Development and validation of a HPLC method for the determination of amoxicillin trihydrate, colistin sulphate, nipasol and nipagin in an injectable suspension

Dezvoltarea și validarea unei metode HPLC pentru determinarea amoxicilinei trihidrat, colistinului sulfat, nipaginului și nipasolului dintr-o suspensie injectabilă

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Cuvinte cheie: *amoxicilina trihidrat, colistin sulfat, nipagin, nipasol, metoda HPLC.*

Abstract

A liquid chromatographic method has been developed and validated for simultaneous determination of amoxicillin trihydrate, colistin sulphate, nipasol and nipagin in injectable suspension. Efficient chromatographic separation was achived on a Hypersil Gold (150mm x 4.6mm, 5.0 µm) with mobile phase containing 4.46 g‰, pH 2.5 (adjusted with dilute sulphuric acid) in gradient with acetonitrile at a flow rate of 1.0 mL/min. detection of the analyses was performed at different wavelengths using a DAD detector. The elution was a seven step gradient elution program in 42 minutes. The proposed HPLC method was statistically validated with respect to specificity, linearity, limits of detection and quantification, ranges, precision and accuracy. The HPLC method was applied to injectable suspension in which the analyses were successfully quantified with no interfering peaks from excipients.

Rezumat

A fost dezvoltata si validată o metoda cromatografică de determinare simultană a amoxicilinei trihidrat, colistinului sulfat, nipaginului și nipasolului dintr-o suspensie injectabilă. Separarea cromatografică eficientă a fost realizata pe o coloana Hypersil Gold (150mm x 4.6mm, 5.0 µm) cu o faza mobile formata din sulfat de sodiu 4.46 g‰, pH 2.5 (ajustat cu acid sulfuric diluat) și acetonitril, folosind un debit de 1 mL/min. Detecția compusilor s-a facut la diferite lungimi de undă folosid un detector DAD. Eluția s-a făcut în 42 de minute cu un gradient în sapte etape. Metoda HPLC propusă a fost validată urmarind parametrii: specificitate, liniaritate, limită de detecție și de cuantificare, precizie si acuratețe. Metoda HPLC a fost aplicată unei suspensii injectabile în care analiții au fost cuantificați cu success fără interferențe din partea excipientilor.

Introduction

Amoxicillin is a broad-spectrum low-toxic synthesis penicillin with strong bactericide effect.

Colistin sulphate (polymyxin class) is an elution antibiotic for Gram-negative germs.

The mixture of these two antibiotics is indicated in acute, primary or secondary infections: colibacillosis, salmonellosis, sepsis, infectious enteritis, urogenital infections.

One milliliter of injectable suspension contains various amounts of active substances, namely 100 mg amoxicillin trihydrate and 12.5 mg colistin sulphate.

Therefore, the main objective of this study was to develop a method for the quantification of both active substances and nipagin and nipasol at the same time.

After validation, the method has been successfully used for the analysis of Amoxicolistin -suspension for injection.

1. Materials and method

1.1.Reference materials and reagents

Amoxicillin trihydrate, colistin sulphate, nipagin, nipasol standards were purchased from the European Pharmacopeia.

The pharmaceutical product in the study, Amoxicolistin – suspension for injection was provided by Romvac Company.

- Ultrapure water obtained with a Milli-Q Integral (Merck Millipore) system was used for the preparation of all solutions.
- The HPLC-grade Acetonitrile was provided by Merck.
- Sodium sulphate was provided by Sigma.
- Sulfuric acid 50% used for pH correction was provided by Fluka.

1.2. Chromatographic system and conditions

The chromatographic system Ultimate 3000 Thermo Scientific LC Surveyor (Thermo Electron Corporation, USA) consists of high pressure quaternary pump provided with degasser, auto sampler with 100 µL loop, column thermostat, auto sampler thermostat and UV-VIS– diode array detector.

The entire chromatographic system is controlled with the Chromeleon soft.

The chromatographic separation was carried out on Hypersil Gold, L= 150 mm, ID=4,6 mm, 5 µm column. The mobile phase consists of a mixture of acetonitrile: sodium sulphate 4.46 g‰, pH=2.5 with sulfuric acid.

The 1 mL/min flow and 10 µL injection volume are the parameters used in this method.

1.3 Preparation of standard stock solutions

Standard stock solution of amoxicillin trihydrate (1 mg/mL) – In a 10 ml flask, weigh 10 mg amoxicillin trihydrate, add 4 mL water, dissolve and then bring to volume with water. Ultrasonate the solution for homogenization.

Standard stock solution of colistin sulphate (5 mg/mL) – In a 5 ml flask, weigh 25 mg colistin sulphate, add 4 mL acetonitrile/ water 2/8, dissolve and then bring to volume with the mixture. Ultrasonate the solution for homogenization.

Standard stock solution of nipagin (0.6 mg/mL) – In a 20 ml flask, weigh 12 mg nipagin, add 4 mL methanol, dissolve and then bring to volume with methanol. Ultrasonate the solution for homogenization.

Standard stock solution of nipasol (0.2 mg/mL) – In a 25 ml flask, weigh 5 mg nipasol add 4 mL methanol, dissolve and then bring to volume with methanol. Ultrasonate the solution for homogenization.

1.4. Preparation of samples

Stock solution of Amoxicolistin-suspension for injection.

In a 50 ml flask, dissolve 1 mL suspension for injection, (equal to 100 mg amoxicillin trihydrate, 12.5 mg colistin sulphate, 0.75 mg nipagin and 0.25 mg nipasol), add 25 mL acetonitrile/ water 2 / 8, dissolve and then bring to volume with acetonitrile/ water 2 / 8. Ultrasonate the solution for homogenization.

For the quantitative determination of colistin sulphate, nipagin, nipasol, inject the stock solution of Amoxicolistin as such.

For the quantitative determination of amoxicillin, dilute 1 mL from the stock solution of Amoxicolistin with acetonitrile/ water 2/8 into a 10 mL flask.

Before injecting in the chromatographic system, filter the solution through PVDF 0,45 µm filter.

1.5.Validation of chromatographic method

The validation of HPLC method for the simultaneous determination of amoxicillin trihydrate, colistin sulphate, nipagin and nipasol in the veterinary medicinal product was carried out in accordance with the ICH guidelines.

The assessed parameters were:

- specificity,
- linearity,
- limits of detection and quantification,
- precision and
- accuracy.

Specificity was checked by calculating parameters such as: retention time, theoretical plates, asymmetry and resolution.

The linearity of the proposed method was determined based on the calibration curves obtained by graphical representation of areas vs. active substance concentrations, in the 10% - 200% range.

The obtained calibration curves were used for the determination of limits of detection and quantification.

The precision of the method was checked regarding both repeatability and intermediate reproducibility by calculating the standard deviation.

The accuracy of method can be assessed by the recovery percentage of active substances.

The recovery study was carried out using sample solutions with concentrations in the 80%-120% range.

2. Results and discussions

The development of an HPLC method in order to obtain an efficient separation of the four substances in Amoxicolistin involved various columns, composition and pH of mobile phase.

The initial isocratic separation proved to be insufficient and therefore a gradient was used for a better separation of the active substances.

Table 1 is showing the gradient elution in seven stages.

Table 1

The gradient elution in seven stages

Time (min)	Flow (mL/min)	% solvent B	%solvent C
		acetonitrile	Na_2SO_4
0	1	5	95
20	1	25	75
26	1	25	75
30	1	50	50
33	1	50	50
37	1	5	95
42	1	5	95

The specificity of method was confirmed by the chromatographic separation of both active substances in the presence of excipients. The peaks obtained are well-defined and separated at the baseline, as shown in figure 1.

The identification of active substances was carried out with the soft which enabled the determination of other performance parameters like resolution, asymmetry, retention times and theoretical plates.

The results are shown in table 2.

The calibration curves for amoxicillin trihydrate, colistin sulphate, nipagin and nipasol were drawn under the above experimental conditions in the 10%-200% range against working concentrations.

The statistical calculations made based on calibration curves: regression equation and correlation coefficient are shown in table 3.

Results on precision are shown in table 4, with the observation that the relative standard deviation values are within specified limits (%RSD < 2).

The accuracy, represented by the recovery percentage of the three active substances, is shown in Table 5.

Recovery percentage for amoxicillin trihydrate, colistin sulphate, nipagin and nipasol are higher than 90%.

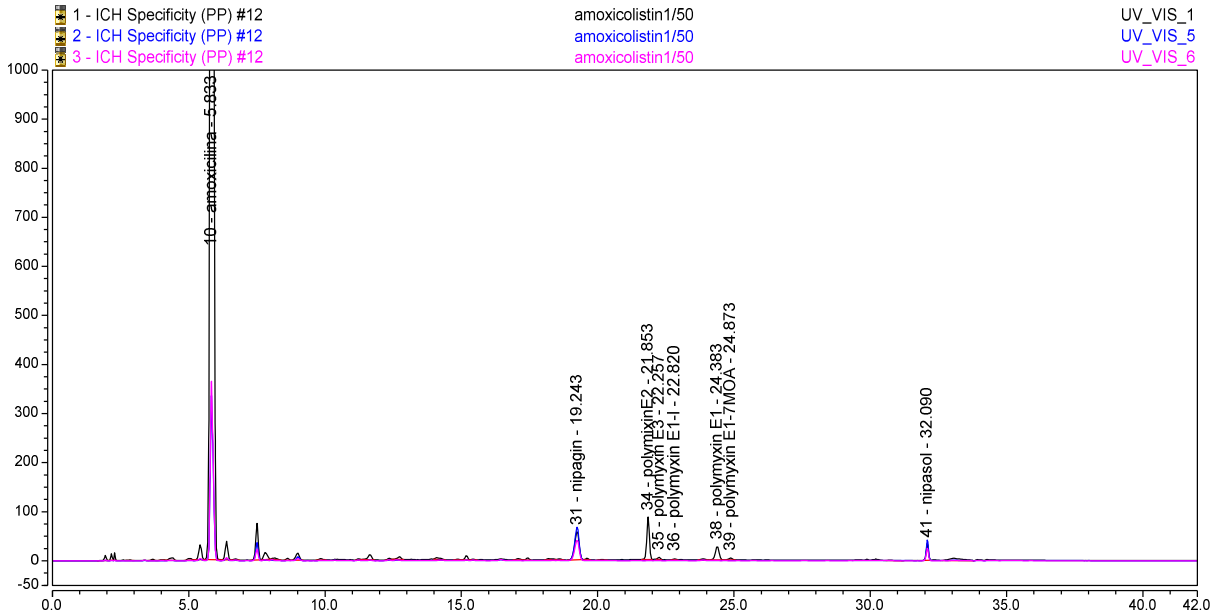

Figure 1. Typical chromatogram of Amoxicolistin –suspension for injection

Table 2

Parameters calculated for the mixture in figure 1.

Peak Name	Retention Time	Resolution (EP)	Asymmetry (EP)	Plates (EP)
	min			
UV_VIS_1	UV_VIS_1	UV_VIS_1	UV_VIS_1	UV_VIS_1
polymixinE2	21.853	2.41	0.95	269142
polymyxin E3	22.257	3.22	0.97	284806
polymyxin E1-I	22.82	4.64	0.92	245353
polymyxin E1	24.383	1.95	0.94	165231
polymyxin E1-7MOA	24.873	20.63	0.93	141957
Peak Name	Retention Time	Resolution (EP)	Asymmetry (EP)	Plates (EP)
	min			
UV_VIS_5	UV_VIS_5	UV_VIS_5	UV_VIS_5	UV_VIS_5
amoxicilina	5.833	3	1.22	13922
Peak Name	Retention Time	Resolution (EP)	Asymmetry (EP)	Plates (EP)
	min			
UV_VIS_6	UV_VIS_6	UV_VIS_6	UV_VIS_6	UV_VIS_6
nipagin	19.243	56.22	0.9	60444
nipasol	32.09	n.a.	0.93	782498

Table 3

Analytical parameters

Peak Name	Regression equation	Corr.Coeff.
Amoxicillin trihydrate	Y = 28.2494 x + 0.1135	0.99552
Polymixin E1	Y = 89.977x -0.7361	0.9992
Polymixin E2	Y = 86.6788x -0.3997	0.9998
Polymixin E3	Y = 49.7700x -0.0188	0.9998
Polymixin E1-I	Y = 88.8748x -0.0550	0.9992
Polymixin E1-7	Y = 88.4039x -0.0162	0.9997
Nipagin	Y = 657.6518x - 0.1883	1.00
Nipasol	Y = 657.6518x - 0.1883	0.998

Table 4

Results on precision

Parameter	Mean retention time	Concentration	%RSD	Admissibility criterion
Amoxicillin trihydrate				
Repeatability	5.842	95.7494	0.4	%RSD<2,0
Reproducibility	5.848	96.1613	0.5	%RSD<2,0
Parameter	**Mean retention time**	**Mean area**	**%RSD**	**Admissibility criterion**
Colistin sulphate				
Repeatability	4,989	12.0301	0.3025	%RSD<2,0
Reproducibility	4,982	12.1296	1.4576	%RSD<2,0
Parameter	**Mean retention time**	**Mean area**	**%RSD**	**Admissibility criterion**
Nipasol				
Repeatability	4,989	0.2387	0.6482	%RSD<2,0
Reproducibility	4,982	0.2411	1.1160	%RSD<2,0
Parameter	**Mean retention time**	**Mean area**	**%RSD**	**Admissibility criterion**
Nipagin				
Repeatability	4,989	0.7217	0.6604	%RSD<2,0
Reproducibility	4,982	0.7220	0.4597	%RSD<2,0

Table 5

Results on accuracy

Concentration level %	Amoxicillin trihydrate concentration * mg/mL	% Amoxicillin trihydrate recovery *	% recovery mean
80	73.6656	92.08	93.86
100	95.016	95.02	
120	113.380	94.48	
Concentration level %	**Colistin sulphate concentration * mg/mL**	**% Colistin sulphate recovery ****	**% recovery mean**
80	9.533	95.33	96.78
100	11.86	94.94	
120	14.011	100.077	
Concentration level %	**Nipagin concentration * mg/mL**	**% Nipagin recovery ****	**% recovery mean**
80	0.57403	95.66	98.92
100	0.7203	96.04	
120	0.84046	105.05	
Concentration level %	**Nipasol concentration * mg/mL**	**% Nipasol recovery ****	**% recovery mean**
80	0.18723	93.59	94.38
100	0.23863	95.45	
120	0.2823	94.11	

* mean of three determinations

3. Conclusions

An HPLC-DAD method was validated in this study for the administration of a suspension for injection containing amoxicillin trihydrate, colistin sulphate, nipagin and nipasol indicated in acute, primary or secondary infections.

The four substances were successfully separated within 42 minutes, using a Hypersil Gold 150x4.6 mm column.

The proposed HPLC method proved to be selective, fast, precise and specific for the simultaneous determination of the two active substances and excipients in Amoxicolistin suspension for injection.

The recovery percentage obtained revealed that the excipients have no influence on determination.

The relative standard deviation values lower than value 2 indicate a high precision degree.

In conclusion, this study proves that the described analytical method can be used for the determination of amoxicillin trihydrate colistin sulphate, nipagin and nipasol in Amoxicolistin – suspension for injection.

References

1. **European Pharmacopoeia 8.0 (2014)**,
2. **Nomenclature of Romvac Products**, 171