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

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

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

INTRODUCTION

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

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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 almost white, crystalline powder. They are practically insoluble in water and sparingly soluble in alcohol. The sodium salt of the phosphate or succinate ester is generally used to provide water-soluble forms for injections or solutions.
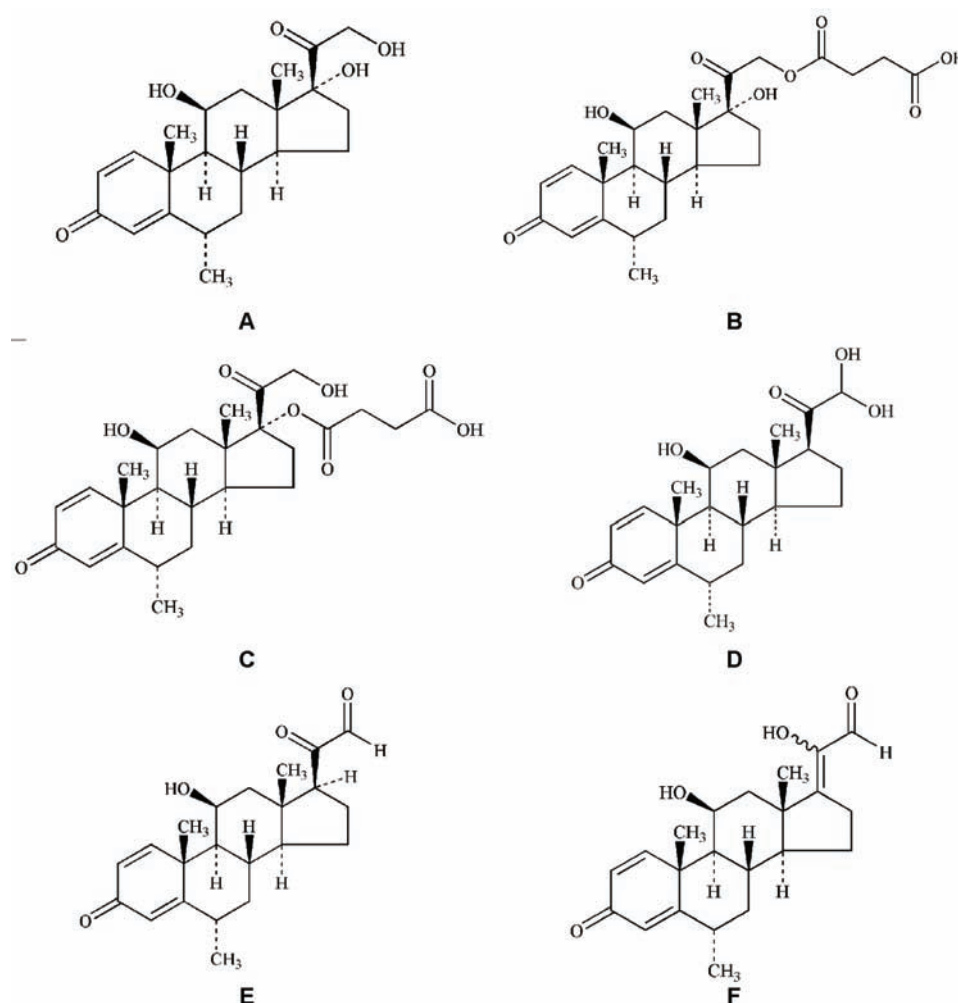

Fig. 1. Structure of MP (A), MP 21-HS (B), MP 17-HS (C), impurity **B** (D), impurity **B1** (E), and impurities **C** and **D** (F).

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 the cited substances, only MPNaS may be given intravenously. MPNaS is so extremely soluble in water[3] 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 $(6\alpha,11\beta)$-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.[6–10] This is the main reason for using the lyophilization process for production of injections. A problem associated with this type of injection is the storage of the medications (powder and solution for reconstitution) that should be administered as separate component parts and mixed prior to injection. Therefore, dual chamber vials have been developed to facilitate the storage and mixing of such two-component medications. This container closure system consists of a glass vial with two chambers separated by an intermediate rubber closure and an upper closure on the top. The specific feature of this type of the products is that both the 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 the literature as well.[11–13] Although a number of studies of the physical and chemical stabilities of MPNaS have been reported,[14–19] no published information is available on its stability in a 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 of MP. Special consideration was given to changes in the concentration of the active substance (total MP), the concentration of free MP, determination of related substances as possible degradation products and also their identification.

EXPERIMENTAL

Materials

A lyophilized injectable preparation of MPNaS was purchased by Hemofarm, Vršac, Serbia. The solution for reconstitution was water for injection/ 0.9 % benzyl alcohol. Two milliliters of reconstituted solution contained MPNaS equivalent to 125 mg MP, phosphate buffer and benzyl alcohol. This product was tested in the early development phase in accordance with relevant guidelines.[20–26]

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 the International Conference on Harmonization Standards for Stability (Q1A)[21] and Photostability (Q1B UV).[26] The following chambers were used: Weiss 2000, Weiss Gallenkamp, Weiss 600, Weiss Gallenkamp, Sanyo PSC 062, Sanyo Gallenkamp (Great Britain) and Weiss Umwelttechnik 140, Weiss Umwelttechnik, Germany.

Procedure for stability testing

A stability study was designed to increase the rate of chemical degradation of the finished pharmaceutical product (FPP) by using exaggerated storage conditions as part of the formal stability studies. The acceleration conditions used in this study are presented in Table I. During the study, the vials were kept in an upright or in a horizontal position. The horizontal position was chosen for compatibility assessment, in other words to ensure maximal contact of the diluent of the subject drug product with the elastomeric closures.

TABLE I. Storage conditions and testing frequencies

Storage conditions		Storage orientation	Testing time points, months
Accelerated	40±2 °C/75±5 % RH[a]	Upright	0, 1, 2, 3 and 6
		Horizontal	0, 3 and 6
Intermediate	30±2 °C/65±5 % RH	Upright	0, 3, 6, 9 and 12
		Horizontal	0, 6 and 12
Long-term	25±2 °C/60±5 % RH	Horizontal	0, 12, and 24

[a]Relative humidity

The in-use stability[24,25] was evaluated by examining the quality parameters of the subject drug product susceptible to change at the end of the proposed in-use shelf life (*i.e.*, 48 h after reconstitution, kept at room temperature). The samples were tested immediately after production (initial test point), at the end of the accelerated and intermediate conditions and at 12 and 24 month-testing points during long-term testing.

Photostability testing was performed using a light source – Option 2.[26] This means that a cool white fluorescent lamp and a near UV fluorescent lamp were used. The vials were exposed to light providing a defined energy (1.2×10^6 lx h) and then reconstituted. At the same time, protected samples (vials wrapped in aluminum foil or kept in a cardboard box) were used as the dark controls. At the end of this phase, the concentration of MP and related substances was determined.

Methods

HPLC Method for MP determination. Analytical procedures for the determination of both the total MP and free MP were in accordance with USP 32. The concentration of total MP is expressed as the sum of the concentration of free MP, MP 21-HS and MP 17-HS. The concentration of free MP is separately expressed as it is an important degradation product. The analyses were performed on an HPLC system, Agilent Technology 1100 Series, equipped with a 254 nm detector and a column packed with porous silica particles (L3). The flow rate was 1.0 mL min^{-1}. The mobile phase was prepared by mixing butyl chloride, water-saturated butyl chloride, tetrahydrofuran, methanol and glacial acetic acid (95:95:14:7:6; volume ratio).

An internal standard solution of concentration 3 mg mL^{-1} was obtained by dissolving the required amount of fluorometholone (RS) in tetrahydrofuran.

An MP solution was prepared by transferring an accurately weighed amount of MP RS (about 7.5 mg) to a 25-mL volumetric flask and dissolving to the mark with solvent. The solvent was 3 % glacial acetic acid in chloroform.

A standard solution was prepared by transferring an accurately weighed amount of MP 21-HS RS (about 32.5 mL) 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.

A test solution was prepared as follows. The 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 100-mL volumetric flask. The internal standard solution (10 mL) was added and the mixture was diluted with solvent to the mark. After 5 minute shaking, the layers were separated and the upper one was discarded.

The injection volume was 10 µL of each sample.

HPLC Method for determination of related substances. The related substances were determined by an isocratic HPLC method using an Agilent 1100 Series instrument with a variable wavelength UV–Vis detector. Separation was realized on a C18 column (250 mm×4.6 mm, 5 µm) column at a flow rate of 1.2 mL min^{-1}. The mobile phase was prepared by combining acetic acid, acetonitrile, and purified water (2:30:75, volume ratio).

A test solution was prepared as follows. Reconstituted solutions prepared from the contents of 10 vials of MPNaS for injection were mixed and 0.4 mL was transferred to a 20-mL volumetric flask and diluted with mobile phase up to 20 mL. The concentration of the obtained solution was 0.1 % w/v.

A standard solution was prepared by transferring 1 mL of test solution to a 100-mL volumetric flask and diluting with mobile phase. Then 1 mL of this solution was diluted with mobile phase to 10 mL. The concentration of the standard solution was 0.0001 % w/v.

The injection volume was 20 µl of each sample.

Methods for identification of related substances. Samples were treated with 5 mol L^{-1} HCl for 30 min at 80 °C in order to force degradation and obtain higher concentrations of the degradation products.

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

The high resolution electrospray ionization in a time of flight (HR-ESI-TOF) mass spectra of MP and three degradation products were measured on an Agilent 6210 LC/MS instrument. The mass spectrometer was operated under the following conditions: source – ESI in the 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.

The ^{1}H-NMR spectra of impurities **C** and **D** were measured on a Varian Gemini 2000 instrument at 200 MHz in CDCl$_3$. The NMR spectra of MP and impurity **B** (^{1}H-, ^{13}C-, DEPT--135, COSY, NOESY, HSQC and HMBC) were measured on a Bruker Avance III instrument at 500 MHz in CDCl$_3$ (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 the elution of peaks is the internal standard

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peak, MP 21-HS peak and successive smaller peaks of free MP and methylpred-nisolone 17-hydrogen succinate (MP 17-HS). The concentration of total MP is expressed as the sum of the concentration of free MP, MP 21-HS and MP 17-HS (Fig. 1C). The concentration of total MP in the initial sample (in the form of so-dium succinate) was defined as 100 %, and subsequent sample concentrations were expressed as percentage of the initial concentration.
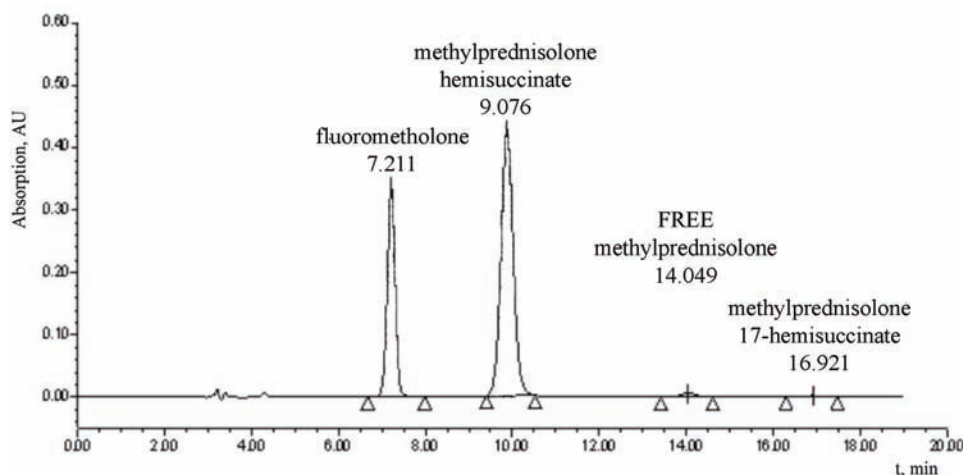

Fig. 2. Chromatogram of the MP test solution; fluorometholone was used as the internal standard.

The HPLC method for the determination of related substances confirmed the existence of four specified and not identified impurities marked as impurity **A**, **B**, **C** and **D**. A typical chromatogram related to the impurities is presented in Fig. 3. The second part of this study was focused on the identification of these im-purities.

The influences of different temperature in terms of the 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 can be seen, increasing the temperature was followed by an increase of free MP (because of hydrolysis) but it was still significantly below the maximum allowed level of 6.6 %. The obtained results in-dicated that there were no changes in the content of total MP, which could in-fluence the shelf life.

An increase in the concentrations of the related substances was also regis-tered. After 6 months conditioning at 40 °C/75 % relative humidity (RH), the concentrations of impurity **B** and impurity **D** were above 0.15 %, which is the identification level in accordance with relevant guidelines.[27] The increase in the concentration of impurity **A** was not significant.
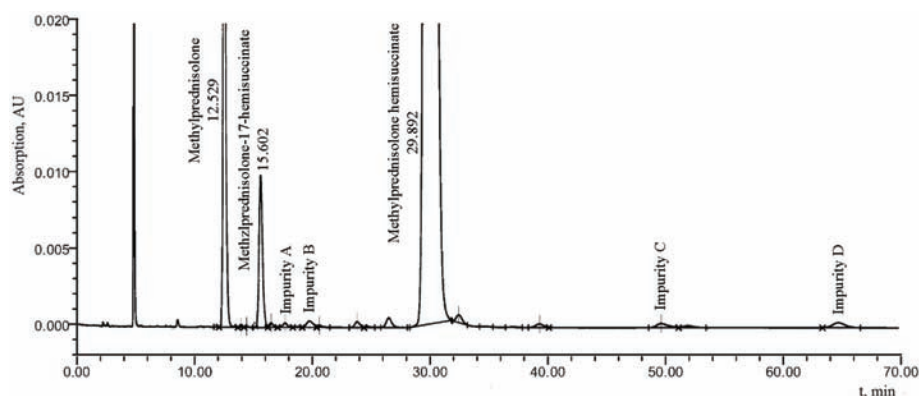
Fig. 3. Chromatogram of related substances – impurities **A**, **B**, **C** and **D** are considered as related substances: the sample was treated at 40 °C/75 % RH for 3 months and prepared as described in the method for determination of related substances (test solution).

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

Storage conditions	Initial result	Intermediate result	Final result
40 °C/75 % RH[a]	101.9/1.51	101.3/2.70	100.4/3.12
30 °C/65 % RH	101.9/1.51	100.9/2.21	102.1/2.89
25 °C/60 % RH	101.9/1.51	101.8/2.04	101.3/2.68

[a]Relative humidity

TABLE III. Results of the determination of the related substances (presented as percent of labeled amount of MP)

Storage conditions	Related substance	Initial result	Intermediate result	Final result
40 °C/75 % RH[a]	**A**	*<LOD*[b]	0.03	0.04
	B	0.03	0.22	0.25
	C	0.03	0.11	0.14
	D	0.04	0.15	0.21
30 °C/65 % RH	**A**	*<LOD*	*<LOD*	0.05
	B	0.03	0.10	0.14
	C	0.03	0.06	0.08
	D	0.04	0.10	0.12
25 °C/60 % RH	**A**	*<LOD*	0.03	0.06
	B	0.03	0.10	0.13
	C	0.03	0.06	0.08
	D	0.04	0.09	0.12

[a]Relative humidity; [b]limit of detection

For the testing of the in-use stability, all samples are conditioned according to ICH requirements and reconstituted. The samples were analyzed initially (immediately after production in use) and at the end after conditioning under defined conditions. The measurements on day 0 served as the references. The obtained

results are presented in Table IV. There were no statistically significant changes in the tested parameters – if the solution was kept at room temperature, there was no influence of water during 48 h; hence, only the results immediately after production (initial test point) and after 6 months conditioning at 40 °C/75 % RH are presented.

TABLE IV. Results of the in-use testing

Tested parameters	Immediately after production (initial test point)		Final test (after 6 months conditioning at 40 °C/75 % RH)	
	0 h	48 h	0 h	48 h
Total MP concentration, %	101.9	99.6	100.4	100.2
Free MP concentration, %	1.51	3.62	3.12	4.98
Related substances, %				
Impurity **A**	<*LOD*[a]	0.01	0.04	0.03
Impurity **B**	0.03	0.04	0.25	0.25
Impurity **C**	0.03	0.04	0.14	0.14
Impurity **D**	0.04	0.06	0.21	0.20

[a]Limit of detection

The results of the photostability testing are summarized in Table V. It was concluded that there was a change in the tested parameters. It is obvious that impurity **A** increased while the concentration of total MP decreased. When vials are kept in the dark (wrapped in aluminum foil or kept in a cardboard box), the increase in the concentration of impurity **A** was not significant; hence, impurity **A** was not the subject of further identification.

TABLE V. Results of the photostability testing

Tested parameters	Initial	Directly exposed vials	Protected vials
Total MP concentration, %	101.9	96.3	100.3
Free MP concentration, %	1.51	1.58	1.64
Related substances, %			
Impurity **A**	<*LOD*[a]	0.32	0.01
Impurity **B**	0.03	0.03	0.05
Impurity **C**	0.03	0.04	0.04
Impurity **D**	0.04	0.07	0.06

[a]Limit of detection

Identification of the structures of the degradation products

From the retention times of the impurities, it was concluded that impurity **B** was less polar then MP, and impurities **C** and **D** were significantly less polar.

Previous investigations of impurity **B** by MS showed that this compound has a mass of 374 amu, *i.e.*, the same as MP. The most abundant ion in HR-ESI-TOF mass spectra of MP is the ion at *m/z* 375.2464 (M+H$^+$), followed by 771.4071 (2M+Na$^+$) and 357.2057 (M+H$^+$–H$_2$O). The same ions appear in the mass spec-

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trum of impurity **B**, which means that impurity **B** and MP have the same molecular formula, *i.e.*, they are isomers. The presence of other ions (343, 345, 387...) in the mass spectrum of **B** showed that impurity **B** was impure and started to decompose. Hence, additional analyses (*i.e.*, ^{1}H-, ^{13}C-, DEPT-135, COSY, NOESY, HSQC and HMBC NMR spectroscopy) were required in order to elucidate the structure of impurity **B**.

Compound **B** had same mass and similar polarity as MP, so its structure could be 17-deoxy-21,21-dihydroxy-6α-methylprednisolone (Fig. 1D). The same compound was defined as impurity **B** of MP in EP.[1] In the European Pharmacopoeia this compound was defined as an impurity of MP and designated as **B**.

It was assumed that MP initially gave impurity **B**, which was later transformed (by standing and/or solvent exchange) into impurity **B1**. Since the structure of impurity **B1** was not compatible with the chromatographic behavior and mass spectra of impurity **B**, the structure of impurity **B1** was determined unequivocally by NMR spectroscopy.

The ^{1}H-NMR spectrum of **B1** (Fig. 4) is similar to that of MP, showing that they have the same basic structure. Based on all the spectra, it can be concluded that instead of a –CH$_2$OH group at 21 and an –OH group at 17 in MP, impurity **Bl** has a –CHO group at 21 and an H-atom at 17. Thus **Bl** has the structure of 17--deoxy-21-dehydro-6α-methylprednisolone (Figure 1E). The same impurity is known in the literature as an impurity of 6α-methylprednisolone acetate.[28]

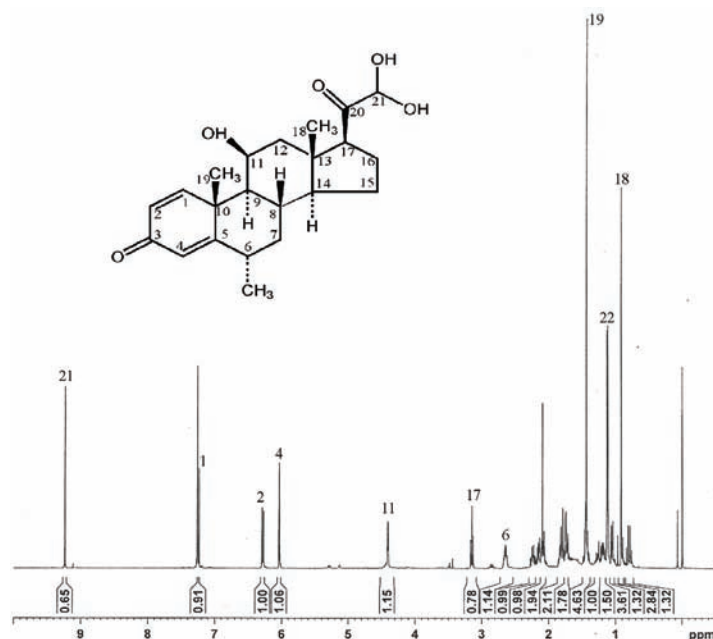
Fig. 4. ^{1}H-NMR Spectrum of impurity **B**.

The ^{1}H-NMR spectra of impurities **C** and **D** were very similar; they differed slightly in their chemical shifts. Compared to the proton spectrum of MP, the signals from the diastereotopic protons at C-21 were missing and a new signal appeared at 9.71 ppm in the spectrum of **C** and at 9.56 in the spectrum of **D**. This means that **C** and **D** are isomeric aldehydes (Fig. 1 F). The 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 the *E* and *Z* (respectively) 20-hydroxy-17(20) *E*-ene-21-al derivatives of 6α-methylprednisolone, which was defined as impurity **D** of MP in EP.[1]

According to the obtained results, the proposed degradation profile of MP is shown in Fig. 5.
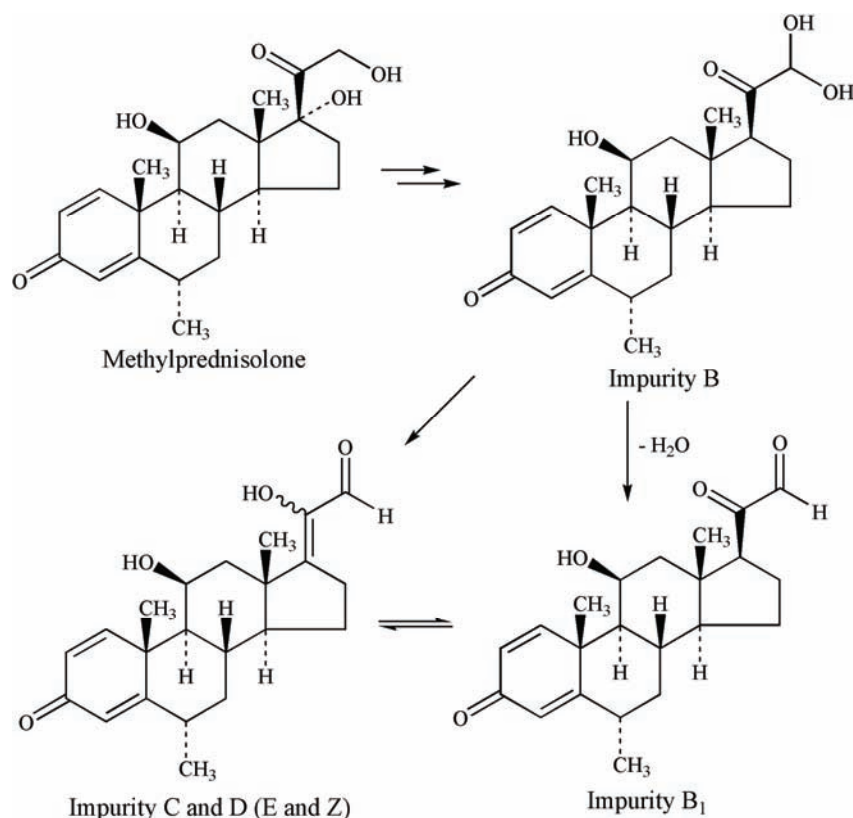

Fig. 5. Proposed degradation profile of MP.

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. The concentration of impurity **A** increases in the presence of light.

ИЗВОД

МЕТИЛПРЕДНИЗОЛОН И ЊЕГОВЕ СРОДНЕ СУПСТАНЦЕ У ЛИОФИЛИЗАТУ ЗА РАСТВОР ЗА ИНЈЕКЦИЈЕ

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У овом раду испитиване су особине фармаколошки активне супстанце метилпреднизолона (у облику метилпреднизолон-натријум-сукцината) у готовом производу – лиофилизату за раствор за инјекције. Циљ рада је испитивање хемијске стабилности метилпреднизолон-натријум-сукцината у двокоморној бочици, као специфичном систему контактног паковања. Испитан је ефекат различитих параметара: температуре, влаге и светлости. За одређивање концентрације метилпреднизолона, као збирне концентрације метилпреднизолон естара (17-хидроген-сукцината и 21-хидроген-сукцината) и слободног метилпреднизолона, коришћена је метода описана у Америчкој фармакопеји. За испитивање сродних супстанци примењена је HPLC метода. Уочена су 4 деградациона производа. Доказано је да повећање температуре има највећи значај на процес деградације и утиче на повећање садржаја нечистоћа **B**, **C** и **D**, док присуство светлости доводи до повећања садржаја нечистоће **A**. Нечистоће **B**, **C** и **D** су идентификоване применом масене и NMR спектроскопије. Све три нечистоће су идентификоване као сродне супстанце метилпреднизолона.

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