The present invention regards a HPLC method useful for analyzing colistimethate sodium (CMS) or for the separation of CMS components. It consists of a HPLC method using a hydrophobic stationary phase and a mobile phase comprising an aqueous buffer and an organic solvent whose concentration in the mobile phase is increased during the elution. The aqueous buffer is preferably a phosphate buffer and the organic solvent preferably acetonitrile.
Characterization of Colistimethate Sodium (CMS)

Field of the Disclosure
The following disclosure enables characterization of Colistimethate Sodium (CMS). The purpose of the disclosure is to develop a method for the chromatographic characterization of CMS.

Background
CMS is manufactured by chemical modification of the antibiotic mixture Colistin, also called Polymyxin E. The conversion of Colistin to CMS involves chemical modification of primary amino groups of Colistin with methyl sulphonate groups (Barnette et al, Brit. J. Pharmacol. (1964), 23, 552). According to the US Pharmacopoeia (USP), CMS consists of only two components with defined molar mass originating from Colistin A (1749.82 Da) and Colistin B (1735.80 Da).

In the European Pharmacopoeia (E.P.), it is stated that Colistin comprises five Polymyxin components (El, E2, El-i, E1-7MOA, and E3). According to the E.P., the sum of the mentioned five components in Colistin should be at least 77%, and El-i, E3 or E1-7MOA should individually not exceed 10%. Hence, the main part of Colistin is Polymyxin E1 and Polymyxin E2 (Colistin A and B, respectively). The ratio between Polymyxin E1 and Polymyxin E2 in Colistin varies depending on manufacturing conditions and the producing strain.

Colistin comprises a cyclic heptapeptide and a tripeptide side chain acylated at the N-terminus by a fatty acid. Besides the components already mentioned, at least 30 different other components have been detected in Colistin (Orwa et al., J. Antibio. (2001). 51, 433) with the main components being Colistin A (Polymyxin El) and B (Polymyxin E2). Colistin A and Colistin B differ in the fatty acid side chain, see Polymyxin E1 in Figure 1.

The prior art is insufficient with respect to providing analytical methods that render it possible to separate and identify the various components of CMS. One HPLC method for the analysis of CMS in water has previously been described in the literature (Li et al, Antimicro. Agents. Chemo (2003) 47, 4). Although the HPLC quality reported by Li et al. was limited, it was possible to investigate how CMS was degraded in vivo and in aqueous solutions. Li et al. shows that CMS is degraded very quickly in solution resulting in a mixture of partly sulphonmethylated Colistin. Although Li et al. was unsuccessful in determining the structure using MS analysis of the peaks in the chromatogram, the group managed to show that all peaks in the chromatogram originated from Colistin A and B.
To the inventor's knowledge, there is hitherto no reliable analytical method available for determining the quality and composition of CMS. According to the requirement of the USP, IR is used to identify the CMS. However, analyzing a sample by IR does not provide any information about modification, degree of substitution, the position of substituents, degradation etc., or the actual composition of the CMS. In addition, a microbial assay is used to ensure that the sample shows a required level of antimicrobial potency. However, free Colistin also exerts antimicrobial activity, i.e. in general reporting having higher potency compared with the potency measured for CMS. Free Colistin furthermore is in general reported to be more toxic and lack the prodrug characteristics of CMS. Thus, there are limited knowledge of the actual quality, stability and the composition of CMS.

The complex composition of Colistin and the different possibilities for substitution makes characterization of CMS difficult. During the analytical development of an HPLC method, it has been discovered that fresh solutions of CMS consist of more than 100 different components (see Figure 4). Thus, the HPLC profile described by Li et al. in 2003 seems incomplete and is only showing a minor part of the actual components and composition of CMS.

Bai et al. J Chromatograph Separat Techniq 2011, 2:1 mentions a HPLC method for separation of two CMS components involving an acidic mobile phase comprising TFA. Aqueous solutions comprising 0.05% TFA have a pH of ca 2.2 (Heyrman and Henry, Keystone Technical Bulletin TB99-06, Importance of Controlling Mobile Phase pH in Reversed Phase HPLC).

Due to the lack of proper analytical tools enabling characterization of the quality and composition of CMS preparation, there are considerable concerns about dosing and safety of some CMS product (see e.g. the NAN Alert, "Risk of serious or fatal medication error", 29th June 2011). Thus, there is a staggering need for an analytical method useful in determining the content and quality of CMS compositions.

Summary

The present disclosure concerns the characterization of CMS using HPLC. It also provides an analytical method for determining the quality and stability of a CMS preparation thereby ensuring that CMS is not degraded during manufacturing, handling and storage, and that the manufacturing conditions used provides a CMS preparation with the desired prodrug characteristics.

The present disclosure concerns a chromatographic method for separating components of CMS.

In particular, a chromatographic method for separating components of CMS is provided, comprising loading of a sample comprising CMS onto a stationary phase,
and eluting with a mobile phase; wherein the mobile phase comprises 50-95% v/v aqueous buffer with pH 5-8 and 5-50% of an organic solvent and initially has a low content of organic solvent which is increased to a higher level during the elution.

In one preferred embodiment the method is a HPLC method with a binary mobile phase system and a hydrophobic resin.

According to one embodiment, a HPLC method for separating CMS components comprises loading of a sample comprising CMS onto a stationary phase and eluting with a mobile phase, wherein

- the mobile phase comprises a mixture of solvent A and solvent B, wherein solvent A comprises 90-99% v/v phosphate buffer and 1-10% v/v acetonitrile and wherein solvent B comprises 40-60% v/v phosphate buffer and 40-60% v/v acetonitrile; and
- wherein the mobile phase initially comprises 70-90% v/v of solvent A and 10-30% v/v of solvent B; and wherein
- the mobile phase composition is gradually changed to 40-60% v/v of solvent A and 40-60% v/v of solvent B; and
- wherein the stationary phase is a hydrophobic resin.

According to one aspect of this embodiment, solvent A comprises 92-97% v/v phosphate buffer and 3-8% v/v acetonitrile.

According to another aspect of this embodiment, solvent A comprises 95% v/v phosphate buffer and 5% v/v acetonitrile.

According to another aspect of this embodiment, solvent B comprises 45-55% v/v phosphate buffer and 45-55% v/v acetonitrile.

According to another aspect of this embodiment, solvent B comprises ca. 50% v/v phosphate buffer and ca. 50% v/v acetonitrile.

According to another aspect of this embodiment, the concentration of the phosphate buffer is 0.01-0.1M.

According to another aspect of this embodiment, the concentration of the phosphate buffer is 0.03-0.07M.

According to another aspect of this embodiment, the concentration of the phosphate buffer is 0.05M.

According to another aspect of this embodiment, the pH of the phosphate buffer is 6-7.
According to another aspect of this embodiment, the pH of the phosphate buffer is 6.3-6.8.

According to another aspect of this embodiment, the pH of the phosphate buffer is 6.5.

According to another aspect of this embodiment, the stationary phase is a hydrophobic C8-C24 resin.

According to another aspect of this embodiment, the stationary phase is a hydrophobic C15-C20 resin.

According to another aspect of this embodiment, the stationary phase is a hydrophobic C18 resin.

According to another aspect of this embodiment, the detection wavelength is 190-230 nm.

According to another aspect of this embodiment, the detection wavelength is 200-220 nm.

According to another aspect of this embodiment, the detection wavelength is 210 nm.

According to another aspect of this embodiment, the column temperature is 20-40 °C.

According to another aspect of this embodiment, the column temperature is 25-35 °C.

According to another aspect of this embodiment, wherein the column temperature is about 30 °C.

According to another aspect of this embodiment, the flow is 0.1-0.60 ml/min.

According to another aspect of this embodiment, the flow is 0.2-0.40 ml/min.

According to another aspect of this embodiment, the flow is about 0.30 ml/min.

According to one embodiment of the disclosure, the particle size of the stationary phase is in the range 0.5-5 micrometer.
According to one preferred embodiment of the disclosure, the particle size of the stationary phase is approximately 1 - 2 micrometers.

According to one preferred embodiment of the disclosure, the particle size of the stationary phase is approximately 1.7 micrometers.

According to one embodiment of the disclosure, the length of the column comprising the stationary phase is more than 5 to 30 cm.

According to one preferred embodiment of the disclosure, the length of the column comprising the stationary phase is approximately 10-20 cm.

According to one preferred embodiment of the disclosure, the length of the column comprising the stationary phase is approximately 15 cm.

According to one embodiment of the disclosure, the diameter of the column comprising the stationary phase is between 1 to 5 mm.

According to one embodiment of the disclosure, the diameter of the column comprising the stationary phase is between 2.1 to 4.6 mm.

According to one embodiment of the disclosure, the diameter of the column comprising the stationary phase is more than 2.1 mm.

According to another aspect of the present disclosure, the loaded sample comprises CMS dissolved in a solution comprising 90-99% v/v methanol and 1-10% v/v water.

According to yet another aspect of the present disclosure, the loaded sample comprises CMS dissolved in a solution comprising 95% v/v methanol and 5% v/v water.

According to another embodiment of the present disclosure, an HPLC method is provided for analyzing CMS comprising a binary gradient system wherein the mobile phase comprises a mixture of:

A) about 95% v/v phosphate buffer pH 6-7 and about 5% v/v acetonitrile;
B) about 50% v/v phosphate buffer pH 6-7 and about 50% v/v acetonitrile; and

wherein the mobile phase initially comprises about 80% v/v of A) and about 20% v/v of B); and

wherein the mobile phase composition is gradually changed to about 50% v/v of A) and about 50% v/v of B); and

wherein the stationary phase is a hydrophobic C18 resin.
Brief Description of the Figures

Figure 1 shows the structure of Colistin A in accordance with the teaching of the prior art suggesting a structure of one component of Colistimethate Sodium (CMS) according to the Coly-Mycin® label.

Figure 2 shows a chromatogram of blank.

Figure 3 shows a chromatogram of the Quantitation limit solution.

Figure 4 shows a chromatogram of a CMS USP standard.

Detailed description

CMS is used herein as any composition resulting from the treatment of Colistin with formaldehyde and bisulphite. Thus, CMS is meant to embrace the mixture of compounds emerging when sulphomethylating Colistin, regardless of the method used. Chemical abstract has assigned such a composition the number 8068-28-8.

Colistin is used herein as any composition comprising Polymyxin E1 and Polymyxin E2. Chemical abstract has assigned the number 1066-17-7 for Colistin.

Polymyxin E1 is used herein as the compound having the CAS no 7722-44-3. Polymyxin E1 is synonymous to Colistin A.

Polymyxin E2 is used herein as the compound having the CAS no 7239-48-7. Polymyxin E2 is synonymous to Colistin B.

"Substantially pure" is used herein as compositions comprising more than 90% of a component according to integrated area under a HPLC chromatogram.

"HPLC" (High-performance liquid chromatography) is used herein as any chromatographic technique that can separate a mixture of compounds to identify, quantify or purify the individual components of the mixture. HPLC usually employ pressure to percolate a mobile phase through a column comprising a stationary phase.

"Binary gradient system" is used herein as a chromatographic method employing a mobile phase comprising two different solvents for a gradient elution.
By "Gradually changed" as used herein, it is to be understood that the composition of the mobile phase is changed in more than one step e.g. continuously, linearly or in small steps.

By "organic solvent" as used herein, it is to be understood to include organic solvents like C₁-C₅ alcohols, C₂-C₅ ethers, C₂-C₆ esters, acetonitrile or their mixtures, etc.

By "aqueous buffer" as used herein, it is to be understood to include buffers comprising water as the main solvent and weak acids and their corresponding weak bases.

According to one embodiment, a sample comprising CMS is loaded onto the column. The sample could be dissolved in organic solvents including, but not limited to, methanol, ethanol and acetonitrile.

The sample loaded onto the stationary phase is according to one aspect of the present invention a mixture comprising CMS dissolved in an aqueous buffer or organic solvent or their mixtures.

According to one embodiment of this aspect, said solution comprising 80-100% v/v organic solvent and 0-20 % v/v water.

According to another aspect of the present invention, the sample loaded onto the stationary phase is preferably a mixture comprising CMS dissolved in a solution comprising 95% v/v organic solvent and 5% v/v water.

According to another embodiment of this aspect, the sample loaded onto the stationary phase is a mixture comprising CMS dissolved in a cold solution comprising 95% v/v methanol and 5% v/v water.

According to yet an embodiment of this aspect, the sample comprises 1-10% water to facilitate dissolution of CMS. The most preferred loading samples comprise CMS dissolved in 95% v/v methanol and 5% v/v water.

According to one embodiment, the two different solvents of the mobile phase comprises different amounts of acetonitrile and phosphate buffer, e.g. wherein the first solvent (solvent A) comprises 90-99% v/v phosphate buffer and 1-10% v/v acetonitrile and wherein a second solvent (solvent B) comprises 40-60% v/v phosphate buffer and 40-60% v/v acetonitrile.
In order to obtain a binary gradient system, the mobile phase is gradually changed, e.g. by having a mobile phase initially comprising 70-90% v/v of solvent A and 10-30% v/v of solvent B, and wherein the mobile phase composition is gradually changed to 40-60% v/v of solvent A and 40-60% v/v of solvent B.

The solvents used in the binary gradient system may be of gradient grade. Acetonitrile is preferably used as one of the two solvents in the binary gradient system according. It is to be understood that the acetonitrile used is of HPLC gradient grade. Acetonitrile of gradient grade is available from various manufacturers, e.g. LiChrosolv® gradient grade (Merck no. 1.00030).

The phosphate buffer is also preferably of HPLC gradient grade. Phosphate buffer of gradient grade is available from various manufacturers, such as e.g. Sodium dihydrogenphosphate dihydrate (Merck no. 1.06498).

The hydrophobic resin suitable as the stationary phase according may be a hydrophobic C8-C24 resin, such as a hydrophobic C15-C20 resin, e.g. such as a hydrophobic C18 resin. According to one embodiment, a hydrophobic resin corresponds to the Acquity UPLC CSH C18, 1.7 μη, 150 x 2.1 mm available from Waters Corp. Other useful resins would include Brigded Ethyl Hybrid (BEH) from Waters Corp.

CMS comprises of a mixture compounds originating from Polymyxin E1 and E2. E1 and E2 CMS accounts for around 80% of the content in CMS, which is approximately the same as in Colistin.

Colistin in an aqueous solution is considered to be stable for more than 120 hours at 37°C [Li et al., Antimicro. A and Chemoth, 2003, vol 47, No. 4, pl364]. However, CMS is readily hydrolysed to a variety of derivatives. Even freshly dissolved CMS is a complex mixture composed of a variety of derivatives. A multicomponent composition has also been shown by Li et al. in 2003. The prior art concludes that CMS is a Colistin prodrug that eventually is hydrolysed to Colistin in vitro/in vivo (Bergen et al, Antimicr. Agents and Chemoth. 50(6), 2006, 1953).

Description of the HPLC method according to the present invention is outlined below. It is to be understood that the examples provided is not to be contemplated as limiting to the scope of the present invention.
Examples

5.1 Apparatus
A binary gradient HPLC system with a pressure limit of at least 11000 PSI and with UV detector capable of performing measurements at 210 nm.

5.2 Chemicals and Reagents
Sodium dihydrogenphosphate dihydrate (Merck no. 1.06345), or equivalent.

Sodium hydroxide pellets (Merck no. 1.06498), or equivalent.

Acetonitrile, LiChrosolv gradient grade (Merck no. 1.00030), or equivalent.

CMS Control sample, USP Reference standard or equivalent.

1 M sodium hydroxide solution.
4.0 g sodium hydroxide is added to 100 ml Milli-Q water and the mixture is stirred until all sodium hydroxide is dissolved.

0.05 M phosphate buffer, pH 6.5
7.8 g sodium dihydrogenphosphate dihydrate is dissolved in ca. 980 ml Milli-Q water. pH is adjusted to 6.5 with 1 M sodium hydroxide solution and Milli-Q water is added to 1000 ml.

LC solvent A.
475 ml 0.05 M phosphate buffer, pH 6.5 is added 25 ml acetonitrile. The mixture is filtered through 0.22 μη membrane filter and degassed in ultrasonic bath for 10 min.

LC solvent B.
250 ml 0.05 M phosphate buffer, pH 6.5 is added 250 ml acetonitrile. The mixture is filtered through 0.22 μη membrane filter and degassed in ultrasonic bath for 10 min.

5.3 System and Method Parameters
Column: Waters Acquity UPLC CSH C18, 1.7 μη, 150 x 2.1 mm, or equivalent.

Precolumn: Waters VanGuard UPLC CSH C18, 1.7 μη, 5 x 2.1 mm, or equivalent.

Column temperature: 30 °C

Flow: 0.30 ml/min
Flow type: Gradient

LC Solvent A 0.05 M phosphate buffer pH 6.5/acetonitrile, 95/5 v/v.

LC Solvent B 0.05 M phosphate buffer pH 6.5/acetonitrile, 50/50 v/v.

Gradient: Initial, 20 % B; 0 - 10 min, linear to 32 % B; 10 - 35 min, linear to 47 % B; 35 - 36 min, linear to 20 % B; 36 min - 44 min, 20 % B.

Equilibration: At least 30 min. Make at least 2 control sample injections to check that retention times are constant.

Wavelength: 210 nm.

Injection volume: 2.0 µL.

Run time: 44 min.

Auto sampler temp.: 5°C.

5.4 Preparation of Test Sample Solution

For 1 million unit vial: Add 2.00 mL of MilliQ water (approximately 21°C) and dissolve (approximately 37.5 mg/mL of CMS). Directly after dissolution, transfer 1 mL into a 20 ml volumetric flask and fill to mark with methanol and store solution at 2-8°C (approximately 1.875 mg/mL of CMS).

For 2 million unit vial: Add 4.00 mL of sterile water (approximately 21°C) and dissolve (approximately 37.5 mg/mL of CMS). Directly after dissolution, transfer 1 mL into a 20 ml volumetric flask and fill to mark with methanol and store solution at 2-8°C (approximately 1.875 mg/mL of CMS).

For Finished Dosage Form (Corresponding to 150 mg of Colistin Base): Add 2.0 mL of water for injections (approximately 21°C) or equivalent and dissolve. Directly after dissolution, transfer 200 µL of this solution into a 20 mL volumetric flask, fill to mark with methanol and store solution at 2-8°C. Final concentration of CMS in Test Sample solution is approximately 2 mg/mL.

5.5 Preparation of Control Sample Solution

Accurately weigh 10 mg of CMS control sample (USP Reference Standard or equivalent) into a 5 mL volumetric flask (2.0 mg/mL). Add 0.25 mL MilliQ water
and dissolve CMS. Directly after dissolution, dilute to volume with methanol and store solution at 2-8°C.

5.6 Preparation of Quantitation Limit (QL) Sample Solution

Transfer 1.5 ml of the control sample solution prepared in 5.5 (2.0 mg/mL) into a 25 mL volumetric flask. Dilute to volume with methanol (0.12 mg/mL).

5.7 HPLC Analysis

5.7.1 Analysis Set up

The samples are analyzed as shown in table 1.

Table 1. Typical sequence of injections.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank, Methanol:Water (95:5)</td>
<td>Equilibration of the system and to establish interfering peaks from the system or solvent, if any.</td>
</tr>
<tr>
<td>Quantition Limit (QL) Sample</td>
<td>Min. 1 injection of Control Sample (USP Reference Standard) diluted to QL (0.12 mg/mL)</td>
</tr>
<tr>
<td>Control Sample</td>
<td>Min. 2 injections of Control Sample (USP Reference Standard) as system suitability evaluation.</td>
</tr>
<tr>
<td>Test Sample</td>
<td>Measurement, Min. 1 injection per sample</td>
</tr>
<tr>
<td>Control Sample</td>
<td>1 injection (system suitability evaluation)</td>
</tr>
</tbody>
</table>

5.7.2 Acceptance Criteria for System Suitability

Blank: There should be no peaks that interfere with the CMS peaks from the blank

Quantitation limit (QL): S/N of the most abundant peak (RRT 1.00) in QL Sample should not be lower than 10 times the noise.
Repeatability: The difference in retention times of the peak RRT 1.00 in two consecutive injections in Control Sample should be less than 0.5 min.

Plate number (N): USP Plate number of the peak with RRT 1.00 in Control Sample should be not less than 20,000 and RRT 2.61 in Control Sample should be not less than 100,000

5.7.3 Analysis of Test Samples - Peak Identification and Quantitation
- Integrate all peaks above 0.05% (area %).
- The most abundant peak in the range of 10.5 to 12.5 is set as Identification reference peak (RRT 1.00). Calculate RRT of all other peaks in chromatogram.

Figure 2 shows the chromatogram of blank, whereas figure 3 shows the chromatogram of the Quantitations limit solution and figure 4 shows the CMS USP standard, respectively.

The results presented above shows that the method of the present invention provides a longed for analytical tool for determining the content and quality of a CMS composition.
CLAIMS

1. A chromatographic method for separating components of CMS comprising loading of a sample comprising CMS onto a stationary phase, and eluting with a mobile phase;

   wherein

   the mobile phase comprises 50-95% v/v aqueous buffer with pH 5-8 and 5-50% of an organic solvent and initially has a low content of organic solvent which is increased to a higher level during the elution.

2. A method according to claim 1, wherein the mobile phase comprises 60 - 90%, v aqueous buffer with pH 5.5-7.5 and 10-40% of an organic solvent.

3. A method according to claim 2, wherein the mobile phase comprises 74% to 86% aqueous buffer with pH 6.0-7.0 and 14% to 26% organic solvent.

4. A method according to any of the above claims, wherein the mobile phase is gradually changed from 14% to 26% of organic solvent.

5. A method according to any of the above claims, wherein the mobile phase buffer is a phosphate buffer and organic solvent is acetonitrile.

6. A method for separating components of CMS comprising loading of a sample comprising CMS onto a stationary phase, and eluting with a mobile phase;

   wherein

   the mobile phase comprises a mixture of solvent A and solvent B, wherein solvent A comprises 90-99% v/v phosphate buffer and 1-10% v/v acetonitrile, and wherein solvent B comprises 40-60% v/v phosphate buffer and 40-60% v/v acetonitrile; and wherein the mobile phase initially comprises 70-90% v/v of solvent A and 10-30% v/v of solvent B; and wherein

   the mobile phase composition is gradually changed to 40-60% v/v of solvent A and 40-60% v/v of solvent B; and

   wherein the stationary phase is a hydrophobic resin.

7. A method according to claim 1, wherein solvent A comprises 92-97% v/v phosphate buffer and 3-8% v/v acetonitrile.

8. A method according to claim 1, wherein solvent A comprises 95% v/v phosphate buffer and 5% v/v acetonitrile.
9. A method according to claim 1, wherein solvent B comprises 45-55% v/v phosphate buffer and 45-55% v/v acetonitrile.

10. A method according to claim 1, wherein solvent B comprises about 50% v/v phosphate buffer and about 50% v/v acetonitrile.

11. A method according to claim 1, wherein the concentration of the phosphate buffer is 0.01-0.1M.

12. A method according to claim 1, wherein the concentration of the phosphate buffer is 0.03-0.07M.

13. A method according to claim 1, wherein the concentration of the phosphate buffer is 0.05M.

14. A method according to claim 1, wherein the pH of the phosphate buffer is 6-7.

15. A method according to claim 1, wherein the pH of the phosphate buffer is 6.3-6.8.

16. A method according to claim 1, wherein the pH of the phosphate buffer is 6.5.

17. A method according to claim 1, wherein the stationary phase is a hydrophobic C8-C24 resin.

18. A method according to claim 1, wherein the stationary phase is a hydrophobic C15-C20 resin.

19. A method according to claim 1, wherein the stationary phase is a hydrophobic C18 resin.

20. A method according to claim 1, wherein the detection wavelength is 190-230 nm.

21. A method according to claim 1, wherein the detection wavelength is 200-220 nm.

22. A method according to claim 1, wherein the detection wavelength is 210 nm.

23. A method according to claim 1, wherein the column temperature is 20-40 °C.
24. A method according to claim 1, wherein the column temperature is 25-35 °C.

25. According to another aspect of this embodiment, the column temperature is about 30 °C.

26. A method according to claim 1, wherein the flow is 0.1-0.60 ml/min.

27. A method according to claim 1, wherein the flow is 0.2-0.40 ml/min.

28. A method according to claim 1, wherein the flow is about 0.30 ml/min.

29. A method according to claim 1, wherein the loaded sample comprises CMS dissolved in a solution comprising 90-99% v/v methanol and 1-10% v/v water.

30. A method according to claim 1, wherein the loaded sample comprises CMS dissolved in a solution comprising 95% v/v methanol and 5% v/v water.

31. A HPLC method for analyzing CMS comprising a binary gradient system wherein the mobile phase comprises a mixture of
   A) about 95% v/v phosphate buffer pH 6-7 and about 5% v/v acetonitrile;  
   B) about 50% v/v phosphate buffer pH 6-7 and about 50% v/v acetonitrile; and
   wherein the mobile phase initially comprises about 80% v/v of A) and about 20% v/v of B); and
   wherein the mobile phase composition is gradually changed to about 50% v/v of A) and about 50% v/v of B); and
   wherein the stationary phase is a hydrophobic C18 resin.
Figure 1

Polyoxin E1
Colistin A

Derivatization

CMS
Colistin methanesulfonate
Figure 2:
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. B01D15/16 B01D15/32 C07K7/62
ADD.

According to International Patent Classification (IPC) or both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
B01D C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<td>E</td>
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<td>A</td>
<td>wo 2011/051070 AI (XELLIA PHARMACEUTICALS APS [DK] ; KOCH TORBEN [DK] ; PEDERSEN CARSTEN 0V) 5 May 2011 (2011-05-05) examples</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:
  * "A" document defining the general state of the art which is not considered to be of particular relevance
  * "E" earlier application or patent but published on or after the international filing date
  * "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  * "O" document referring to an oral disclosure, use, exhibition or other means
  * "P" document published prior to the international filing date but later than the priority date claimed
  * "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  * "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  * "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  * "A" document member of the same patent family

Date of the actual completion of the international search
5 August 2014

Date of mailing of the international search report
18/08/2014

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax. (+31-70) 340-3016

Authorized officer
Fourgeaud, Dami
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