Determination of New Long-term Metabolites of Methylprednisolone In Human Urine by HPLC-ESI(-)/MS

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ABSTRACT

Methylprednisolone synthetic corticosteroid (MP, Medrol, Canada - 8 mg) was orally administered to healthy male volunteer. Urine samples were collected up to three days post-dose. Ten new metabolites have been identified in addition to the seven ones reported previously. LC-MS analysis have shown well-known 11-keto – 11-hydroxy interconversion and the formation of methylprednisolone 20-hydroxy, 6,7-dihydro and 20-hydroxy-6,7-dihydro metabolites. In addition formation a number of mono-hydroxylated metabolites of MP have been observed. We conclude that the new metabolites can be used for the detection of methylprednisolone abuse since the parent drug MP readily disappears in urine.

Keywords: LC-MS; doping analysis; methylprednisolone; metabolism

INTRODUCTION

Methylprednisolone (MP; 11β,17α,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione) is a synthetic glucocorticosteroid. It is mainly used in therapeutic treatment, particularly for their anti-inflammatory and immunosuppressive actions, or for substitution therapy in the case of insufficiency of adrenal cortex secretion. The systematic use of synthetic corticosteroids is often associated with significant side effects, ranging from skin fragility to full-blown iatrogenic Cushing syndrome [1-4]. Prolonged use may lead to suppression of the hypothalamic-pituitary-adrenal axis. Consequently, because glucocorticoids may produce harmful side-effect, their use during competitions has been prohibited by the World Anti Doping Agency (WADA) [5]. Hence the methods to detect this compound or its metabolites in human urine are required.

Metabolic studies of MP in human urine by GC-MS have been first published by Rodchenkov et al [6]. Authors have found that the 6,7-dihydro and 20-hydroxy metabolites are the main metabolites. Therefore, for the screening of urine steroids these metabolites and unchanged MP were recommended for identification purpose.

GC-MS methods of has been widely applied in the doping control, but for analysis of metabolism of corticosteroids is not successful. The most serious problems encountered in the GC-MS analysis of corticosteroids are low volatility of these compounds as MO-TMS or TMS derivatives [7-9].

High-performance liquid chromatography (HPLC) presents the conditions which are well suited for the separation of corticosteroids and its metabolites and the use of mass spectrometry for detection provides a good specificity and sensitivity [10-15].

This work presents HPLC-MS method for qualitative identification of long-term metabolites of methylprednisolone in human urine. Structural assignments of metabolites were based on changes in molecular masses and retention time. The sensitive and specific method for the simultaneous detection of methylprednisolone, its metabolites and endogenous steroids in human urine was developed.

EXPERIMENTAL

1. Reagents and chemicals

Cortisone, hydrocortisone, tetrahydrocortisole, tetrahydrocortisone, α- and β-cortol, α- and β-cortolone, 6β-hydroxycortisol, methylprednisolone, fluoxymesterone, β-glucuronidase/arylsulphatase from *Helix pomatia* were purchased from Sigma-Aldrich (Germany). All chemicals were of analytical grade purity. Acetonitrile (G Chromasolv for HPLC, supergradient grade, min. 99.9%) was obtained from Sigma-Aldrich (Germany), methanol (LiChrosolv for HPLC grade, min. 99.8%) from Merck (Germany). Toluene and diethyl ether (AnalaR) were purchased from BDH (UK). Ammonium acetate (min. 98%) and formic acid (min. 99%) were purchased from Acros Organics (Belgium). Sodium sulphate, potassium hydrocarbonate and potassium carbonate were obtained from ChimMed (Russia). All chemicals were of analytical grade purity. Purified water for HPLC was obtained with a "Milli-Q plus" purification system (Millipore, France).

2. LC-ESI/MSD Ion trap-analysis

Analysis was performed using an HPLC system 1100 series (Agilent Technologies, USA) configurated of binary pump with on-line degasser and autosampler. The analytical column was a 100 × 2.1 mm I.D. Ultra C18 from Restek (USA), 5 μm particle size, connected to a guard-column 4 × 12.5 mm I.D. The mobile phase was 0.2 mM ammonium acetate with 0.05% formic acid (A) – acetonitrile (B) at a flow rate of 0.2 ml/min. The solvent gradient program was as follows: 0 min – 15% B; 10 min – 60% B; 15 min – 75% B; 25 min – 85% B.
Spectral detection was carried out on Ion Trap mass spectrometer Superior Line System (Agilent Technologies, USA) equipped with an atmospheric pressure electrospray ionization source (ESI). Electrospray conditions of the mass spectrometer were set: nebulizer gas nitrogen: 30 psi; drying gas nitrogen: 9 L/min, 350ºC; capillary voltage – 3500V. The mass spectrometer was operating in the negative ion mode for analysis of the analytes.

3. Sample preparation

3.1. Administration

One healthy volunteer (male, 32 years, 80 kg) gave their informed consent to participate in the study. Blank urine was collected before the administration of a single oral dose of Methylprednisolone (2 tablets, 4 mg); Medrol, Pharmacia & Upjohn, Canada. The samples urine were collected during 3 days and immediately frozen at -30°C.

3.2. Sample preparation

To 5 ml of urine 5 μl of internal standard (fluoxymesterone, 20 μg/ml) was added. Then 1 ml of phosphate buffer (pH = 7.4) and 30 μl of β-glucuronidase//arylsulphatase *Helix pomatia* were added prior to enzymatic hydrolysis. The hydrolysis process was carried out at 55°C for 1 hour. The solution was cooled to room temperature and the pH was adjusted to 9 by adding solid buffer (Na_2CO_3/$NaHCO_3$, 1:1 mixture). Then, *ca*. 2 g of anhydrous sodium sulfate were added, and the mixture was extracted with 5 mL of mixture of diethyl ether/toluene (1:1, v/v). After shaking (2 min, Vortex) and centrifugation (5 min, 3000 rpm), the organic layer was separated and taken to dryness at 60°C. For HPLC-MS analysis the residue was dissolved in 50 μl of methanol, and 5 μl of this solution was injected into the LC-MS ion trap system.

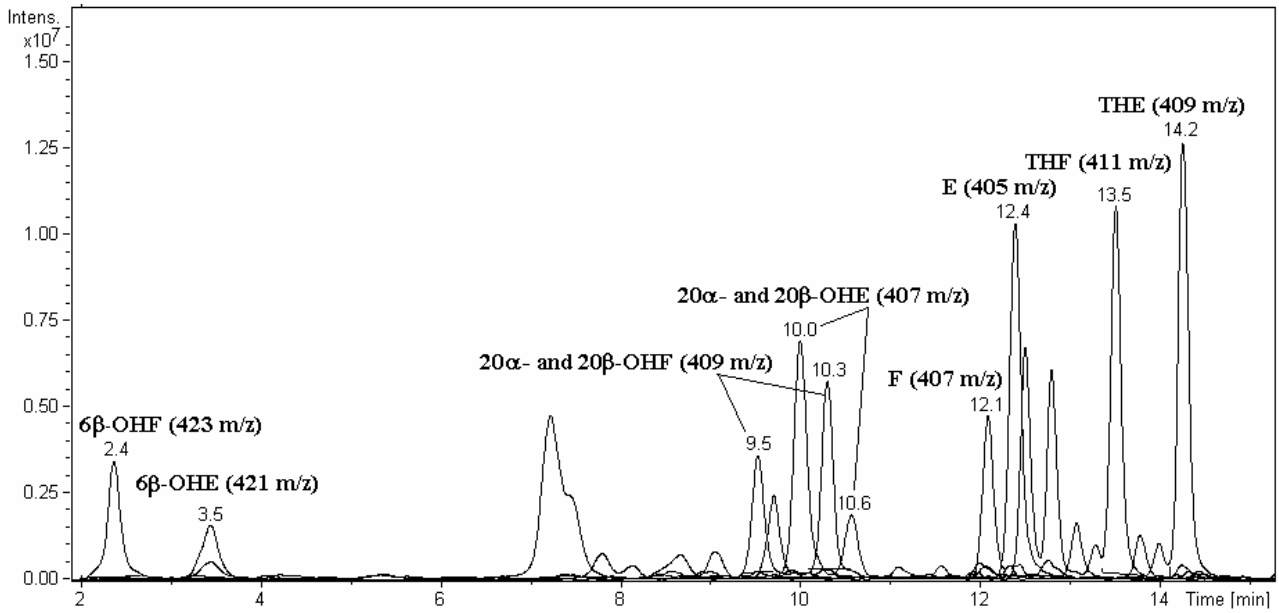
RESULTS AND DISCUSSION

In this study, it was possible to identify sixteen urinary metabolites of methylprednisolone (**MP**) including already reported [6] metabolites, namely 11-keto-metylprednisolone (**11-KMP**), 20α- and 20β- hydroxy-methylprednisolone (**20α-OHMP** and **20β-OHMP**), 6,7-dihydro-11-keto-methylprednisolone (**6,7-dihydro-11-KMP**), 6,7-dihydro-methylprednisolone (**6,7-dihydro-MP**), 20α- and 20β- hydroxy-6,7-dihydro-methylprednisolone (**6,7-dihydro-20α-OHMP** and **6,7-dihydro-20β-OHMP**). Using HPLC-MS method, the ten new metabolites of methylprednisolone were detected in the chromatogram. In the total ion chromatogram many peaks were observed, which were absent in negative control urine.

The identification of metabolites was based on metabolism of a cortisol (**F**) which is a major endogenous glucocorticoid. The main metabolites of cortisol are cortisone (**E**), tetrahydrocortisol (**THF**), tetrahydrocortisone (**THE**), 20α- and 20β-dihydrocortisol (**20α-OHF, 20β-OHF; α- and β-Cortol**), 20α- and 20β-dihydrocortisone (**20α-OHE, 20β-OHE; α- and β-Cortolone**). **F** and **E** are also metabolized to 6β-hydroxycortisol (**6β-OHF**) and 6β-hydroxycortisone (**6β-OHE**). The urinary profile of cortisol and its main metabolites in human blank urine is presented in Fig. 1.

In this study we have created an optimized method to detect MP and its metabolites and main endogen corticosteroids also.

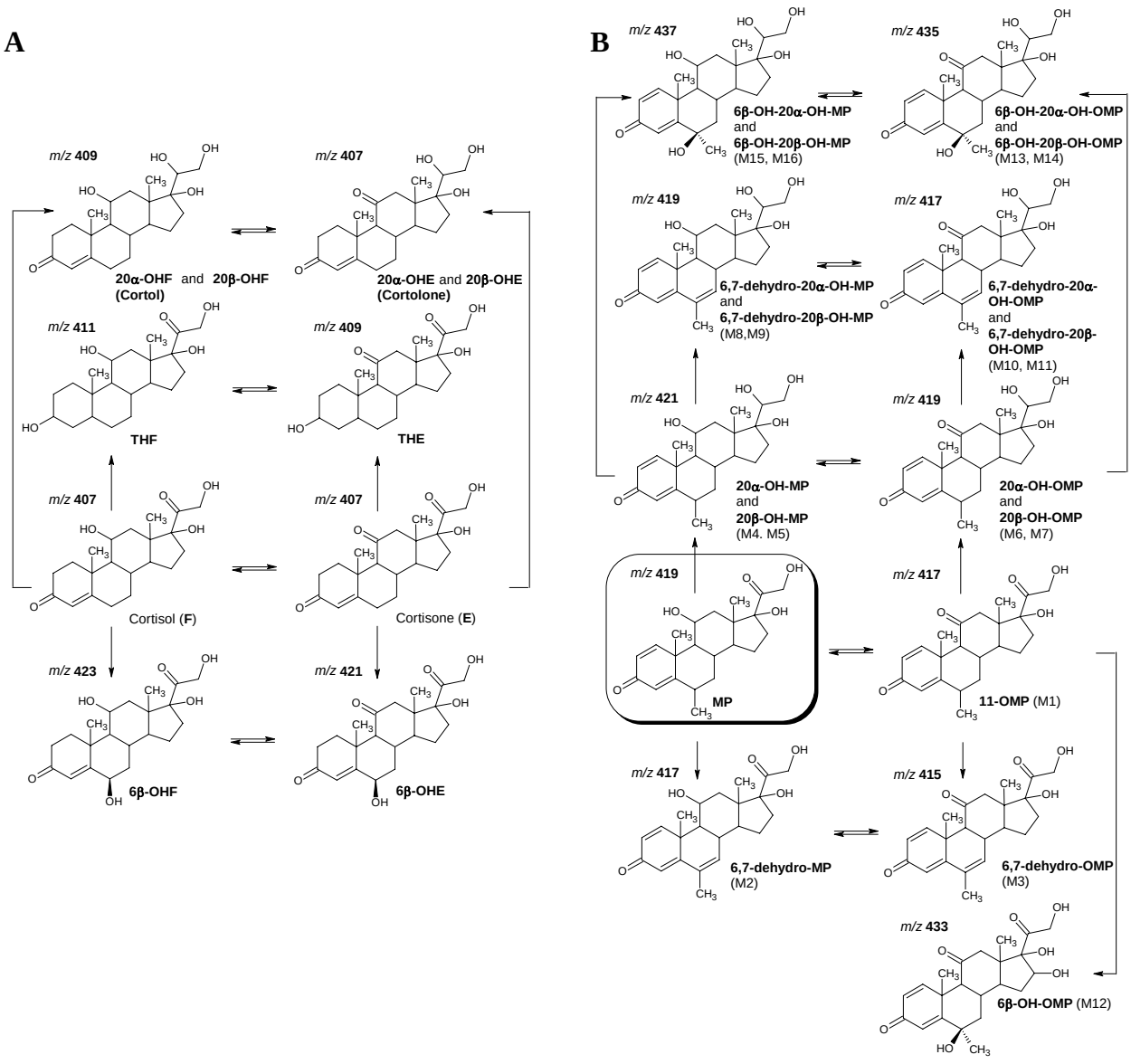
Fig. 1: HPLC-MS urine steroid profile. Ion chromatograms of *m/z* 423, 421, 411, 409, 407 in human blank urine.



1.Metabolism of methylprednisolone

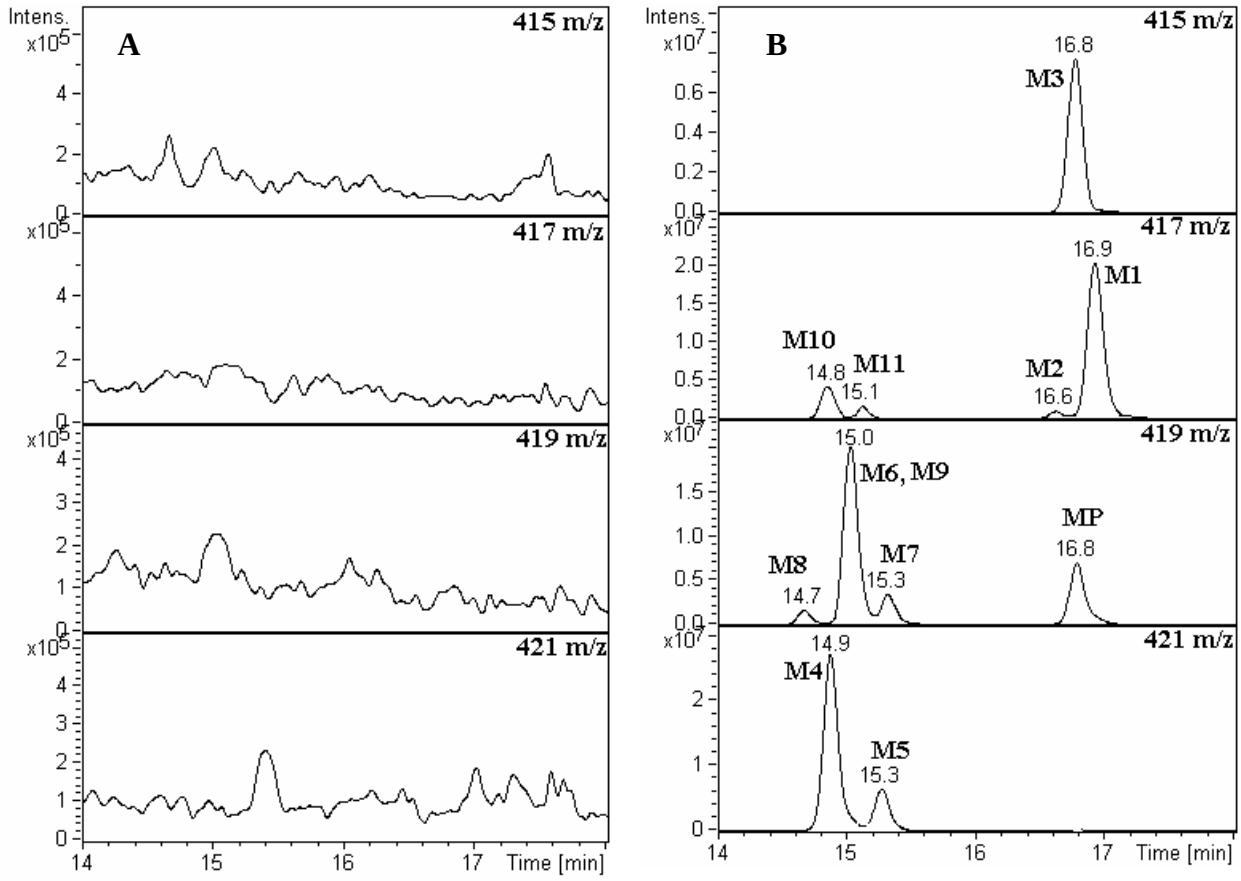
Analysis by LC-ESI(-)/MS have shown that besides the unchanged parent drug (**I**), the following sixteen metabolites were detected: 11-oxo metabolite **M1 (OMP)**; 6,7-dihydro-MP **M2**; 6,7-dihydro-11-OMP **M3**; 20α- and 20β- hydroxy isomers of MP (**M4, M5**), 20α- and 20β- hydroxy isomers of 11-OMP (**M6, M7**), 20α- and 20β-hydroxy isomers of 6,7-dihydro-MP (**M8, M9**), 20α- and 20β- hydroxy isomers of 6,7-dihydro-11-OMP (**M10, M11**), 6β-hydroxy-11-OMP (**M12**), 20α- and 20β- hydroxy isomers of 6β-hydroxy-6,7-dihydro-11-OMP (**M13, M14**), and 20α- and 20β-hydroxy isomers of 6β-hydroxy-6,7-dihydro-MP (**M15, M16**). Metabolites M12-M16 have not been reported before! (Fig. 2).

Fig. 2: (**A**) Metabolic pathway of cortisol; (**B**) Proposed metabolism of Methylprednisolone (MP)



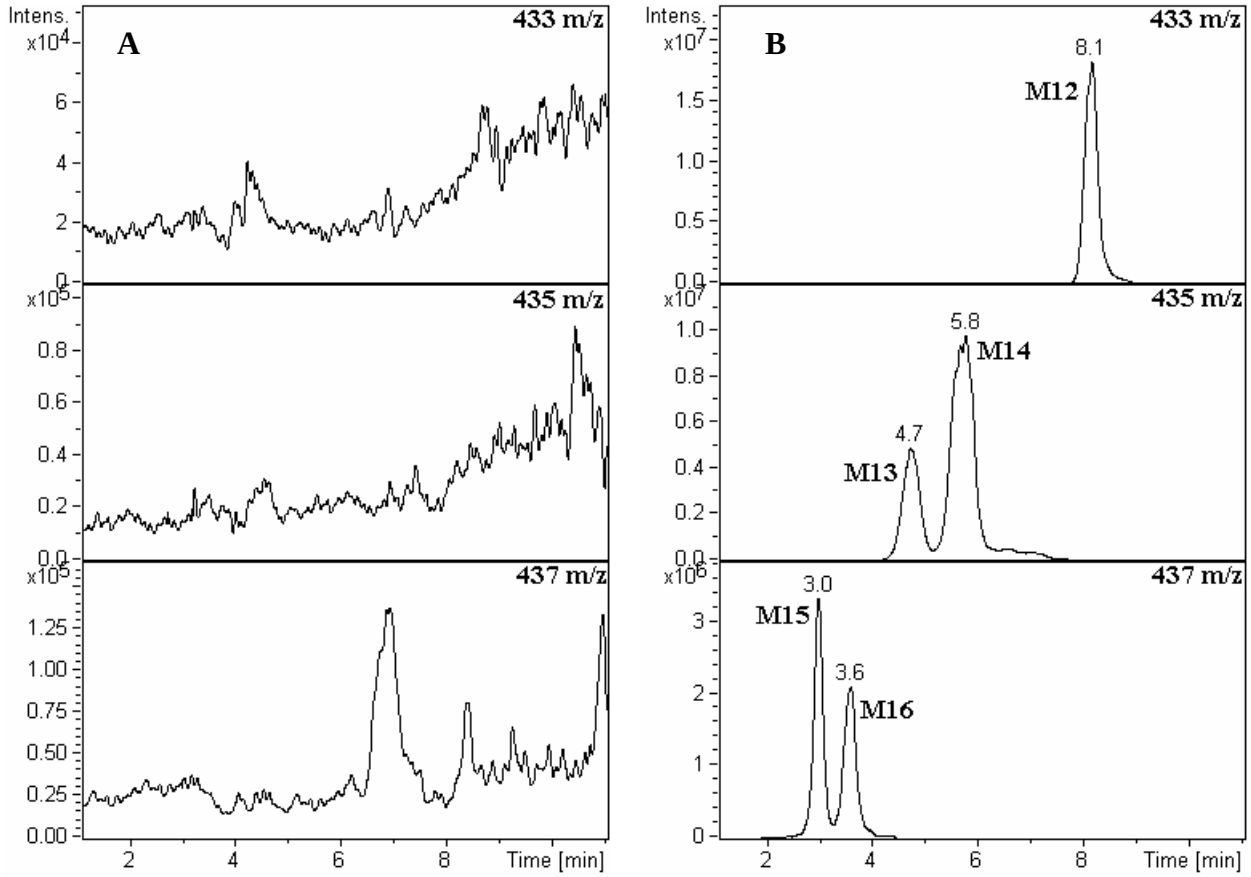
LC-MS analysis have shown formation of adduct ions with formic acid from mobile phase, are characterized by peak $[(M-H)^-+46]$ (e.g. for MP: Mw = 374, $[(M-H)^-+46]$ = 419). This peak is present as a base peak in all endogenous and exogenous corticosteroids and their metabolites. MS-MS mass spectra of these compounds are not informative, and further MS-MS fragmentation results in formation of two new peaks $(M-H)^-$ and $[(M-H)^--30]$ due to the loss of CH_2O.
The review of total ion chromatogram of human urine after drug administration has shown a number of peaks with similar mass spectra: *m/z* 415 (one peak), *m/z* 417 (four peaks), *m/z* 419 (four peaks), *m/z* 421 (two peaks), *m/z* 433 (one peak), *m/z* 435 (two peaks) and *m/z* 437 (two peaks). Chromatograms by selected characteristic ions before and after administration of methylprednisolone are presented in Fig. 3-4.

Fig. 3: Ion chromatograms of *m/z* 421, 419, 417, 415 in (**A**) blank urine; (**B**) after administration of a single oral dose (8 mg), after 10 hours.



The unchanged drug (**MP**) and **M1** metabolite are slightly separated and were eluted at 16.8 min and 16.9 min in the methylprednisolone positive urine, respectively. The elution order of **MP** (*m/z* 419) and **M1** (*m/z* 417) was assumed to be similar to endogenous **F** (*m/z* 407) and **E** (*m/z* 405), where the cortisol (**F**) elutes first. Therefore, by analogy with cortisol-cortisone elution, elution order **M1** corresponds to metabolite with C-11 hydroxy group oxidation (11-oxo-metabolite of **MP**) - methylprednisone.

Fig. 4: Ion chromatograms of *m/z* 433, 435, 437 in (**A**) blank urine; (**B**) after administration of a single oral dose (8 mg), after 10 hours.

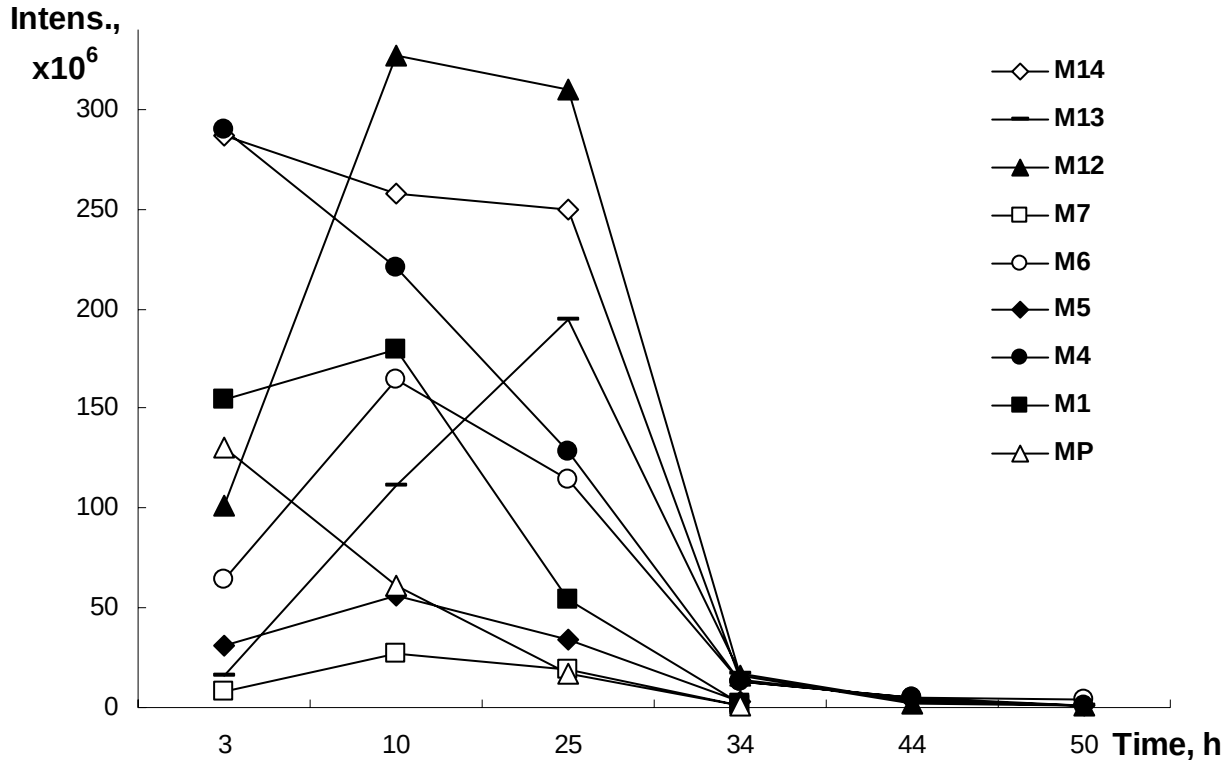


Two 6,7-dihydro metabolites of **MP** and **M1** (11-OMP) were detected. Metabolites **M2** (*m/z* 417) and **M3** (*m/z* 415) are forming a second pair with 11-hydroxy – 11-keto interconversion and they have shown a shorter retention time than methylprednisolone (**MP**) – methylprednisone (**M1**) pair, 16.6 min and 16.8 min, respectively. The characteristic ions $[(M-H)^-+46]$ of these metabolites were decreased by 2 Da in comparison with that of the MP and 11-KMP.

Four metabolites with reduced C=O bond at C-20 position were detected. Metabolites **M4**, **M5** (*m/z* 421) and **M6**, **M7** *(m/z* 419) were eluted at 14.9 min, 15.3 min and 15.0 min, 15.3 min, respectively. Peaks **M4** and **M5** are considered to be isomeric 20α- and 20β-hydroxy metabolites of **MP**. Peaks **M6** and **M7** are considered to be isomeric 20α- and 20β-hydroxy metabolites of **M1**. The elution order of 20α- and 20β- isomers of **MP** and **M1** were assumed to be similar to endogenous 20α- and 20β- isomers **F** and **E** (20α- and 20β-OHF, 20α- and 20β-OHE, Fig. 1). The increase in base ions $[(M-H)^-+46]$ by 2 Da, shorter retention time compared to that of **MP** and **M1**, and formation of two peaks (20α- and 20β- isomers) with *m/z* 421 and *m/z* 419 clearly indicated reduced C=O bond at C-20 position.

The methods of GC-MS [6] and the HPLC-MS have shown, that 6,7-double bond formation is one of the main metabolic ways of MP and 11-OMP. Therefore, we expected formation of four 6,7-dihydro metabolites of 20-OH-MP (**M4**, **M5**) and 20-OH-OMP (**M6**, **M7**). Metabolites **M8**, **M9** (*m/z* 419) and **M10**, **M11** *(m/z* 417) were eluted at 14.7 min, 15.0 min and 14.8 min, 15.1 min, respectively. We did not observed the **M9** metabolite (20α- or 20β-hydroxy-6,7-dihydro MP) but we supposed that it coelutes with **M6** metabolite. The base ions $[(M-H)^-+46]$ of these metabolites were the same with that of **MP** and **M1** but retention time were shorter.

Fig. 5: Excretion of methylprednisolone and its main metabolites by LC-ESI/MSD Ion trap using free fraction in human urine after oral administration of 8 mg of methylprednisolone (*n*=2).



Rodchenkov et. al [6] tried to detect a 6β-hydroxylated metabolites of **MP**, but those compounds were not found. In this study we determined five hydroxylated metabolites with *m/z* 437, *m/z* 435 and *m/z* 433 (Fig. 4). Mono-hydroxylated metabolites of **MP** were found in the first time.

Metabolites **M15** and **M16** with *m/z* 437 might be formed from compounds with *m/z* 421. Metabolites **M15** and **M16** (*m/z* 437) were eluted at 3.0 min, 3.6 min, respectively. In chromatogram after drug administration we observed only two peaks with *m/z* 421 (**M4**, **M5**). Therefore, increase in characteristic ions $[(M-H)^-+46]$ by 16 Da, shorter retention time compared to that of **M4** and **M5** clear indicated an addition of hydroxy-group to these compounds. We could not confidently indicate a position of hydroxy group, but we suppose that the hydroxylation has taken place on 6β-position, although the 6α-methyl group may influence oxidation. Hydroxylation to 6β-position is very character for corticosteroids metabolism. Authors [16] have shown it on the example of prednisone-prednisolone metabolisms.

Metabolites **M13** and **M14** with *m/z* 435 might be formed from any compounds with *m/z* 419. These metabolites were eluted at 4.7 min, 5.8 min, respectively. In chromatograms after drug administration we observed four peaks with *m/z* 419 (**MP**, **M6**, **M7, M8**). By analogy with **M15** and **M16** metabolites we may suggested that it is hydroxylates **M6** and **M7** compounds. The 6β-position is held at a **M8** metabolite. Therefore, increase in characteristic ions $[(M-H)^-+46]$ by 16 Da, shorter retention time compared to that of **M6** and **M7** evidently indicated an addition of hydroxy-group to these compounds.

Metabolite **M12** with *m/z* 433 might be formed from any compounds with *m/z* 417. This metabolite was eluted at 8.1 min. In chromatogram after drug administration we saw four peaks with *m/z* 417 (**M1**, **M2**, **M10**, **M11**). As well as at the **M8** metabolite, the 6β-position is held at a **M2**, **M10** and

M11 metabolites. And that fact that we see only one peak with *m/z* 433 indicates hydroxylation of **M1** compound. Hence, increase in characteristic ions [$(M-H)^-$+46] by 16 Da, shorter retention time compared to that of **M1** evidently indicated added hydroxyl-group to it compound.
Finally, LC-MS analysis have shown together with the well known 11-keto – 11-hydroxy interconversion and the formation of methylprednisolone 20-hydroxy, 6,7-dihydro and 20-hydroxy-6,7-dihydro metabolites, we can observed formation a number of mono-hydroxylated metabolites of methylprednisolone.

2. Excretion study

After MP administration the amount of natural corticosteroid F and its metabolites decreases significantly. In the course of extraction the main metabolites M1, M5, M6, M7, M12 and M13 increase while MP, M4 and M14 decrease. Using LC-ESI/MS Ion Trap, the unchanged MP, M1, M5 and M7 could be detected for only 30 hours. Referring to WADA reporting cut-off level of 30 ng/ml, the urine sample is considered as positive and forming doping rule violations for 15 hrs only. Meantime the other metabolites are present in significantly higher amount for longer time. Fig. 5 shows excretion curves of MP and its metabolites. The metabolites M4, M6, M12, M13 and M14 could be detected for up 50 hours. Mono-hydroxylated metabolites M12, M13 and M14 were the most abundant metabolites of the first day. After 2 days the excreted amount of the metabolites M6 was largest among the all excreted metabolites. The metabolites M4, M6, M12, M13 and M14 were detected at 2-3 days after oral administration single dose – 8 mg of methylprenisolone.

CONCLUSIONS

In this study, it was possible to identify sixteen urinary metabolites of methylprednisolone after oral administration of 8 mg. The main metabolites in human urine and numerous minor metabolites were detected. Metabolites with m/z 437, m/z 435 and m/z 433 must are mono-hydroxylated with a hydroxyl group in 6β-positions but their structure have not yet elucidated. The position of hydroxyl group may be further investigated by GC-MS with MO-TMS derivatives. The principal (**M4, M6, M12, M13** and **M14**) metabolites with *m/z* 421, 419, 433 and 435 can be detected in urine 60 hrs post-administration. We conclude that the new metabolites can be used for the detection of methylprednisolone abuse because of the parent drug MP readily disappears in urine.

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