Simple and rapid method on High Performance Liquid Chromatography for simultaneous determination of benzylpenicillin potassium, streptomycin sulphate and related substances in Ascomicin – a veterinary use ointment

Metodă HPLC simplă și rapidă, pentru determinarea simultană a benzilpenicilinii de potasiu, sulfatului de streptomicină, a substanțelor înrudite chimic și a produsilor de degradare din Ascomicin, unguent pentru uz veterinar

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Cuvinte cheie: benzilpenicilina de potasiu, sulfat de streptomicină, HPLC, validare.

Abstract

A new simple, rapid, accurate and precise High – Performance Liquid Chromatography (HPLC) method for determination of benzylpenicillin potassium and streptomycin sulphate in Ascomicin ointment was developed and validated. The method can be used for the detection and quantification of known and unknown impurities and degradation products in this pharmaceutical product during routine analysis and also for stability studies in view of its capability to separate degradation products. The method was validated for accuracy, precision, specificity, robustness and quantification limits according to ICH Guidelines. The estimation of benzylpenicillin potassium and streptomycin sulphate was done by Waters HPLC 2695. The chromatographic conditions comprised a reverse-phased C18 column (5 µm particle size, 250 mm×4.6 mm i.d.) with a mobile phase consisting of a mixture of solution in water containing 0.025 M of sodium phosphate dibasic and 0.02 of sodium hexansulfonate adjusted to pH 6.0 with 22.5 g/l solution of phosphoric acid and acetonitrile in gradient elution. The flow rate was 0.8 ml/min. Standard curves were linear over the concentration range of 5.00 µg/ml to 5.00 mg/ml for streptomycin sulphate and 3.26 µg/ml to 3.26 mg/ml for benzylpenicillin potassium. Statistical analyses proved the method was precise, reproducible, selective, specific and accurate for analysis of benzylpenicillin potassium, streptomycin sulphate and related substances.

Rezumat

Metoda cromatografica HPLC prezentata este o metoda noua, putin costisitoare şi rapida, pentru determinarea benzilpenicilinii de potasiu ş i a sulfatului de streptomicină, în unguentul pentru uz veterinar, Ascomicin. Metoda poate fi utilizată pentru identificarea substanțelor active şi cuantificarea impurităţilor cunoscute ş i necunoscute ş i a produsilor de degradare, în timpul analizelor de rutină, dar şi pe parcursul studiilor de stabilitate pentru produsul finit Ascomicin. Metoda a fost validată, ş i s-a demonstrat că este exactă, precisa, selectiva/specifică, robustă, ş i îndeplineşte criteriile impuse de ghidul ICH pentru limita de detectie, ş i respectiv pentru limita de cuantificare. Determinările au fost efectuate utilizând aparatul HPLC Waters 2695. Condiţiile cromatografice au fost următoarele: coloană C18 (5 µm dimensiunea particulelor, 250 mm x 4.6 mm i.d.). Faza mobilă constă într-un amestec de soluţie de fosfat dibazic de sodiu 0.025 M şi hexansulfonat de sodiu 0.02 M, ajustat la pH 6.0 cu o soluţie de acid orto-fosforic, ş i acetonitril, cu eluţie în gradient. debitul a fost de 0.8 ml/min. Metoda prezintă linearitate în domeniul de concentraţii de 5.00 µg/ml până la 5.00 mg/ml pentru sulfatul de streptomicină ş i 3.26 µg/ml până la 3.26 mg/ml pentru benzilpenicilină de potasiu. Evaluările statistiche au dovedit că această metodă este precisă, reproducible, selectivă, specifică ş i exactă.

1. Introduction

Benzylpenicillin potassium, chemically, potassium (2S,5R,6R)-3,3-dimethyl-7-oxo-6-
[(phenylacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate is indicated at the beginning of treatment of severe injections induced by penicillin-sensitive pathogens (later the therapy can continue by giving procaine benzylpenicillin). It is a first choice drug in pneumonias and other infections induced by pneumococci (except enterococci), in pneumococcal meningitides (combined with sulfonamides), infections induced by pyogenic streptococci, sensitive golden staphylococci, gonococci and meningococci (in combination with sulfonamides), further in treatment of anthrax, diphtheria, lues, listeriosis, actinomycosis. It is effective in infections induced by Clostridium sp. and Corynebacterium sp. and in treatment of other infections induced by etiological agents sensitive to penicillin.

Streptomycin sulphate, chemically, sulphate 5-(2,4-diguanidino-3,5,6-trihydroxy-cyclohexoxy)-4-[4,5-dihydroxy-6-(hydroxymethyl)-3-methylamino-tetrahydropyran-2-yl]oxy-3-hydroxy-2-methyl-tetrahydrofuran-3-carbaldehyde is an antibiotic drug, the first of a class of drugs called aminoglycosides to be discovered, and it was the first cure for tuberculosis. It is derived from the actinobacteria Streptomyces griseus or obtained by other means. Streptomycin is a bactericidal antibiotic.

Streptomycin is also used to combat the growth of bacteria, fungi, and algae. Streptomycin controls bacterial and fungal diseases of certain fruit, vegetables, seeds and ornamental crops, and it controls algae in ornamental ponds and aquaria. A major use is in the control of fire blight on apple and pear trees. As in medical applications, extensive use can be associated with the development of resistant strains. Several papers have been published proposing HPLC methods for identification, assay and control of the related substances for streptomycin sulphate using UV detection [1, 2], fluorescence [3, 4] and mass spectrometry [5, 6, 7]. Also, for benzylpenicillin potassium were published several papers for identification, assay and control of related substances using UV detection [8, 9]. But there is no indicating high-performance liquid chromatography method for their simultaneous determination in a pharmaceutical ointment. The method was validated for: accuracy, precision, specificity, robustness and quantification limits according to ICH Guidelines [10,11].

The aim of the present work is to develop an accurate, specific, reproducible method for determination of streptomycin sulphate and benzylpenicillin potassium in the same chromatographic condition.

2. Materials and methods

2.1. Chemicals and reagents

Streptomycin sulphate and benzylpenicillin potassium were obtained from EDQM. Acetonitrile (HPLC grade), sodium hexane sulphonate and sodium phosphate dibasic were obtained from Merck. The mobile phase were filtering through 0.45 µm filter membrane and degassed through sonicate for 10 minutes.

The apparatus used was HPLC Waters separation modules 2695 and Waters 2696 PDA detector.

2.2. Chromatographic conditions

The column used was Thermo BDS Hypersil-C18, 250 mm x 4.6 mm, 5 µm particle size. Chromatographic separation was achieved using a mobile-phase consisting of solution (A) (0.025 M of sodium phosphate dibasic and 0.02 M of sodium hexan sulfonate adjusted at pH 6.0 ±0.01 with 22.5 g/l solution of o-phosphoric acid) and Acetonitrile.

Gradient elution was performed slowly from 0 to 27 minutes 90% A; 27-35 minutes 80% A; 35 – 40 minutes 90% A; 40-45 minutes 90% A. The flow rate was kept at 0.8 ml/min and detection was performed at 205 nm for streptomycin sulphate and impurities for streptomycin sulphate and 225 nm for benzylpenicillin potassium and impurities for benzylpenicillin potassium. The injection volume was 10 µl in all HPLC runs.

2.3. Method Validation

Method validation parameters studied were limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, precision, repeatability, robustness and specificity. The LOQ was defined as the lowest
concentration that could be determined with acceptable accuracy and precision.

The LOD and LOQ were determined from signal to noise response. The Linearity was determined by calibration curve. For the construction of calibration curve, five calibration standard solutions were prepared for assay and five calibration standard solutions were prepared for purity. The precision and repeatability was estimated by assaying six replicate samples on day-1 and day-2. The accuracy was evaluated by the recovery determination. The specificity was evaluated by force degradation of the samples. The samples were subjected to acid and basic degradation, thermal degradation and hydrogen peroxide degradation.

2.4. Preparation of Solutions

Standard preparation: A standard solution for assay of streptomycin sulphate and benzylpenicillin potassium was prepared by transferring accurately 16.3 mg benzylpenicillin potassium and 25.0 mg streptomycin sulphate into 10 ml volumetric flask. A standard solution for purity was prepared by diluting standard solution for assay to give final concentration 16.3 µg/ml for benzylpenicillin potassium and 25.0 µg/ml streptomycin sulphate.

Sample preparation: The sample solution was prepared by accurately weighting pharmaceutical product into Erlenmeyer flask and adding 25 ml of chloroform. Vortex until the complete dissolution of paraffin and add 5.0 ml of water five times and shake every time. Separate the aqueous phase and filter using 0.45 µm membrane filter.

3. Results and discussion

3.1. LOD and LOQ

Limit of detection and Limit of quantification were determined.

Results showed that the detection limit was 1.00 µg/ml for benzylpenicillin potassium and 1.52 µg/ml for streptomycin sulphate. The LOQ calculated was 3.264 µg/ml for benzylpenicillin potassium and 5.004 µg/ml for streptomycin sulphate.

3.2. Linearity

The method was found to be linear for the concentration range described in Table 1.

When average peak areas were plotted against concentration levels, good correlation coefficients ($r^2$) were obtained as 0.9998 and 0.9996 for benzylpenicillin potassium and streptomycin sulphate for assay and 0.9998 for benzylpenicillin potassium and streptomycin sulphate for purity.

3.3. Accuracy

The current method is valid and accurate. Accuracy was evaluated by the recovery determination. The method's accuracy was determined by spiking working standards of the benzylpenicillin potassium and streptomycin sulphate into placebo at different concentration levels: 80, 100 and 120% of target concentration of each of the compounds for purity and assay.

Results showed the amount obtained by this method were between 99.1-101.8 and 98.0-100.6 for benzylpenicillin potassium and streptomycin sulphate in the assay. For purity the results were between 99.7-102.5 for benzylpenicillin potassium and 98.2-103.3 for streptomycin sulphate.

The absolute recovery was determined in triplicate by direct comparison of peak area from standard versus sample. The data was analyzed statistically by calculating RSD.

3.4. Precision

Precision was measured in terms of repeatability of application and measurement data. In order to evaluate the method's capability to produce similar results on repetitive test for nominal concentration, six individual samples from Streptomycin sulphate and benzylpenicillin potassium were tested separately.

It showed very low relative standard deviation for the two compounds. Intermediate precision of the method was carried out in six different samples preparations by a different analyst, in two different days.

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Correlation coefficient ($r^2$)</th>
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<tbody>
<tr>
<td></td>
<td>Assay solution</td>
</tr>
<tr>
<td>Benzylpenicillin potassium</td>
<td>0.999</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>0.999</td>
</tr>
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</table>
The value of RSD is below 2% for the two compounds; intermediate precision of the method is established. The main chromatograms are presented in figs 1 to 11.

### Table 2

<table>
<thead>
<tr>
<th>Matrices</th>
<th>Repeatability, %RSD</th>
<th>Intermediate precision, %RSD</th>
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<tr>
<td>Benzylpenicillin potassium</td>
<td>1.1</td>
<td>0.4</td>
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<tr>
<td>Streptomycin sulphate</td>
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</table>

3.5. Specificity

The specificity of the method was ascertained by analyzing spiked samples under stress condition. The specificity has been investigated to demonstrate that there is no interference between excipients, active ingredients, related substances and degradation compounds that may be presents in samples.

The stressed condition samples are evaluated relative to the control sample with respect to assay and related substances / degradation product (%). The presence of other ingredients in the formulations did not cause any interference with the streptomycin sulphate and benzylpenicillin potassium.

3.6. Robustness

Robustness of the method was determined by analyzing same standards at normal operating conditions and also by changing some analytical conditions, mobile phase and flow rate, etc. The results obtained showed that the method is robust.

**Conclusion**

The aim of this study was to develop a selective and sensitive HPLC method for the rapid detection of streptomycin sulphate and benzylpenicillin potassium in the same chromatographic conditions.

The proposed method was found to be rapid, accurate, repeatable, specific and robust.

This method was applied for the analysis of the drug product in marketed formulations and could be used for the finished product at release on market and for validity term of the drug product.

**Fig. 1.** Representative chromatogram for System suitability solution at 205 nm
### Table

<table>
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<tr>
<th>Name</th>
<th>Retention Time</th>
<th>Area</th>
<th>% Area</th>
<th>Height</th>
<th>Resolution</th>
<th>USP Resolution</th>
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<td>4.554</td>
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<td>34421</td>
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Fig. 2. Representative chromatogram for System suitability solution at 225 nm

Fig. 3. Representative chromatogram for phenylacetic acid (impurity B)

Fig. 4. Representative chromatogram for benzylpenicillin potassium
Fig. 5. Representative chromatogram for streptomycin sulphate

Fig. 6. Representative chromatogram for dihydrostreptomycin sulphate

Fig. 7. Representative chromatogram for standard solution for assay at 205 nm
Fig. 8. Representative chromatogram for standard solution for assay at 225 nm

Fig. 9. Representative chromatogram for standard solution for purity at 205 nm

Fig. 10. Representative chromatogram for standard solution for purity at 225 nm
Fig. 11. Representative chromatogram for dosing / identification / chemically related substances / degradation products at 225 nm

<table>
<thead>
<tr>
<th>Denumire substanță</th>
<th>Timp migrație</th>
<th>Aria</th>
<th>% Aria</th>
<th>Înălțime</th>
<th>Rezoluție</th>
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<td>18.3</td>
<td>2.3</td>
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</tbody>
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References

8. Samanidou VF, Nisyriou SA, Papadoyannis IN. Development and validation an HPLC method for the determination of penicillin antibiotics residues in bovine muscle according to the European Union Decision 2002/657/EC