Methylprednisolone and its related substances in freeze-dried powders for injections

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RUNNING TITLE: METHYLPREDNISOLONE AND ITS RELATED SUBSTANCES

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Abstract: In this work behavior of active pharmaceutical substances – methylprednisolone (in a form of methylprednisolone sodium succinate) in finished pharmaceutical dosage form – freeze dried powder for injections was examined. The goal was to evaluate the chemical stabilities of methylprednisolone sodium succinate packaged in dual chamber vial, as a specific container closure system. The effect of different parameters: temperature, moisture and light were monitored. USP method for assay was used to determine concentrations of methylprednisolone, as a sum of the concentration of methylprednisolone esters (17-hydrogen succinate and 21-hydrogen succinate) and free methylprednisolone. HPLC method was used for stability evaluation of active substance and related substances determination. Four main degradation products were noted. Temperature has a major impact on degradation process and appearance of 3 degradation products (impurities B, C and D), while the presence of light caused increasing content of impurity A. Identification of impurity B, C and D has been done using mass and NMR spectroscopy as well. All three substances are related substances of methylprednisolone.

Keywords: methylprednisolone sodium succinate; freeze dried powder; container closure system; stability; impurities.

INTRODUCTION

Methylprednisolone (MP) is a synthetically produced glucocorticoid with structure similar to a natural hormone produced by adrenal glands. Like most adrenocortical steroids, MP is typically used in the replacement therapy for adrenal insufficiency and as an anti-inflammatory and immunosuppressant.

MP (Fig. 1A) belongs to the group of corticosteroids which are hydroxyl compounds (alcohols). Both, free alcohol and ester (Fig. 1B) occur as odorless, white or practically white, crystalline powder. They are practically insoluble in water and sparingly soluble in alcohol. Sodium salt of the phosphate or succinate ester is generally used to provide water-soluble forms for injections or solutions.

In pharmacopeias there are several referenced chemical substances: MP, methylprednisolone acetate, methylprednisolone hydrogen succinate (MP 21-HS)1–4 and methylprednisolone sodium succinate (MPNaS).3

Among all cited substances, only MPNaS may be given intravenously. MPNaS is so extremely soluble in water3 that it may be administered in a small volume of diluents and is especially well suited for intravenous use in situations in which high blood levels of MP are required rapidly and oral therapy is not possible.5 The chemical name for MPNaS is (6α,11β)-
11,17,21-trihydroxy-6-methyl-pregna-1,4-diene-3,20-dione-monosodium salt and the molecular weight is 496.53 g mol\(^{-1}\).

MP is a very unstable substance, especially in the presence of moisture.\(^6\)\(^-\)\(^9\) This is the main reason for using the lyophilization process for production of injections. Problem associated with this type of injections is storing of medications (powder and solution for reconstitution) that should be administered as separate component parts and mixing prior to injection. Therefore, dual chamber vials have been developed to facilitate storage and mixing of such two-component medications. This container closure system consists of the glass vial with two chambers separated with intermediate rubber closure and an upper closure on the top. The specific feature of this type of the products is that both - lyophilized active ingredient (in the lower chamber) and the solution for reconstitution (in the upper chamber) are in the same vial.

MP and its derivatives are well known and defined, not only in Pharmacopeias, but in official documentations of the manufacturers and in literature as well.\(^1\)\(^1\)\(^-\)\(^3\) Although a number of studies of the physical and chemical stabilities of MPNaS have been reported,\(^1\)\(^4\)\(^-\)\(^9\)\(^1\) no published information is available on the stability of it in the dual chamber vial.

The objective of this work was to evaluate the chemical stability of lyophilized injectable preparation of MPNaS. The last step to reach our goal was to check the influence of all critical points on the degradation profile on MP. Special consideration was given to the changing of the concentration of the active substance (total MP), concentration of free MP, related substances determination as possible degradation products and their identification as well.

**EXPERIMENTAL**

**Materials**

Lyophilized injectable preparation of MPNaS was purchased by Hemofarm, Vrsac, Serbia. Solution for reconstitution is water for injection/ 0.9 % benzyl alcohol. Two milliliters of reconstituted solution contain MPNaS equivalent to 125 mg MP, phosphate buffer and benzyl alcohol. This product has been tested in the early development phase in accordance with relevant guidelines.\(^2\)\(^0\)\(^-\)\(^2\)\(^6\)

All solvents: \(n\)-butyl chloride (Merck, Germany), tetrahydrofuran (Sigma-Aldrich, Germany) methanol (Merck, Germany), glacial acetic acid (Sigma-Aldrich, Germany) and chloroform (Merck, Germany) were of a grade suitable for high-performance liquid chromatography (HPLC) analysis. MP 21-HS (Aventis Pharma, France), MP and fluorometholone reference standards (RS) were obtained commercially. The reference standard was used without further purification.

All samples were conditioned according to the requirements of International Conference on Harmonization standards for stability (Q1A)\(^2\)\(^1\) and photostability (Q1B UV).\(^2\)\(^6\) Following chambers were used: Weiss 2000, Weiss Gallenkamp, Weiss 600, Weiss Gallenkamp, Sanyo PSC 062, Sanyo Gallenkamp (Great Britain) and Weiss Umweltechnik 140, Weiss Umweltechnik, Germany.

**Procedure for stability testing**

**Stability study** is designed to increase the rate of chemical degradation of FPP (Finished pharmaceutical product) by using exaggerated storage conditions as part of the formal stability studies. Accelerated conditions are presented in Table I. During the study, the vials were kept in upright, as well as, in horizontal position. Horizontal position was chosen, for compatibility assessment, in other words to assure maximal contact of diluent of the subject drug product with elastomeric closures.

**In-use stability testing**\(^2\)\(^3\)\(^,\)\(^2\)\(^5\) is being evaluated by examine the quality parameters of the subject drug product susceptible to change at the end of the proposed in–use shelf life (i.e., at 48 hours after reconstitution, kept at room temperature). Samples were tested immediately after production (initial test point), at the end of accelerated and intermediate conditions and in 12 month and 24 month testing points during long-term testing.
Photostability testing was performed using light source – Option 2. It means that a cool white fluorescent lamp and a near UV fluorescent lamp were used. Vials were exposed to light providing defined energy (1.2 million lux hours) and then reconstituted. At the same time protected samples (vials wrapped in aluminum foil or kept in a carton box) were used as dark control. At the end of this phase, the concentration of MP and related substances was determined.

Methods

HPLC method for MP determination: Analytical procedures for total MP and free MP determination were in accordance with USP 32. Analytical procedure is the same, concentration of total MP is expressed as a sum of concentration of free MP, MP 21-HS and MP 17-HS. Concentration of free MP is separated expressed due to the fact it is important degradation product. Analyses were performed on HPLC system Agilent Technology 1100 Series equipped with 254 nm detector and column of porous silica particles packing (L3). The flow rate was 1.0 mL min⁻¹. Mobile phase was prepared by mixing butyl chloride, water-saturated butyl chloride, tetrahydrofuran, methanol and glacial acetic acid (95:95:14:7:6; v/v/v/v/v).

Internal standard solution was obtained by dissolving fluorometholone (RS) in tetrahydrofuran. Concentration of solution was 3 mg mL⁻¹.

MP solution: About 7.5 mg of MP RS was accurately weighed transferred to a 25-mL volumetric flask and dilute with solvent to 25 ml.

Solvent: 3% glacial acetic acid in chloroform.

Standard solution: About 32.5 mg of MP 21-HS RS was weighed accurately and transferred to a 50-mL volumetric flask. 5 mL of internal standard solution and 5 mL of MP solution were added. Solvent was added up to 50 mL.

Test solution: Tested product was reconstituted according to the manufacturer's directions. Aliquots of 10 vials were collected and 0.8 mL of this solution was transferred to a volumetric flask of 100 mL. Internal standard solution was added (10 mL) and dissolved with solvent up to the mark. After 5 minute shaking, layers were separated and upper one was discarded.

Injection volume was 10 µl of each sample.

HPLC method for determination of related substances: Related substances were determined by isocratic HPLC method. Apparatus was Agilent 1100 Series, with variable wavelength UV-vis detector. Column C18, 250 x 4.6 mm, 5 µm column was used, flow rate was 1.2 mL min⁻¹. Mobile phase was prepared by combining acetic acid, acetonitrile, and purified water (2:30:75, v/v/v).

Test solution: Reconstituted solutions prepared from the contents of 10 vials of MPNaS for injection were mixed, 0.4 mL was transferred to 20-mL volumetric flask and diluted with mobile phase up to 20 mL. Concentration of obtained solution is 0.1 % w/v.

Standard solution: 1 mL of test solution was transferred to 100-mL volumetric flask and diluted with mobile phase. 1 mL of this solution was diluted with mobile phase to 10 mL. Concentration of standard solution is 0.0001 % w/v.

Injection volume was 20 µl of each sample.

Methods for identification of related substances

Samples were treated with 5 mol/L HCl over 30 min at 80 °C in order to force degradation and get higher concentration of the degradation products.

The samples of the degradation products were obtained from reaction mixture by semi-preparative HPLC. The same apparatus was used (Agilent 1100 Series), with variable wavelength UV-vis detector, but semi-preparative Zorbax Eclipse XDB-C18 (250 × 9.4 mm; 5 µm) column was used, flow rate was 4.0 mL min⁻¹ and injection volume was 1000 µL. Mobile phase was prepared by combining formic acid and acetonitrile (7:3, v/v).

HR-ESI-TOF (High Resolution Electrospray Ionization in a Time of Flight) mass spectra were measured for MP and three degradation products on Agilent 6210 LC/MS instrument. The mass spectrometer was operated under the following conditions: source - ESI in positive/negative mode, dry gas: 12.0 L min⁻¹, dry temperature: 350 °C, nebulizer: 45 psig, scan: 100–1500 m/z, fragmentor: 140 V, capillary voltage: 4000 V.

1H NMR spectra of impurities C and D were measured on Varian Gemini 2000 at 200 MHz in CDCl3. NMR spectra of MP and impurity B (1H-, 13C-, DEPT- 135, COSY, NOESY, HSQC and HMBC) were measured on Bruker Avance III instrument at 500 MHz in CDCl3 (a few drops of CD3OD were added for MP).

RESULTS AND DISCUSSION
**Assay MP and related substances determination**

A chromatogram of the test solution obtained during assay determination is presented in Fig. 2. The order of elution of peaks is the internal standard peak, MP 21-HS peak, successive smaller peaks of free MP and methylprednisolone 17-hydrogen succinate (MP 17-HS). The concentration of total MP is expressed as a sum of concentration of free MP, MP 21-HS and MP 17-HS (Fig. 1C). Concentration of total MP in initial sample (in form of sodium succinate) was defined as 100 %, and subsequent sample concentrations were expressed as percentage of initial concentration.

HPLC method for determination of related substances confirmed existence of four specified and not indentified impurities marked as impurity A, B, C, and D, respectively. Typical chromatogram related to impurities is presented in Fig. 3 Second part of this work was focused on identification of these impurities.

Influences of different temperature in terms of concentration of total MP, free MP and related substances are given in Tables II and III. All results are related to horizontal positioned vials. As it can be seen increasing in temperature is followed by increasing of free MP (because of hydrolysis) but, it is still significantly below allowed level which is 6.6 %. Obtained results indicate that there are no changes in content of total MP which could influence to shelf life.

Increasing of concentration of related substances is noticed as well. After 6 months conditioning at 40 °C / 75 % relative humidity (RH) concentrations of impurity B and impurity D were above on 0.15 % which is identification level in accordance with relevant guidelines. Increasing of impurity A is not significant.

In-use stability: all samples are conditioned according to the ICH requirements and reconstituted. Samples were analyzed initially (immediately after production in use) and at the end after conditioning at defined conditions. The measurements on day 0 served as references. Results are presented in Table IV. There are no statistically significant changes in tested parameters - if the solution is kept at room temperature there is no influence of water during 48 hours, so only results immediately after production (initial test point) and after 6 months conditioning at 40 °C / 75 % RH are presented.

The results of photostability testing are summarized in Table V. It was concluded that there is a change in tested parameters. It is obvious that impurity A is increasing while concentration of total MP decreased. When vials are kept at dark (wrapped in aluminium foil or kept in a carton box) increasing of impurity A is not significantly and impurity A was not subject of further identification.

**Identification of structure of degradation products**

Regarding retention times of impurities, it was concluded that impurity B is less polar then MP, and impurities C and D have significantly lower polarity.

Previous investigations of impurity B by MS showed that this compound has mass 374 amu, same as MP. The most abundant ion in HR-ESI-TOF mass spectra of MP is ion at m/z 375.2464 (M+H+), followed by 771.4071 (2M+Na+) and 357.2057 (M+H+–H2O). The same ions appear in mass spectrum of impurity B, which means that impurity B and MP have the same molecular formula, i.e., they are isomers. Presence of other ions (343, 345, 387...) in mass spectrum of B shows that impurity B is impure and started to decompose. So, additional analyses (such as 1H-, 13C-, DEPT-135,
COSY, NOESY, HSQC and HMBC NMR spectra) were required in order to elucidate the structure of impurity B.

Compound B has same mass and similar polarity as MP so its structure could be 17-deoxy-21,21-dihydroxy-6α-methylprednisolone (Fig. 1D). The same compound is defined such as impurity B of MP in EP. In European Pharmacopoeia this compound is defined as impurity of MP and signed as B.

It was assumed that MP initially gives impurity B which is later transformed (by staying and/or exchanging solvent) into impurity B1. Since structure of impurity B1 is not compatible with chromatographic behavior and mass spectra of impurity B, structure of impurity B1 is determined unequivocally by NMR.

The 1H-NMR spectrum of B1 (Fig. 4) is similar to MP showing that they have the same basic structure. On the basis of all spectra, it can be concluded that instead of -CH2OH at 21 and -OH group at 17 in MP, impurity B1 has -CHO group at 21 and H-atom at 17. Thus B1 has structure of 17-deoxy-21-dehydro-6α-methylprednisolone (Figure 1E). The same impurity is known in literature as the impurity of 6α-methylprednisolone acetate.

The 1H-NMR spectra of impurities C and D are very similar - they differ slightly in chemical shifts. Comparing to proton spectrum of MP signals from diastereotopic protons at C-21 are missing and new signal appears at 9.71 ppm in spectrum of C and at 9.56 in spectrum of D. That means C and D are isomeric aldehydes (Fig. 1 F). Spectral data of C and D are in very good agreement with data for isomeric enol aldehydes obtained from prednisolone. Thus C and D can be identified as E and Z (respectively) 20-hydroxy-17(20) E-ene-21-al derivatives of 6α-methylprednisolone defined as impurity D of MP in EP.

According to the results, degradation profile of MP is proposed (Fig. 5).

CONCLUSIONS

Even if MP is a well known substance, it is still a subject of many studies especially regarding stability. In this work degradation profile in the freeze-dried product is presented. It is obviously clear that the temperature has influence on degradation process, as well as light. Three of four detected degradation products are increasing under the temperature treatment. During this study it is confirmed that they are derivatives of the MP and at the same time defined as MP impurities in the Monograph in EP. Concentration of impurity A increases in a presence of light.

Acknowledgment. We gratefully acknowledge the financial support of the Ministry of Sciences and Environment of the Republic of Serbia (Grant No. 142072).

IZVOD

METILPREDNIZOLON I NJEGOVE SRODNE SUPSTANCE U LIOFILIZATU ZA RASTVOR ZA INJEKCIJE

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primenom masene i NMR spektroskopije. Sve tri nečistoće su identifikovane kao srodne supstance metilprednizolona.

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ICH Steering Committee, ICH Q1B, Stability Testing: Photostability Testing of New Drug Substances and Products, 1996;
TABLE I. Storage conditions and testing frequencies

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Storage orientation</th>
<th>Testing time points / months</th>
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</thead>
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<tr>
<td>Accelerated</td>
<td>upright</td>
<td>0, 1, 2, 3 and 6</td>
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<tr>
<td></td>
<td>horizontal</td>
<td>0, 3 and 6</td>
</tr>
<tr>
<td>Intermediate</td>
<td>upright</td>
<td>0, 3, 6, 9 and 12</td>
</tr>
<tr>
<td></td>
<td>horizontal</td>
<td>0, 6 and 12</td>
</tr>
<tr>
<td>Long-term</td>
<td>horizontal</td>
<td>0, 12, and 24</td>
</tr>
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</table>

RH – relative humidity
TABLE II. Results for concentration of total MP/free MP (percent of the labeled amount of MP) under different storage conditions

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Initial result</th>
<th>Intermediate result</th>
<th>Final result</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 °C / 75 % RH</td>
<td>101.9/1.51</td>
<td>101.3/2.70</td>
<td>100.4/3.12</td>
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<tr>
<td>30 °C / 65 % RH</td>
<td>101.9/1.51</td>
<td>100.9/2.21</td>
<td>102.1/2.89</td>
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<tr>
<td>25 °C / 60 % RH</td>
<td>101.9/1.51</td>
<td>101.8/2.04</td>
<td>101.3/2.68</td>
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</table>

RH – relative humidity
<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Related substance</th>
<th>Initial result</th>
<th>Intermediate result</th>
<th>Final result</th>
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</thead>
<tbody>
<tr>
<td>40 °C / 75 % RH</td>
<td>A</td>
<td>&lt;LOD*</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.03</td>
<td>0.22</td>
<td><strong>0.25</strong></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.03</td>
<td>0.11</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>0.04</td>
<td>0.15</td>
<td><strong>0.21</strong></td>
</tr>
<tr>
<td>30 °C / 65 % RH</td>
<td>A</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.03</td>
<td>0.10</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.03</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>0.04</td>
<td>0.10</td>
<td>0.12</td>
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<tr>
<td>25 °C / 60 % RH</td>
<td>A</td>
<td>&lt;LOD</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.03</td>
<td>0.10</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.03</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>0.04</td>
<td>0.09</td>
<td>0.12</td>
</tr>
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</table>

RH – relative humidity

* LOD – Limit of detection
TABLE IV. Results for in-use testing

| Tested parameters | 0 hour | 48 hours | | Tested parameters | 0 hour | 48 hours |
|-------------------|--------|----------|-------------------|-------------------|
| total MP conc./ % | 101.9  | 99.6     | total MP conc./ % | 100.4  | 100.2     |
| free MP conc./ %  | 1.51   | 3.62     | free MP conc./ %  | 3.12   | 4.98      |

<table>
<thead>
<tr>
<th>Related substances / %</th>
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<th></th>
<th>Related substances / %</th>
<th></th>
<th></th>
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</thead>
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<tr>
<td>Impurity A</td>
<td>&lt;LOD*</td>
<td>0.01</td>
<td>Impurity A</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>Impurity B</td>
<td>0.03</td>
<td>0.04</td>
<td>Impurity B</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Impurity C</td>
<td>0.03</td>
<td>0.04</td>
<td>Impurity C</td>
<td>0.14</td>
<td>0.14</td>
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<tr>
<td>Impurity D</td>
<td>0.04</td>
<td>0.06</td>
<td>Impurity D</td>
<td>0.21</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*A - Immediately after production (initial test point)
*B - Final test (after 6 months conditioning at 40 °C / 75 % RH)
*LOD – Limit of detection
TABLE V. Results of photostability testing

<table>
<thead>
<tr>
<th>Tested parameters</th>
<th>Initial</th>
<th>Directly exposed vials</th>
<th>Protected vials</th>
</tr>
</thead>
<tbody>
<tr>
<td>total MP conc./ %</td>
<td>101.9</td>
<td>96.3</td>
<td>100.3</td>
</tr>
<tr>
<td>free MP conc./ %</td>
<td>1.51</td>
<td>1.58</td>
<td>1.64</td>
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</table>

**Related substances / %**

<table>
<thead>
<tr>
<th>Impurity</th>
<th>Initial</th>
<th>Directly exposed vials</th>
<th>Protected vials</th>
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<tbody>
<tr>
<td>Impurity A</td>
<td>&lt;LOD*</td>
<td>0.32</td>
<td>0.01</td>
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<tr>
<td>Impurity B</td>
<td>0.03</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>Impurity C</td>
<td>0.03</td>
<td>0.04</td>
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</tr>
<tr>
<td>Impurity D</td>
<td>0.04</td>
<td>0.07</td>
<td>0.06</td>
</tr>
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</table>

*LOD – Limit of detection*
FIGURE CAPTIONS:

Fig. 1. Structure of MP (A), MP 21-HS (B), MP 17-HS (C), impurity B (D), impurity B1 (E), and impurities C and D (F).

Fig. 2. Chromatogram of MP test solution; fluorometholone was used as the internal standard.

Fig. 3. Chromatogram of related substances – impurity A, impurity B, impurity C and impurity D are considered as related substances: the sample was treated at 40 °C / 75 % RH for 3 months and prepared as described in the method for determination for related substances (test solution).

Fig. 4. 1H-NMR spectrum of impurity B.

Fig. 5. Proposed degradation profile of MP.
Fig. 1
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.