:

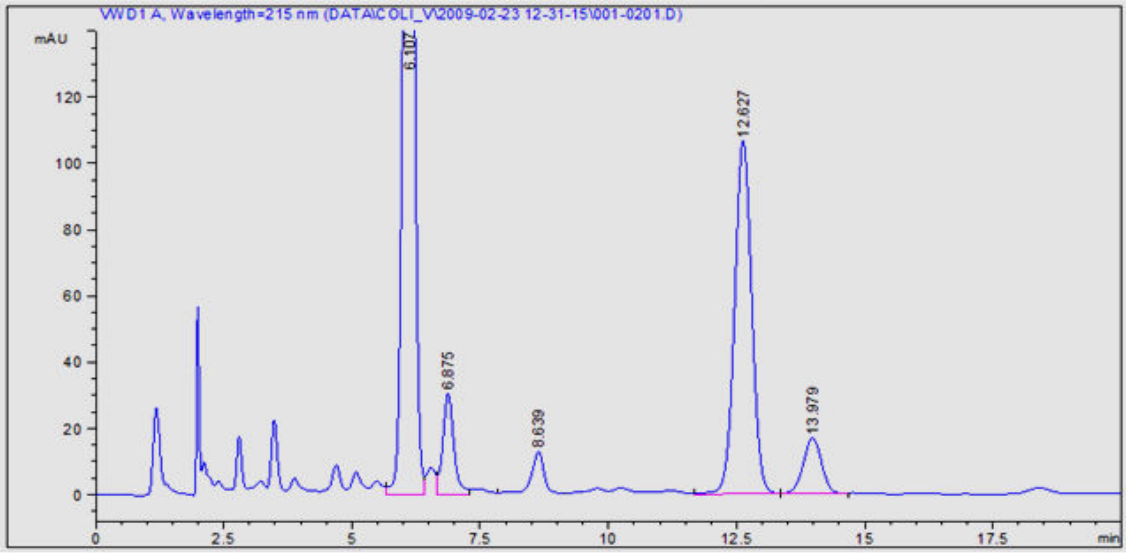
This parameter was evaluated in order to demonstrate that the method is not critically affected by small variations in the composition of the mobile phase.

Operate the system according to the operational parameters described in the method. Use consecutively, the different mobile phase compositions, allowing each time the system to equilibrate.

For each case, inject 20 µl of ***Test solution*** and record the chromatogram.

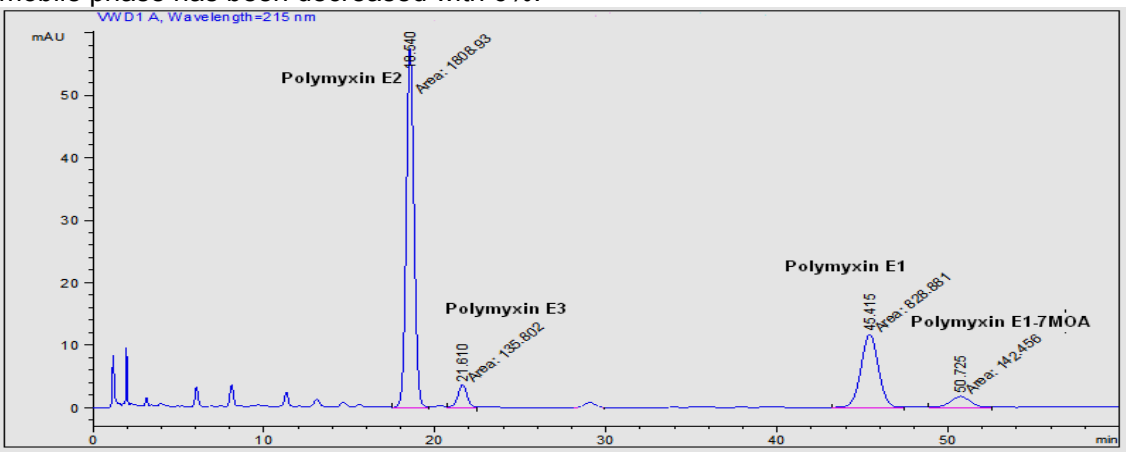
The following chromatograms represent:

1. The chromatogram obtained for normal working conditions



RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
6.107	3.08	5254.75439	412.82892	1.03	0.1917	5623	-	-
6.875	3.60	457.99588	30.52291	0.86	0.2135	5743	2.23	1.17
8.639	4.78	234.83725	12.88445	1.36	0.2354	7462	4.62	1.33
12.627	7.44	2476.41406	106.60281	0.98	0.3543	7037	7.95	1.56
13.979	8.35	419.83685	16.77558	0.99	0.3810	7458	2.16	1.12

2. The chromatogram obtained when the concentration of the organic solvent in mobile phase has been decreased with 6%.

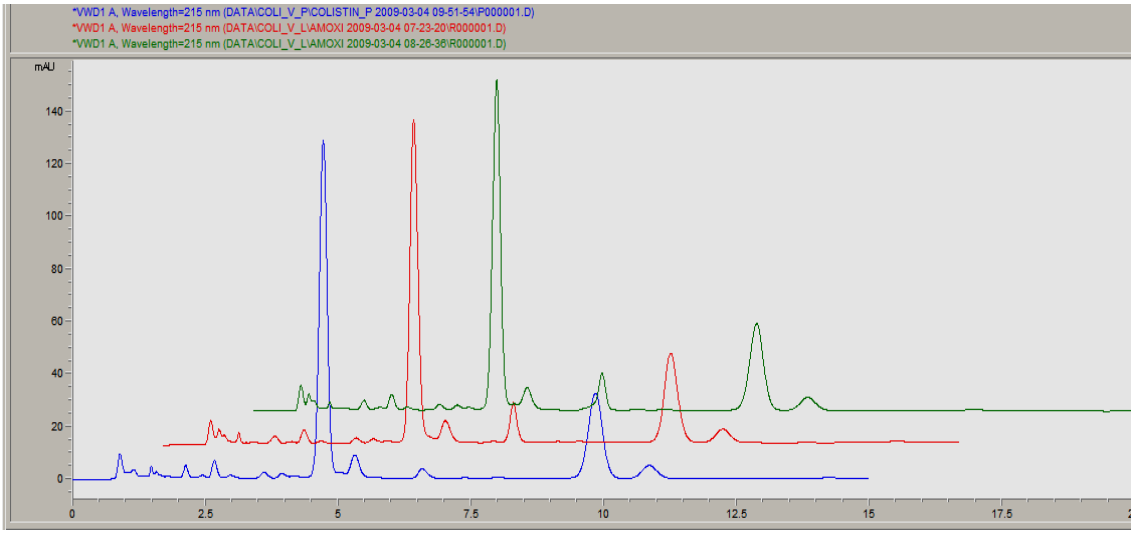


RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
18.540	11.40	1808.93262	57.40648	0.91	0.4805	8249	-	-
21.610	13.45	135.80232	3.66416	1.05	0.5484	8602	3.51	1.18
29.048	18.42	35.22672	8.13555e-1	1.04	0.6843	9982	7.09	1.37
45.415	29.36	828.88068	11.68243	1.00	1.0871	9668	10.86	1.59
50.725	32.91	142.45592	1.77801	1.03	1.2279	9454	2.70	1.12

Result, by the experimental date that a concentration increase of the Solvent A, in mobile phase composition, lead at a significant increase for retention time at the five compounds, in elution order Polymyxins E2, E3, E1-I, E1, E1-7MOA, for colistine sulphate, and it is modify, in the same time, the chromatographic separation quality, in increase sense for these.

D: Modify the ionic force of the Solvent A;

At a pH=2.4 for Solvent A, through modification of the concentration for 4.46g *anhydrous sodium sulphate R* in this way: 2.23g/l, 4.46g/l,6.69g/l, the other parameters remain unchanged, was obtained following experimental date: of the overlapping chromatograms and the report of the integration analyze was observed that the retention times and the chromatographic separation quality are insignificant modified.



The Integration report for concentration of the 6.69 g/l *anhydrous sodium sulphate R* in Solvent A, was following:

RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
4.577	1.39	1347.50208	125.70193	1.05	0.1634	4346	-	-
5.149	1.69	114.41441	8.63543	0.85	0.1893	4099	1.91	1.22
6.561	2.42	157.03004	14.11859	1.40	0.1440	11505	4.98	1.44
9.474	3.94	623.16962	33.16745	1.06	0.2863	6064	7.95	1.63
10.434	4.44	107.22754	5.04837	0.95	0.3227	5790	1.85	1.13

The Integration report for concentration of the 4.46 g/l *anhydrous sodium sulphate R* in Solvent A, was following:

RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
4.716	1.46	1353.99609	128.61497	1.00	0.1602	4803	-	-
5.309	1.77	109.49885	8.92949	0.88	0.1796	4842	2.05	1.21
6.577	2.43	48.47829	3.83526	0.76	0.1796	7432	4.15	1.37
9.840	4.13	630.15387	32.53465	1.02	0.2961	6121	8.06	1.70
10.855	4.66	105.84961	5.09679	0.93	0.3155	6559	1.95	1.13

The Integration report for concentration of the 2.23 g/l *anhydrous sodium sulphate R* in Solvent A, was following:

RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
4.716	1.46	1372.18921	123.65763	0.86	0.1650	4524	-	-
5.315	1.77	122.70803	8.89562	0.87	0.1990	3953	1.94	1.21
6.592	2.44	161.07330	15.65429	0.88	0.1440	11614	4.37	1.38
9.554	3.98	630.60358	34.26896	0.90	0.2766	6608	8.27	1.63
10.535	4.49	108.65997	5.23148	0.96	0.3106	6373	1.96	1.13

Conclusion:

The concentration of the *anhydrous sodium sulphate R* in Solvent A did not change nor the retention times for compounds Polymyxins E1, E2, E3, E1-I, E1-7MOA nor the resolution between the compounds.

E: The temperature of the chromatographic column influence;

At 40°C:

Parameters analysed	E2	E3	E1-I	E1	E1-7MOA
Retention time	5.0897	6.656	9.306	12.415	13.729
Resolution		1.97	6.51	6.27	1.97

At 30°C:

Parameters analysed	E2	E3	E1-I	E1	E1-7MOA
Retention time	6.107	6.875	8.639	12.627	13.979
Resolution		2.23	4.62	7.95	2.16

At 20°C:

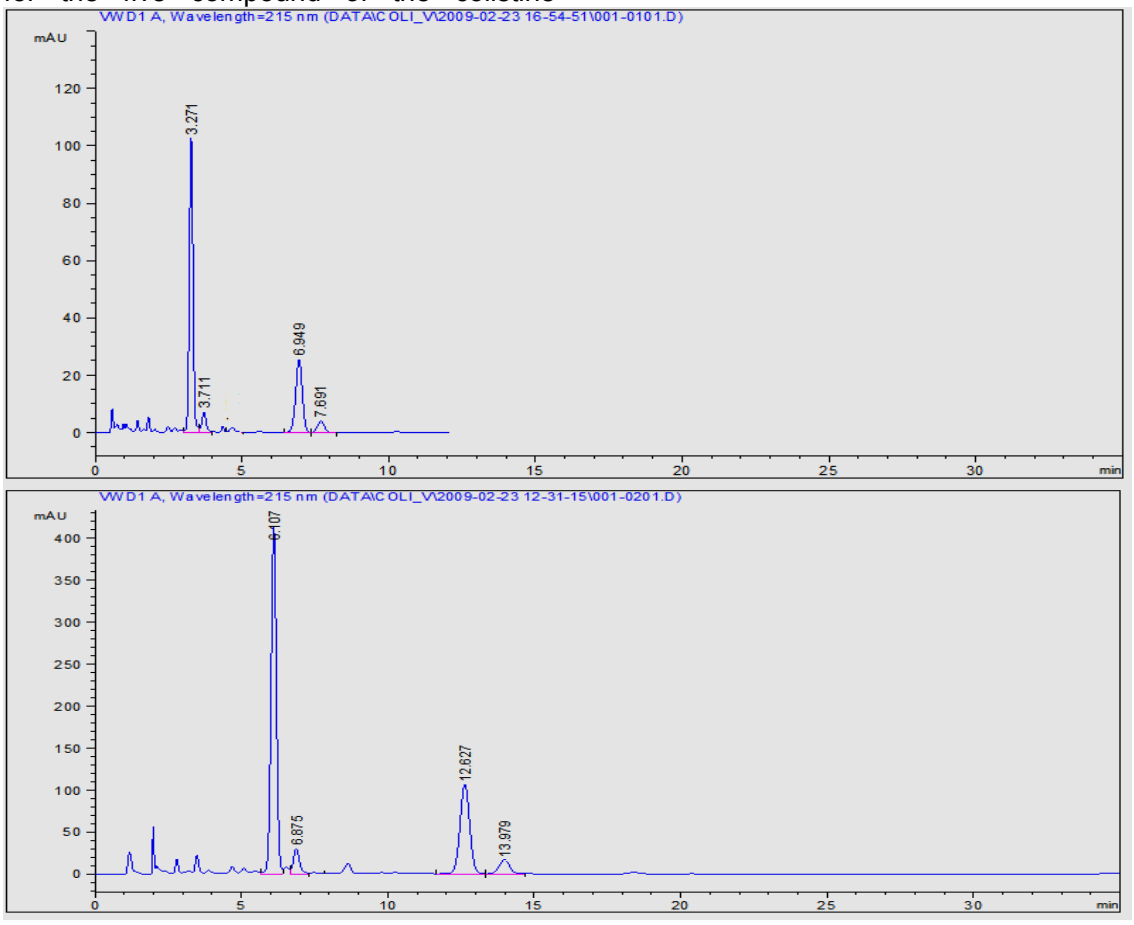
Parameters analysed	E2	E3	E1-I	E1	E1-7MOA
Retention time	6.199	6.974	8.414	12.652	13.956
Resolution	-	2.41	3.89	8.88	2.23

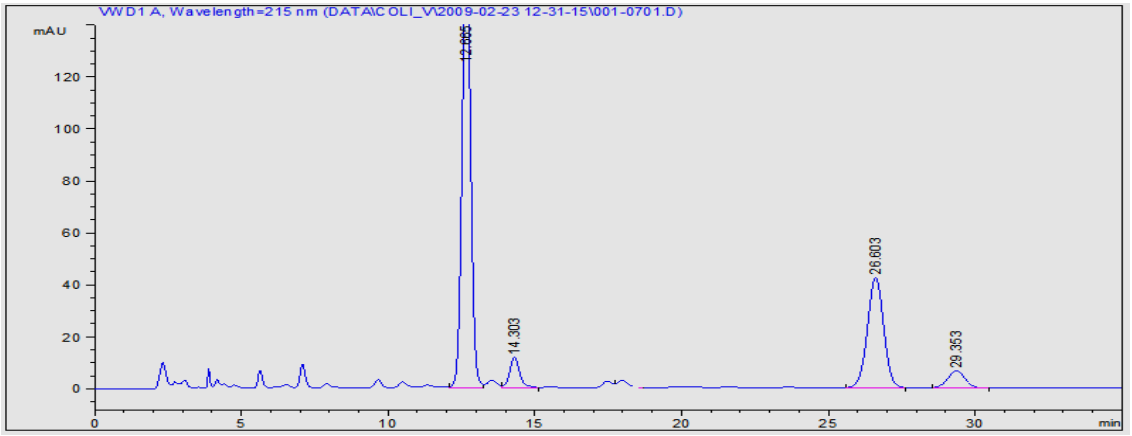
Conclusion: The influence of the temperature on the chromatographic separation is very small but the resolution increase at once with the decrease chromatographic column temperature.

F: The flow of mobile phase influence;

As it was expected, the retention time for the five compound of the colistine sulphate (Polymyxins E1, E2, E3, E1-I, E1-7MOA) are modify function to the flow of the mobile phase, without significant modify for the chromatographic separation quality.

The Chromatograms obtained and the integration report afferent is presented below:





RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
3.271	3.37	894.01123	102.56067	0.94	0.1323	3389	-	-
3.711	3.96	71.28017	7.01757	0.94	0.1505	3370	1.83	1.17
4.671	5.25	20.36927	1.76851	0.83	0.1650	4439	3.58	1.32
6.949	8.29	405.73386	25.45714	1.06	0.2451	4453	6.53	1.58
7.691	9.28	66.71747	3.95955	0.98	0.2597	4861	1.73	1.12

RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
6.107	3.08	5254.75439	412.82892	1.03	0.1917	5623	-	-
6.875	3.60	457.99588	30.52291	0.86	0.2135	5743	2.23	1.17
8.639	4.78	234.83725	12.88445	1.36	0.2354	7462	4.62	1.33
12.627	7.44	2476.41406	106.60281	0.98	0.3543	7037	7.95	1.56
13.979	8.35	419.83685	16.77558	0.99	0.3810	7458	2.16	1.12

RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
12.665	3.23	3515.85181	174.33182	0.97	0.3058	9505	-	-
14.303	3.78	273.08047	11.59746	0.88	0.3446	9544	2.96	1.17
17.978	5.01	78.38828	2.82908	0.81	0.4805	7756	5.23	1.32
29.353	8.81	268.96704	6.55640	0.99	0.6309	11991	12.03	1.76

G: The injection volum influence about the chromatographic separation;

For a injection volume from the 60µl:

Parameter analysed	E2	E3	E1-I	E1	E1-7MOA
Retention time	6.272	7.053	8.467	12.744	14.036
Resolution	-	2.36	3.49	8.52	2.28

For a injection volume from the 20µl:

Parameter analysed	E2	E3	E1-I	E1	E1-7MOA
Retention time	6.199	6.974	8.414	12.652	13.956
Resolution	-	2.41	3.89	8.88	2.23

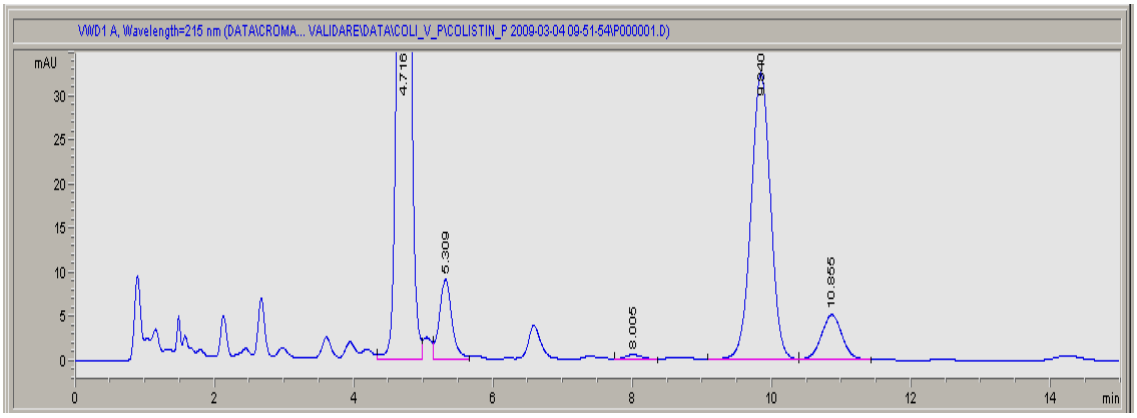
From the experimental date presented above, result that the injection volume has an influence about the chromatographic separation and as expected with decreasing the injection volume the resolution increasing.

The solution stability

It was demonstrated the stability solution through injected the ***System suitability solution*** which represent otherwise the ***Reference solution*** for quantitative and qualitative determination for those five compound of the colistine sulphate (Polymyxins E1, E2, E3, E1-I, E1-7MOA). As how resulted from the chromatograms and the afferent integration report from these, at 12 days after preparation, maintained at 2-8°C, respectively at the ambient temperature the System suitability solution is not damaged.

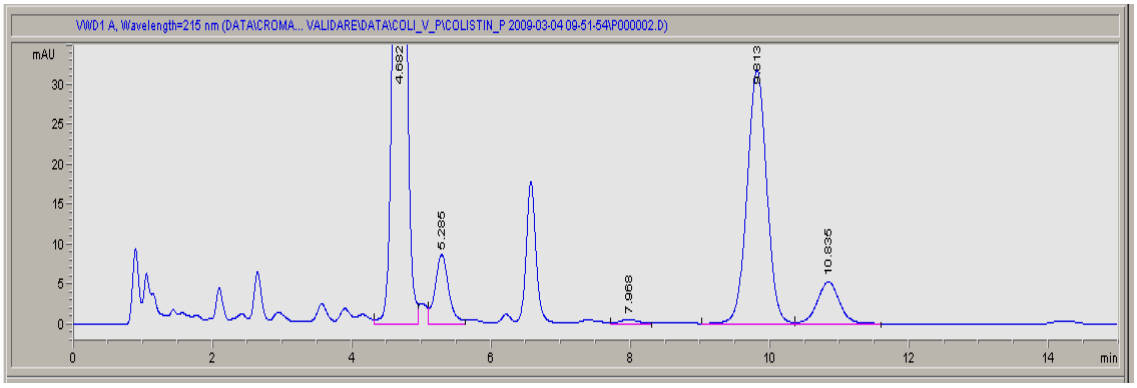
The chromatogram and the integration report, obtained after injecting the ***System***

suitability solution at 12 days from preparation. The solution was maintained at 2-8°C.



#	Time	Area	Height	Width	Symmetry
1	4.716	1365.5	128.9	0.1643	1.004
2	5.309	117.5	9.2	0.1912	0.851
3	8.005	12.8	6.2E-1	0.3094	0.786
4	9.84	638.1	32.6	0.3038	1.024
5	10.855	108.3	5.1	0.3244	0.953

The next chromatogram was obtained after injecting the ***System suitability*** ***solution*** stored 12 days, at room temperature.



#	Time	Area	Height	Width	Area%	Symmetry
1	4.682	1353.4	124.9	0.1672	60.392	0.992
2	5.285	118.8	8.8	0.2035	5.299	0.864
3	7.968	13.2	6.2E-1	0.3092	0.588	0.801
4	9.813	633.1	31.9	0.3073	28.248	1.014
5	10.835	122.6	5.3	0.3462	5.472	0.936

End conclusion:

The proposed analytical method used for API identification assay and relative substances, from pharmaceutical product ***Colidem 50*** – *hydrosoluble powder*, is considerate validated and can be used for the proposed aim.

Bibliography

ICH –Q2A – Text on Validation of Analytical Procedure

ICH-Q2B – Validation of analytical Procedure: Methodology

CPMP/ICH/381/-ICH Q(R1)