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Using complementary mass spectrometric approaches for the determination of methylprednisolone metabolites in human urine

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RATIONALE: The metabolism of methylprednisolone is revisited in order to find new metabolites that could be important for distinguishing between different routes of administration. Recently developed liquid chromatography/tandem mass spectrometry (LC/MS/MS) strategies for the detection of corticosteroid metabolites have been applied to the study of methylprednisolone metabolism.
METHODS: The structures of these metabolites were studied using two complementary mass spectrometric techniques: LC/MS/MS in product ion scan mode with electrospray ionization and gas chromatography/mass spectrometry (GC/MS) in full scan mode with electron ionization. Metabolites were also isolated by semipreparative liquid chromatography fractionation. Each fraction was divided into two aliquots; one was studied by LC/MS/MS and the other by GC/MS after methoxyamine-trimethylsilyl derivatization.
RESULTS: The combination of all the structural information allowed us to propose a comprehensive picture of methylprednisolone metabolism in humans. Overall, 15 metabolites including five previously unreported compounds have been detected. Specifically, 16β, 17α,21- trihydroxy- 6α- methylpregna-1, 4 -diene-3,11,20-trione, 17α,20β,21-trihydroxy-6α-methylpregna-1, 4-diene-3, 11- dione, 11β, 17α, 21-trihydroxy-6α-hydroxymethylpregna-1, 4-diene-3,20-dione, 11β,17α, 20ξ,21-tetrahydroxy-6α-hydroxymethylpregna-1,4-diene-3-one, and 17α,21-dihydroxy-6α-hydroxymethylpregna-1,4-diene-3,11,20-trione are proposed as feasible structures for the novel metabolites. In addition to the expected biotransformations: reduction of the C20 carbonyl, oxidation of the C11 hydroxy group, and further 6β-hydroxylation, we propose that hydroxylation of the 6α-methyl group can also take place.
CONCLUSIONS: New metabolites have been identified in urine samples collected after oral administration of 40 mg of methylprednisolone. All identified metabolites were found in all samples collected up to 36 h after oral administration. However, after topical administration of 5 g of methylprednisolone aceponate, neither the parent compound nor any of the metabolites were detected. Copyright © 2012 John Wiley & Sons, Ltd.

Methylprednisolone (11β,17α,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione, MP) is a moderately potent glucocorticoid with anti-inflammatory and immunosuppressive effects.[1] It has been shown to be effective in a wide range of conditions such as asthma, systemic lupus, rheumatoid arthritis, and renal transplant rejection.[2,3] Its use in sport is prohibited by the World Anti-Doping Agency (WADA). All glucocorticosteroids are prohibited in-competition when administered orally, rectally, intravenously or intramuscularly,[4] and its use requires a therapeutic use exemption approval. However, topical preparations when used for dermatological, auricular, nasal, ophthalmic, buccal, gingival and perianal disorders are not prohibited and do not require any form of therapeutic use exemption.

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In humans, MP undergoes phase I and II metabolism. By using gas chromatography/mass spectrometry (GC/MS), Rodchenkov *et al.* found 11-keto and 20-hydroxy, and 6,7-dehydro as the main metabolites in human urine after oral administration of 20 mg of the drug.[5] A different study, based on liquid chromatography/tandem mass spectrometry (LC/MS/MS) and nuclear magnetic resonance (NMR), concluded that the metabolic pathway of MP after intravenous administration was simple: reduction of the C20 carbonyl group, further oxidation of the C20-C21 side chain, and C6 oxidation.[6] In a recent study performed by LC/MS/MS, metabolites detected after intramuscular and intra-articular administrations were described.[7] Metabolites obtained after 11-oxidation, reduction of C20 and oxidation of the C20-C21 side chain were also detected. Some metabolites conjugated with glucuronic acid were also identified.

In spite of the knowledge that we have about glucocorticoid metabolism, detection of the use of MP in sport is routinely performed by methods aimed at detecting the presence of the parent drug in urine.[8–10] The characterization of minor

corticosteroid metabolites can be of great importance in the doping control field as it may improve the detection of drug misuse, or allow for differentiation between different routes of administration.

Recently, different non-targeted LC/MS/MS methods able to detect anabolic steroids, glucocorticosteroids and their metabolites, based on a specific structural characteristics, have been studied.[7,11,12] LC/MS/MS in triple quadrupole instruments can be applied in different acquisition modes such as neutral loss (NL) or precursor ion (PI) scan modes. The use of these modes allows for the detection of untargeted compounds containing a specific structure which is very useful for metabolic studies. Several methods based on these scan modes have been developed and successfully applied for the detection of steroid metabolites.[11,13,14] Based on these results, different approaches for the non-targeted detection of corticosteroids in urine were evaluated in this study. These screening methodologies may provide a list of potential metabolites of MP with unknown structures. A more comprehensive study should then allow us to propose feasible structures for those candidate metabolites. The combination of classical GC/MS strategies in full scan mode[15] and LC/MS/MS strategies based on the integration of different mass spectrometric scan modes[16] can provide the most comprehensive picture of the metabolism of corticosteroids in general, and MP in particular.

The goals of the present work were: first to enhance our knowledge of the oral metabolism of MP by using complementary mass spectrometric techniques, and second to develop specific and sensitive LC/MS/MS qualitative methods in selected reaction monitoring (SRM) mode for the detection of these metabolites in human urine. These methods were then applied to the detection of potential metabolites of MP after topical use, in order to estimate the differences in metabolism between routes of administration.

EXPERIMENTAL

Chemicals and reagents

Methylprednisolone, sulfatase from *Helix pomatia* type H-1, β-glucuronidase/arylsulfatase from *Helix pomatia* type H-2, sodium borohydride, and methoxyamine hydrochloride were obtained from Sigma (St. Louis, MO, USA). The β-glucuronidase preparation (type *E. coli* K12) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Analytical grade disodium hydrogen phosphate, sodium hydrogen phosphate, ethyl acetate, cyclohexane, and pyridine were obtained from Merck (Darmstadt, Germany). Trimethylsilylimidazole (TMSI) was from Macherey-Nagel (Düren, Germany).

Acetonitrile and methanol (LC gradient grade) and formic acid (LC/MS grade) were purchased from Merck. Milli-Q water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Sep-Pak® C18 cartridges were obtained from Waters (Milford, MA, USA).

LC/MS/MS instrumentation

SRM, precursor ion (PI), neutral loss (NL) and product ion scans were carried out using a triple quadrupole (Quattro Premier XE) mass spectrometer provided with an orthogonal Z-spray electrospray ionization (ESI) interface (Waters) interfaced to an Acquity ultra-performance liquid chromatography (UPLC) system (Waters) for the chromatographic separation. Nitrogen (N_2) was used as the drying gas, nebulizing gas, cone gas and desolvation gas. The desolvation gas flow rate was approximately 1200 L/h and the cone gas flow rate was 50 L/h. A cone voltage of 25 V and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set to 450 °C and the source temperature to 120 °C. In the PI scan methods three different collision energies were used (50, 55 and 60 eV) and a precursor ion window from *m/z* 300 to 600 was monitored. In the PI scan methods two different collisions energies were used (20 and 30 eV) and a window from *m/z* 50 to 400 was monitored. In negative ionization mode, a cone voltage of 25 V and a capillary voltage of 2.5 kV were used. The nitrogen desolvation temperature was set to 400 °C and the source temperature to 120 °C. In the NL scan methods, a collision energy (CE) of 15 eV was used and a precursor ion window from *m/z* 300 to 600 was monitored.

The LC separation was performed using an Acquity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 µm particle size) at a flow rate of 400 µL/min. Water (solvent A) and acetonitrile (solvent B), both with formic acid (0.01%), were selected as the mobile phase solvents. A gradient program was used: the percentage of organic solvent was linearly changed as follows: 0 min, 10% B; 0.6 min, 10% B; 5 min, 20% B; 12 min, 30% B; 16.5 min, 90% B; 17 min, 90% B; 17.5 min, 10% B; 20 min, 10%B.

GC/MS instrumentation

GC/MS analysis was carried out on a 6890 N gas chromatograph coupled with a 5975 quadrupole mass spectrometer with a triple-axis detector (Agilent Technologies, Palo Alto, CA, USA). The steroids were separated on a HP-Ultra1 cross-linked methyl-silicone column (16.5 m × 0.2 mm i.d., 0.11 µm film thickness; J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a constant pressure of 5 psi. A 2 µL aliquot of the final derivatized extract was injected into the system operated in splitless mode (valve opened at 2 min). The GC column temperature was ramped as follows: initial 50 °C, held for 3 min, increased to 230 °C at 30 °C min^{-1}, thereafter increased to 285 °C at 2 °C min^{-1}. The injector and transfer line were kept at 280 °C. In full scan mode, the mass range scanned was from *m/z* 70 to 850.

Semipreparative HPLC

In order to isolate the metabolites, the urine sample (15 mL) was extracted as described previously for the LC/MS/MS analysis. The extract was reconstituted in 250 µL of a water/methanol mixture (90:10, v/v) and 200 µL were injected into the HPLC system. An HP1090 liquid chromatograph with automatic injection system and diode-array detector (Hewlett-Packard, Waldbronn, Germany) was used. The LC column was a Hypersil C18 BDS (100 mm × 4.6 mm i.d., 3 µm particle size). The mobile phase was water/methanol (90:10, v/v) at a flow rate of 1 mL/min. The gradient elution program was as follows (where B is methanol): 0 min, 10% B; 0.5 min, 10% B; 15 min, 50% B; 18 min, 100% B; 20 min, 100% B; 20.5 min,

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10% B; 22 min, 10% B. Along the whole chromatogram time frame, fractions of 30 s were collected in separate glass tubes.

Sample preparation

Urine samples (5 mL) were passed through a C_{18} solid-phase extraction (SPE) cartridge, previously conditioned with 4 mL methanol and 4 mL water. The column was then washed with 4 mL water and finally the steroids were recovered with 4 mL methanol.

The methanolic extract was evaporated under a stream of nitrogen and 3 mL of sodium acetate buffer (0.1 M, pH 4.5) were added. After adding 10 mg of sulfatase type H-1 and 12 μL of β-glucuronidase type H-2, hydrolysis was allowed to proceed for 16 h at 55 °C. After the sample had cooled to room temperature, liquid-liquid extraction was performed by the addition of 5 mL ethyl acetate. After centrifugation, the organic layer was separated and evaporated to dryness.

For LC/MS/MS analysis, the residue was dissolved in 150 μL of a mixture of water/acetonitrile (50:50, v/v) and 10 μL were directly injected into the system.

For GC/MS analyses, the residue was dissolved in 100 μL of a 2% (w/v) methoxyamine hydrochloride solution in pyridine, and derivatization allowed to proceed for 60 min at 60 °C. The pyridine was blown off and 50 μL of TMSI were added. The silylation proceeded for 16 h at 80 °C. The involatile reagents were removed prior to GC/MS analysis by a cyclohexane/water extraction. The organic layer was transferred to an injection vial taking care not to transfer any of the aqueous layer.

Chemical reduction of methylprednisolone

Chemical reduction of the C20 carbonyl group was performed in order to compare authentic materials with the putative 20-hydroxy metabolites extracted from the urine. Reduction was performed by adding 100 mg of $NaBH_4$ to a solution of a MP standard (50 μg) in 6 mL of methanol and 1 mL of water. After the mixture had been stirred at room temperature for 5 h, a few drops of acetic acid were added. The mixture was then diluted with ethyl acetate, washed with saturated $NaHCO_3$ solution and water, and dried with anhydrous Na_2SO_4. The organic layer was evaporated to dryness, and the mixture of products obtained analyzed by LC/MS/MS and GC/MS. It is known that the C20 carbonyl group present in side chains of the pregnane and 21-hydroxypregnanes series is reduced preferentially to the 20β configuration.[17]

Administration study samples

One oral dose of 40 mg of methylprednisolone (Urbason, Sanofi-Aventis, Paris, France) was administered to two healthy male volunteers. Blank samples were collected twice before administration. Urine samples were collected from 0–8 h, 8–24 h, 24–36 h, and 36–48 h, 48–60 h, and 60–72 h after drug intake. All samples were stored at −20 °C until analysis.

Methylprednisolone aceponate (5 g; Adventan®, Schering, Berlin, Germany) were administered to two volunteers (one male, one female) as a cream (2.5 g in each arm). Urine samples were collected from 0–4 h, 4–8 h, 8–24 h, 24–32 h, and 32–48 h, and stored at −20 °C until analysis. Six blank samples, collected from volunteers who did not take any corticosteroid, were used for evaluation of the selectivity of the method.

Ethical approval for the study was granted by Comité Ètic d'Investigació Clínica of our institute (CEIC-IMAS no.94/467) and the Spanish Health Ministry (DGFPS no.95/75). All the subjects participating in the study gave their written informed consent.

Structural elucidation

Data from several mass spectrometric experiments were evaluated in order to propose putative metabolite structures.

By GC/MS, electron ionization mass spectra of the methyloxime-trimethylsilyl (MO-TMS) derivatives of steroids typically give the following fragment ions: $[M–31]^+$ (loss of $-OCH_3$ from the oxime group), sequential losses of 90 Da (HOTMS group), loss of 103 Da ($-CH_2$-OTMS group), and combinations thereof (Fig. 2(D)).[18] Two features of steroid oximes have to be taken into account; first, an 11-carbonyl group is sterically hindered and is thus not derivatized. Second, each carbonyl can generate both the *syn* and the *anti* isomers when derivatized. These isomers may or may not be resolved by GC.[18]

By LC/MS/MS, the use of ESI in both positive and negative mode was evaluated. The occurrence of a $[M+H]^+$ ion in positive ion mode was indicative of the presence of a conjugated 3-keto function[19] while the presence of abundant $[M+H–nH_2O]^+$ ions was considered as symptomatic of reduction in the A ring.[16] Corticosteroids are mainly ionized in negative ion mode by the formation of $[M+HCOO]^-$ ions. This adduct is formed by interaction between the formate ion and the carbonyl and hydroxyl groups at C20 and C21, respectively. Therefore, only corticosteroids containing a keto group in the C20 position and two oxygen atoms at C17 and C21 will ionize in negative ion mode.[16]

In addition, the ions produced in the product ion spectra at collision energies of 20 and 30 eV were studied. At 20 eV, the ions appearing between *m/z* 275 and the $[M+H]^+$ ion were evaluated. The number of losses of water from a protonated steroid is related to the number of hydroxyl groups in the steroid molecule and to the conjugation of the keto group.[20] A loss of CO to form ($[M+H–28–nH_2O]^+$) product ions is related to the presence of a keto function at C11[16] and other losses (e.g. of 26 Da, 30 Da, and 58 Da) can provide further structural information on the steroid.[20] At a CE of 30 eV, the most abundant product ions are derived from the steroidal skeleton.[20] The ions produced for one metabolite were compared with those obtained for the parent drug in order to yield valuable structural information.

RESULTS AND DISCUSION

Screening of possible MP metabolites by LC/MS/MS

Several methods based on both PI and NL scans were applied (NL of 76 Da in negative ion mode; PI scan of *m/z* 77, 91 and 105 and PI scan of *m/z* 237 in positive ion mode).[16] In a previous, preliminary approach four major metabolites were found, and feasible structures were proposed,[16] and in this present work a more in-depth study was carried out in order to identify more metabolites.

Using these approaches, more than 30 peaks were detected in MP post-administration urines. After combining the information from all the PI and NL methods, and contrasting this with the results from pre-administration samples, the list of possible metabolites was reduced to 15 potential MP metabolites candidates (Table 1). In positive ion mode, the occurrence of $[M+H]^+$ and $[M+H\text{-}nH_2O]^+$ ions is possible for corticosteroids while in negative ion mode, $[M–H]^-$ and $[M+HCOO]^-$ ions are possible. Combining the information obtained from both positive and negative ion techniques molecular mass for each metabolite could be calculated (Table 1).

Proposal of structures for the metabolites

The proposed metabolism for MP is depicted in Fig. 1. In addition to the unchanged drug, 15 metabolites were detected in all urine samples collected in the first 36 h after oral intake, and some of them were detected even up to 72 h after intake. These compounds were labelled M1 to M15 and tentatively identified as shown below.

Parent compound (MP)

In the GC/MS analyses the MP standard gave two peaks (at 25.1 and 25.8 min), representing the *syn* and a*nti* steroisomers of the C3 oxime group. The GC/MS spectrum for the MO-TMS derivatives showed a molecular ion at *m/z* 648, and distinctive fragment ions at *m/z* 617, 558, 527, 496, 468, and 437 were formed by losses of the oxime, and silylated groups (Table 2). In addition, B ring fragment ions at *m/z* 163 and *m/z* 189 were also detected. The proposed fragmentation pathways of the molecular ion of the MO-TMS derivative of MP are shown in Fig. 2.

In the LC/MS/MS runs, the MP standard eluted at 11.3 min and the $[M+H]^+$ ion at *m/z* 375 showed the expected fragmentation in a product ion scan at a CE of 20 eV with four losses of water to form product ions at *m/z* 357, 339, 321 and 303 (Table 2). At a CE of 30 eV, the most abundant product ions were found at *m/z* 185, 161 and 135. These ions were equivalent to those obtained typically for 1,4-diene steroids (*m/z* 171, 147 and 121) but with a difference of 14 *m/z* units due to the presence of the methyl group at C6 in MP.[14,17,21] In addition, the product ion at *m/z* 211 can be explained as containing rings C and D together with carbons C20 and C21 (Fig. 2).

When urine samples were fractionated by LC, MP was collected between 7.5 and 8 min. When this fraction was analyzed, the same retention times and equivalent relative abundances were obtained for MP as from the MP standard. The isomer in the C11α configuration that was found previously in rat urine[22] has not been detected in our study.

M1

In the LC fractionation experiments this compound was mainly collected between 7.5 and 8 min. In the LC/MS/MS analyses this compound eluted at 11.1 min. According to the data obtained by the precursor ion scan methods, M1 had a molecular mass of 372 (Table 1). M1 ionized in both positive and negative ion mode, indicating the presence of a keto function at C20 and a hydroxyl group at C21. The product ion spectrum of the $[M+H]^+$ ion at *m/z* 373 at a CE of 20 eV showed three losses of water (*m/z* 355, 337, 319 in Table 2) and several peaks related to losses of CO (*m/z* 327 $[M+H–CO–H_2O]^+$, 309 $[M+H–CO–2H_2O]^+$, and 291 $[M+H–CO–3H_2O]^+$) suggesting the presence of a keto function at C11. At 30 eV (Table 2), the same product ions as were detected in the parent drug were obtained (*m/z* 185, 161 and 135) indicating that both the A and the B rings remained unaltered. Based on these data, 17α,21-dihydroxy-6α-methylpregna-1,4-diene-3,11,20-trione is suggested as the structure of M1.

In the GC/MS analyses this steroid gave two peaks (at 23.5 and 24.1 min), representing the *syn* and a*nti* steroisomers of the C3 oxime group. The GC/MS spectrum for the MO-TMS derivative of M1 showed a molecular ion at *m/z* 574, characteristic fragment ions formed by loss of the oxime and O-TMS groups at *m/z* 543, 453, 484 and 453 (Table 2) and ions at *m/z* 202, and 229 arising from C ring fragmentations. This is in agreement with published data.[5]

Table 1. Metabolites of methylprednisolone (MP). Retention times obtained by LC/MS/MS, protonated molecules $[M+H]^+$ and formate adduct ions $[M+HCOO]^-$ in LC/MS/MS in positive and negative ESI modes, EI molecular ion detected in GC/MS after MO-TMS derivatization and molecular mass (MW)

Metabolite	Retention time (min)	*m/z* $[M+H]^+$	*m/z* $[M+HCOO]^-$	*m/z* MO-TMS	MW
MP (parent drug)	11.3	375	419	648	374
M1	11.1	373	417	574	372
M2	9.4	377	-	693	376
M3	10.1	377	-	693	376
M4	10.5	373	417	646	372
M5	9.1	375	-	691	374
M6	9.7	375	-	-	374
M7	3.5	391	435	736	390
M8	4.3	389	433	-	388
M9	2.4	393	-	781	392
M10	2.9	393	-	781	392
M11	9.4	375	-	-	374
M12	9.9	375	-	-	374
M13	3.9	391	435	-	390
M14	3.3	393	-	-	392
M15	4.5	389	433	-	388

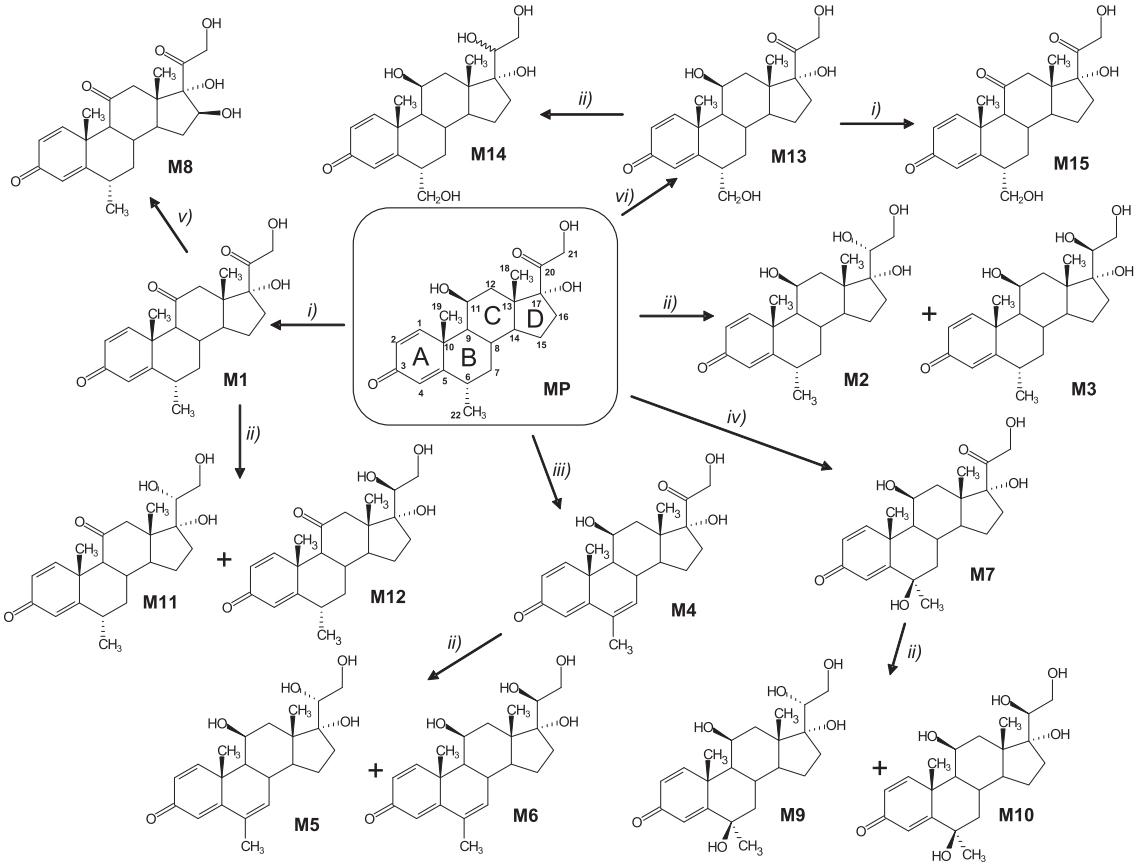

Figure 1. Proposed methylprednisolone metabolism in humans. Metabolic pathways: (i) 11-oxydation, (ii) 20-reduction, (iii) 6,7-dehydrogenation, (iv) 6-hydroxylation, (v) 16-hydroxylation, and (vi) 22-hydroxylation.

Production of such a metabolite was expected since 11-oxidoreduction is one of the principal catabolic reactions undergone by steroid hormones and it was also found after intra-articular or intramuscular administration;[7] however, this metabolite was not detected after the administration of a high intravenous dose for therapeutic treatment in multiple sclerosis patients.[6]

M2 and M3

In the LC fractionation experiments M2 appeared at 7–8 min and M3 was mainly collected between 7.5 and 8 min.

GC/MS analyses showed that these two steroids were stereoisomers. Each of them gave two peaks which correspond to the *syn* and *anti* forms. The four peaks (25.8, 26.4, 26.8 and 27.5 min) exhibited the same electron ionization (EI) mass spectrum; one of them is shown in Fig. 3. The molecular ion was at *m/z* 693, and distinctive fragment ions were formed at *m/z* 662 (M–31), *m/z* 572 (M–31–90), *m/z* 482 (M–31–90–90), and *m/z* 392 (M–31–90–90–90). The base peak is the side-chain-derived ion at *m/z* 205, characteristic of steroids containing hydroxyl groups at C20 and C21.[23] The presence of fragment ions at *m/z* 163 and 189 pointed towards the presence of a methyl group at C6. Previous GC/MS studies obtained similar results and it was concluded that those peaks corresponded to the 20-hydroxy metabolites.[5]

In the LC/MS/MS run, the retention times for the M2 and M3 metabolites were 9.4 and 10.1 min, respectively. M2 and M3 exhibited ionization only in positive mode (Table 1), indicating a change in either C20 or C21. Since the molecular weight of these metabolites is 2 Da more than that of the parent compound, reduction of the keto moiety at C20 seems to be the most feasible alternative. In addition, the product ion spectra of the $[M+H]^+$ ions at *m/z* 377 for both metabolites exhibited four losses of water at a CE of 20 eV (*m/z* 359, 341, 323, 305) and similar ions to those yielded by the parent drug at a CE of 30 eV (Table 2), supporting the proposed structure. The most intense isomer in the biological samples was the 20α, as previously reported;[24] however, in an other study the 20β structure was proposed for the only isomer reported.[6]

In order to establish a comparison with authentic material, sodium borohydride reduction was used to produce the 20α- and 20β-hydroxy metabolites from the MP standard. The mixture of several reaction products was then analyzed by GC/MS. The most intense pair of peaks, previously reported as the 20β isomer,[17] had retention times of 25.8 and 26.4 min. The other pair eluted at the same retention time as M2 (26.8 and 27.5 min). As expected, the spectra obtained were in good agreement with the spectrum obtained from the urine (Fig. 3).Thus, the structures for M2 and M3 are probably 11β,17α,20α,21-tetrahydroxy-6α-methylpregna-1,4-diene-3-one and 11β,17α,20β,21-tetrahydroxy-6α-methylpregna-1,4-diene-3-one, respectively.

Table 2. Main fragment ions (relative abundance in brackets) obtained by GC/MS and main product ions of $[M+H]^+$ obtained by LC/MS/MS for the detected metabolites at the indicated collision energies

Metabolite	GC/MS	LC/MS/MS (20 eV)	LC/MS/MS (30 eV)
MP	648 (10) 617(60), 558(20), 527(30), 496(25), 468(15), 437(25), 366(30), 307(35), 276(60), 233(50), 189(40), **163**(100), 129(55), 103(50)	375 (10), **357** (100), 339 (45), 321 (45), 303(20), 293 (20), 279 (30), 253 (40)	211 (30), 185 (55), 173 (20), **161** (100), 135 (80), 121 (45)
M1	574 (15), 543(20), 484(5) 453(10), 414 (20), 323(70), 292(25), **229**(100), 202(25), 103(50)	373 (55), **355** (100), 337 (65), 327 (50), 319 (45), 309 (45), 291 (25), 281 (55)	211 (25), 185 (70), 173 (50), **161** (100), 135 (40), 121 (35)
M2	693(2), 662(15), 603(5), 590(5), 572(10), 513 (10), 500(15), 482(15), 423(5), 392(15), **205** (100), 163(30)117(30)	377 (5), 359 (35), 341 (30), 323 (15), 305 (20), **281** (100)	185 (50), 173 (20), 161 (45), **135** (100), 121 (50)
M3	693(2), 662(15), 603(5), 590(5), 572(10), 513 (10), 500(15), 482(15), 423(5), 392(15), **205** (100), 163(30)117(30)	377 (10), 359 (55), 341 (30), 323 (25), 305 (25), **281** (100)	185 (80), 173 (30), 161 (60), **135** (100), 121 (50)
M4	646(85), 615(80), 600(20), 556(10), 525(20), 435(10), 291(35), 272(70), 182(55), **129** (100), 103(10)	373 (15), **355** (100), 337 (5), 319 (5), 309 (10), 291 (5), 280 (60)	**185** (100), 173 (25), 161 (80) 159 (75), 135 (25), 121 (20)
M5	691(10), 660(5), 601(10),511(10), 486(30), 396(20), 291(50), **205**(100), 129(60)	375 (35), 357 (10), 339 (25), 321 (40), 303 (30), 279 (55),	263 (45), **185** (100), 173 (10), 161 (15), 159 (20), 135 (15), 121 (10)
M6	N.D.[a]	375 (30), 357 (30), 339 (25), 321 (70), 303 (30), 279 (60),	263 (30), **185** (100), 173 (10), 161 (25), 159 (30), 135 (15), 121 (15)
M7	736(30), 707(5), 646(20), 615(5), 556(5), 525 (5), 470(5), 380(45), 251(45), **219**(100), 191 (20), 103(25)	391 (10), 373 (5), 355 (65), 337 (55), 319 (50)301 (15), 279 (90)	251 (50), 211 (40), **185** (100), 161 (45), 159 (65), 147 (50), 135 (35), 131 (40), 121 (60)
M8	N.D.[a]	389 (10), 371 (20), 353 (25), 335 (10), 331 (25), 325 (20)	**227** (100), 185 (10), 173 (15), 161 (30), 159 (15), 121 (15)
M9	781(25), 750(2), 691(15), 601 (5), 511 (5), 425(30), 396(20), 335(85), 251(50), 205(70), 191(35), **147**(100), 117(30), 103(25)	393 (10), 375 (5), 357 (15), 339 (30), 321 (35), 303 (20), 297 (20), **279** (100)	211 (15), 197 (30), 189 (30), **185** (100), 161 (20), 159 (15), 147 (70), 135 (20), 131 (40), 121 (20)
M10	781(25), 750(2), 691(10), 601 (7), 595(10), 425(30), 396(20), 335(65), 251(35), 205(90), 191(45), **147**(100), 117(40), 103(40)	393 (30), 375 (5), 357 (15), 339 (40), 321 (65), 303 (20), 297 (10), **279** (100)	211 (15), 197 (20), 189 (20), **185** (100), 161 (25), 159 (20), 147 (50), 135 (40), 131 (25), 121 (20)
M11	N.D.[a]	375 (55), 357 (30), 339 (30), 329 (5), 321 (20), 311 (15), 303 (10), 297 (25), 293 (10), **279** (100)	201 (20), 185 (60), 173 (50), **161** (100), 135 (30), 121 (30)
M12	N.D.[a]	375 (35), 357 (40), 339 (40), 329 (10), 321 (35), 311 (20), 303 (15), 297 (15), 293 (20), **279** (100)	201 (30), 185 (60), 173 (60), **161** (100), 135 (30), 121 (30)
M13	N.D.[a]	391 (40), 373 (20), 355 (60), 343 (30), 337 (70), 325 (25), 319 (50), 307(60), 301 (40), 291 (40), **277** (100)	185 (60), **171** (100), 159 (80), 147 (70), 121 (50)
M14	N.D.[a]	393 (10), 375 (5), 357 (10), 339 (10), 327 (5), 321 (10), 309 (5), 303 (20), 297 (25), **279** (100)	185 (90), 171 (100), 159 (40), **147** (100), 121 (35)
M15	N.D.[a]	389 (45), 371 (5), 353 (90), **341** (100), 335 (40), 325 (5), 323 (15), 317 (20), 309 (20), 307 (10), 305 (30), 277 (40)	185 (30), **171** (100), 159 (20), 147 (10), 145 (10), 121 (20)

[a]Not detected.

M4

In the LC fractionation experiments, M4 eluted between 7.5 and 8 min. LC/MS/MS analysis of the LC fraction showed the presence of a steroid with a protonated mass of *m/z* 373 (Table 1). Ionization in negative mode suggested the presence of an unaltered C20-C21 part of the molecule. The product ion spectrum of *m/z* 373 at a CE of 20 eV showed, in addition to three losses of water, an odd-electron ion at *m/z* 280. The presence of odd-electron ions is uncommon in the fragmentation of steroids and they are present normally in steroids with high conjugation of the 3-keto function.[20] At CE of 30 eV, in addition to the ions common to the steroid skeleton of the $[M+H]^+$ ion of the parent compound (*m/z* 185, 173, 161, 135 and 121), an ion at *m/z* 159 was observed (Table 2). This ion (2 *m/z* units less than the parent compound ion at *m/z* 161) suggests that there is a double bond situated in the B ring.

Thus, the most likely structure for M4 is 11β,17α,21-trihydroxy-6-methylpregna-1,4,6-triene-3,20-dione. Although 6,7-dehydration is not considered a major steroid metabolic

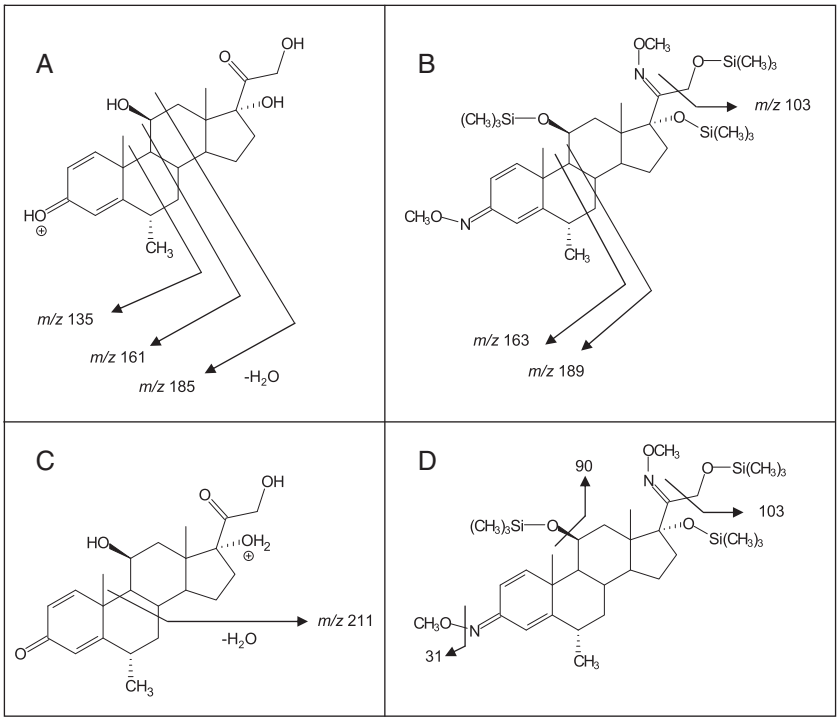

Figure 2. Proposed fragmentation origin of the most abundant ions for methylprednisolone. A, C, CID fragmentation of the $[M+H]^+$ ion; in LC/MS/MS; B, EI fragments of the MO-TMS derivative in GC/MS; D, typical losses 31, 90 and 103 Da of steroids, caused by fragmentation of oxime, HOTMS, and $-CH_2OTMS$ groups in GC/MS analysis.

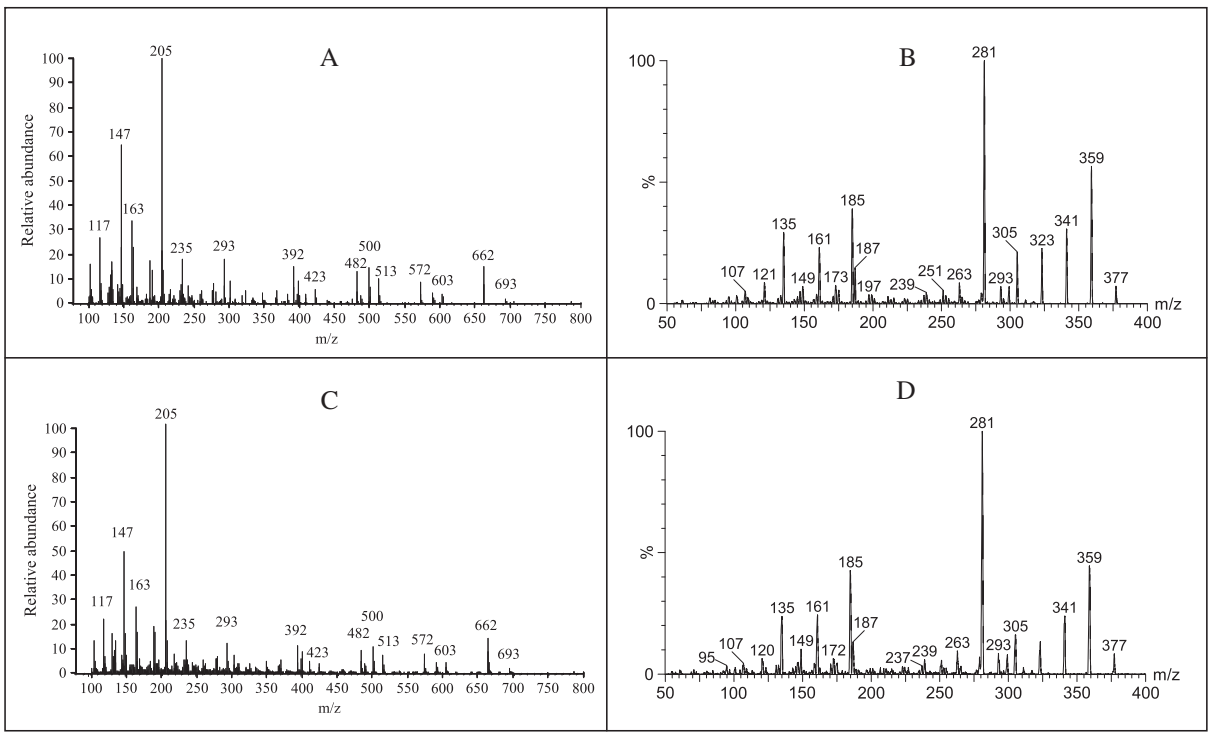

Figure 3. Mass spectra of M2 obtained from urine (A, B) and chemically reduced from MP standard (C, D); (A, C) as MO-TMS derivatives obtained by EI in GC/MS, and (B, D) CID spectra obtained from $[M+H]^+$ ion at 20 eV collision energy in LC/MS/MS analysis.

biotransformation, several 6,7-dehydro metabolites have been recently reported for some steroids,[21,25,26] but they have not been reported for MP after intra-articular or intramuscular administration[7] or after an intravenous high-dose pulse.[6]

In the GC/MS analysis of the LC fraction, two peaks (25.1 and 25.6 min) were detected. The mass spectrum of the second peak is shown in Fig. 4. The molecular ion was at *m/z* 646, and distinctive fragment ions formed by loss of the oxime group and silylated hydroxyl groups at *m/z* 615, 556, 525, 435 were also detected. The base peak was the ion at *m/z* 129. This is in accordance with previously reported data.[5]

Both GC/MS and LC/MS/MS proved that M4 eluted between 7.5 and 8 min in the LC fractionation. Interestingly, in the GC/MS analysis of the 4–4.5 min fraction, M4 was also observed. Thus, it is likely that the same steroid was formed as a derivatization artifact as suggested by Gallicano *et al.*[27] This artifact probably had its origin from the 6β-hydroxy metabolite (M7, see below), since M7 was detected in the same 4–4.5 min fraction.

M5 and M6

The M5 and M6 metabolites were collected between 7.5 and 8 min during LC fractionation. By LC/MS/MS, both M5 and M6 ionized only in positive mode (Table 1), suggesting a modification at either C20 or C21. In agreement with the GC/MS data, the reduction of C20 seems to be the most likely option. Fragmentation of the $[M+H]^+$ ion at *m/z* 375 at a CE of 20 eV (Table 2) showed the expected four losses of water (*m/z* 357, 339, 321 and 303). At 30 eV CE, in addition to the common ions for MP (*m/z* 185, 173, 161, 135 and 121), the ion at *m/z* 159 also appeared with a similar abundance to the *m/z* 161 ion. This situation was analogous to that presented above for M4, suggesting the presence of an additional double bond in the B ring. Similarly to M4, a 6,7-dehydro structure was suggested for M5 and M6.

It was not possible to determine the stereochemistry for M5 and M6. However, if we assume that the chromatographic behaviour of the C20α/β isomer products is not altered by the presence of a C6-C7 unsaturation, M6 (shorter retention time in the LC run) should be identified as 11β,17α,20α,21-

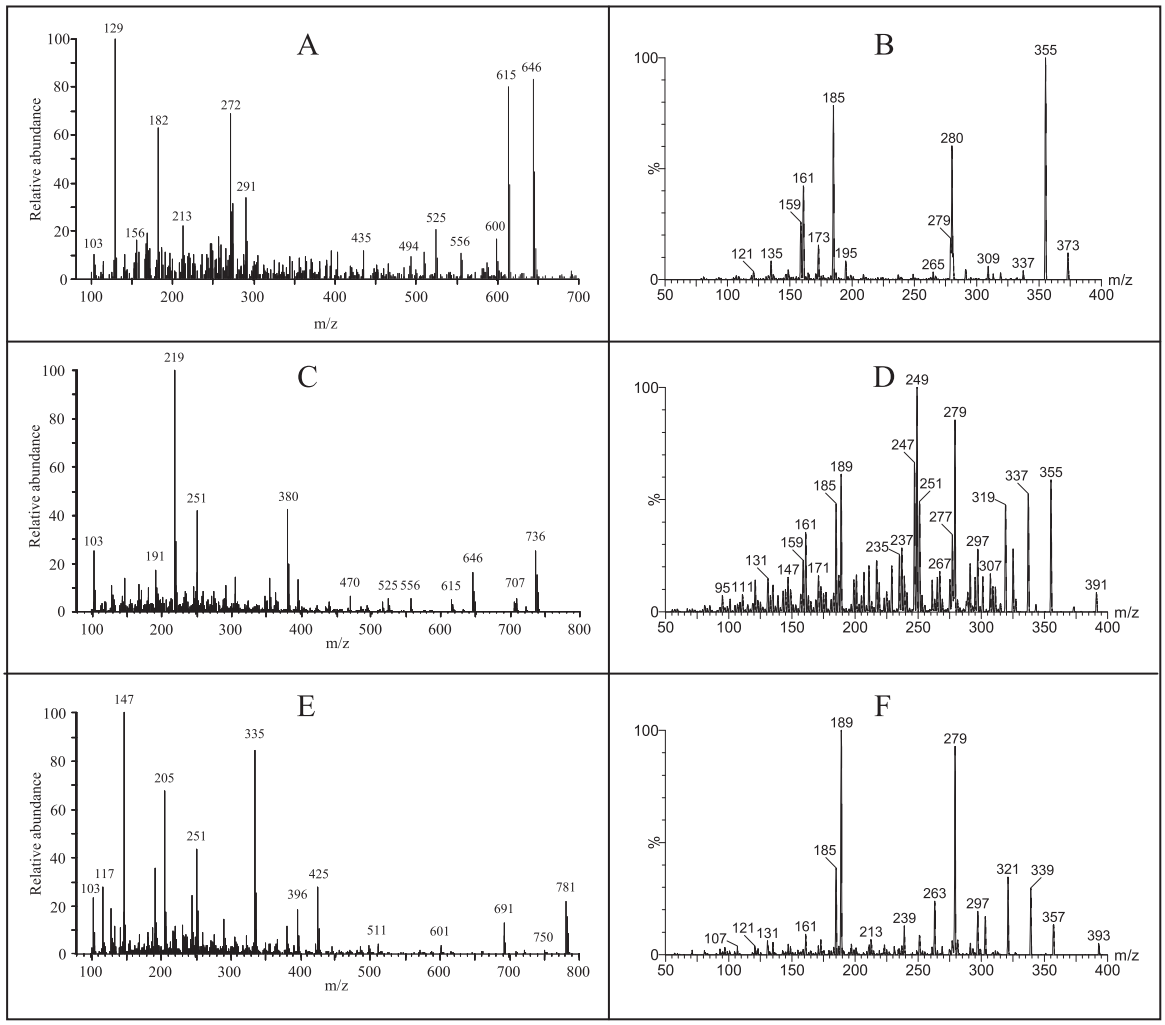

Figure 4. Mass spectra of putative M4 (A, B), M7 (C, D) and M9 (E, F) (A, C, E) as MO-TMS derivatives obtained by EI in GC/MS analysis and (B, D, F) CID spectra obtained from $[M+H]^+$ ion at 20 eV CE in LC/MS/MS analysis.

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tetrahydroxy-6-methylpregna-1,4,6-triene-3-one. Thus, M5 will then be 11β,17α,20β,21-tetrahydroxy-6-methylpregna-1,4,6-triene-3-one.

During GC/MS analysis, only two of the four expected peaks were detected. In a previous study, Rodchenkov and co-workers found the four peaks, but the intensities of two of them were very low.[5] Low intensity signals showing a molecular ion at *m/z* 691 were present at 26.7 and 27.2 min. The rest of the diagnostic ions are summarized in Table 2. This mass spectrum resembles those obtained for M2 and M3 with a 2 *m/z* units shift. Since the 20β metabolites seems to be excreted in a lesser amount than the 20α isomers (M2 vs. M3), it is likely that both peaks correspond to M5.

M5 and M6 have not been reported after intra-articular or intramuscular administration[7]or after intravenous high-dose pulse[6] for MP.

M7

This metabolite was collected between 4 and 4.5 min in the LC fractionation, symptomatic of some polar groups being added to the MP structure. M7 ionized in both positive and negative mode, suggesting that the C20 and C21 positions were unaltered (Table 1). Fragmentation of the $[M+H]^+$ ion at *m/z* 391 at a CE of 20 eV showed five losses of water (*m/z* 373, 355, 337, 319 and 301, see Table 2), confirming the presence of an additional hydroxyl group. At 30 eV CE, in addition to the ions at *m/z* 185, 161, 135 and 121 common to MP, the ion at *m/z* 159 suggested a modification in the B ring. This ion was also present in M4, M5 and M6 and it was associated with the presence of a double bond in position 6. Therefore, C6 and C7 are the most feasible positions for the additional hydroxyl group in M7.

The GC/MS spectrum for the MO-TMS derivative of M7 is displayed in Fig. 4, showing the molecular ion at *m/z* 736. Apart from the distinctive ions formed by losses of the oxime and silylated groups, the characteristic ion at *m/z* 103 for the primary TMS group at C21 was detected. The ion at *m/z* 251 corresponds to the B-ring fragmentation at C9-C10 and C6-C7 (equivalent to the *m/z* 163 ion seen in the MP spectrum). Similar to the other metabolites described, two peaks (27.5 and 28.4 min) were present in the GC run. Thus, 6β-hydroxymethylprednisolone was selected as a feasible structure for M7.

Although 6β-hydroxylation has been described as a pathway in MP metabolism,[6] previous attempts to detect this metabolite by GC/MS in SIM mode have failed.[5]

M8

M8 was not detected by GC/MS. Therefore, a putative structure was established based only on the LC/MS/MS data. The molecular weight of M8 was 14 Da higher than that of the parent drug (Table 1) suggesting a hydroxylation and an oxidation. M8 ionized in both positive and negative mode and, therefore, no modification was proposed for C20 and C21. In addition to several losses of water, the product ion spectrum of the $[M+H]^+$ ion at *m/z* 389 at a CE of 20 eV exhibited a loss of CO (*m/z* 325, $[M+H–CO–2H_2O]^+$) which can indicate the presence of a carbonyl group at C11. The product ion spectrum of *m/z* 389 at 30 eV CE was dominated by an ion at *m/z* 227. This ion, 16 *m/z* units higher than the ion at *m/z* 211, suggests the presence of the hydroxyl group in either the C or D ring. The occurrence of the ions at *m/z* 185, 173, 161, 135 and 121 indicated an unchanged A-B-C ring structure. Therefore, the D ring was selected as the most feasible place for the hydroxylation.

16β-Hydroxylation has not been reported in the metabolism of MP,[6,7] although this is a common metabolic pathway for several steroids[28] and, therefore, 16β,17α,21-trihydroxy-6α-methylpregna-1,4-diene-3,11,20-trione is the most plausible structure for M8. In addition, the presence of an additional hydroxyl group at C16 can make derivatization difficult, thus explaining the inability to detect M8 by GC/MS.

M9 and M10

In the LC fractionation experiments M9 appeared between 3.5 and 4 min, whereas M10 was mainly collected between 4 and 4.5 min. Thus, both metabolites seem to be more polar than MP.

M9 and M10 were only observed to ionize in positive mode, indicating a change at either C20 or C21 (Table 1). Taking into account that the molecular weights of M9 and M10 are 392, reduction of C20 seems to be the most plausible option for this change. In the fragmentation of the $[M+H]^+$ ions at *m/z* 393 at a CE of 20 eV, the five losses of water corroborated the presence of an extra hydroxyl group. At 30 eV CE, the product ion spectra of protonated M9 and M10 showed the characteristic ions of MP (*m/z* 185, 161, 135 and 121), but also ions at *m/z* 159 and 147. These ions were also present in M7 (proposed as 6β-hydroxy-methylprednisolone) indicating that there is the same modification in rings A or B. Peaks corresponding to losses of water, and also a peak at *m/z* 297, are in agreement with ions in the product ion mass spectra described for these compounds at a CE of 25 eV,[6] although in that report mass spectra were not obtained at higher CEs so it is not possible to compare information related to the steroidal skeleton. Therefore, 6β,11β,17α,20α,21-pentahydroxy-6α-methylpregna-1,4-diene-3-one and 6β,11β,17α,20β,21-pentahydroxy-6α-methylpregna-1,4-diene-3-one were proposed as feasible structures for M9 and M10, respectively.

Similarly to M5 and M6, and in agreement with previous results,[6] the proposed stereochemistry of the hydroxyl group at C20 was based on the elution order. M9 eluted first and therefore it was selected as the 20α isomer while a β stereochemistry was proposed for M10.

GC/MS analyses showed that these two steroids were stereoisomers, and each gave two peaks corresponding to the *syn* and *anti* forms. The four peaks (27.8, 28.7, 29.1 and 30 min) exhibited the same mass spectrum; as an example, the spectrum of M9 is shown in Fig. 4. The molecular ion was at *m/z* 781 and the distinctive fragment ions are listed in Table 2. The ion at *m/z* 251 was characteristic of a B ring containing an extra O-TMS group. The ion at *m/z* 396 probably originates from the (M–90–90) ion at *m/z* 601 by losing 205 Da (side chain). By retention analogy, the peaks at 27.8 and 28.7 min would correspond to M10, and the peaks at 29.1 and 30 min to M9. Again, the 20α isomer was of higher intensity than its 20β isomer. Previous studies by GC/MS were not able to identify these metabolites.

M11 and M12

M11 and M12 eluted in the fractions between 7 and 7.5 min during LC fractionation and were not detected by GC/MS. By LC/MS/MS it was found that the molecular weight of both analytes was the same as the parent drug. This could be associated to a combination of oxidation and reduction processes. Both metabolites were only ionized in positive mode indicative of a change at either C20 or C21. The reduction of the keto function in C20 seems to be the most feasible metabolic pathway for this change. The fact that the $[M+H]^+$ ions of the two metabolites displayed the same product ion spectra (as described before for M2/M3, M5/M6 and M9/M10, all of which corresponded to the 20α/20β metabolites) supports this assignation.

The product ion spectra of the $[M+H]^+$ ions at *m/z* 375 for M11 and M12 at a CE of 20 eV exhibited four losses of water together with ions involving a loss of CO (*m/z* 329, 311 and 293) which can be associated with the presence of a carbonyl group at C11. The presence at CE of 30 eV of the same ions as for MP (Table 2) supports the absence of additional changes in the A, B, C rings.

Therefore, 17α,20α,21-trihydroxy-6α-methylpregna-1,4-diene-3,11-dione and 17α,20β,21-trihydroxy-6α-methylpregna-1,4-diene-3,11-dione were proposed as feasible structures for M11 and M12, respectively. Similarly to M5/M6 and M9/M10, the proposed stereochemistry of the hydroxyl group at C20 was based on the retention time. Only one of these two compounds has been reported previously (by Panusa *et al.*[71]) probably the C20α isomer due to it being present at a higher concentration than the C20β isomer which was below their detection limit.

M13

This metabolite was collected between 5 and 5.5 min during LC fractionation; and it was not detected by GC/MS.

The molecular weight of M13 (390 Da, see Table 1) corresponded to a hydroxylated metabolite. The ionization of this metabolite in both positive and negative mode suggested that no modification had taken place at C20 and C21. The product ion spectrum of the $[M+H]^+$ ion at *m/z* 391 for M13 at a CE of 20 eV exhibited five losses of water combined with losses of 30 Da (*m/z* 343, 325 and 307). This neutral loss of 30 Da is characteristic of steroids containing a hydroxymethyl group.[20,29] Thus, a hydroxylation at C18, C19 or C22 seems to be the most feasible metabolic pathway for M13.

In the product ion scan of *m/z* 391 at CE of 30 eV, some of the most abundant ions (*m/z* 171, 147 and 121) were similar to those obtained for steroids and corticosteroids without the methyl group at C6.[7,12,30,31] These ions can be produced after the loss of the hydroxymethyl group in C6. For this reason, hydroxylation at C22 was selected as the most plausible metabolic pathway for the formation of M13. In order to corroborate this, a urine sample collected after the administration of prednisone (same structure as MP, but without the methyl group at C6) was analyzed. No metabolite having losses of 30 Da in the product ion scan of the $[M+H]^+$ ion was found, supporting the hypothesis that the M13 extra hydroxylation takes place at C22.

Although no metabolite of MP with a hydroxyl group in the C22 position has been reported previously, 11β,17α,21-trihydroxy-6α-hydroxymethylpregna-1,4-diene-3,20-dione was selected as a feasible structure for M13.

M14

This metabolite was collected between 4.5 and 5 min during LC fractionation and it was not detected by GC/MS. A molecular mass of 392 Da was obtained for M14 (Table 1) with a retention time of 3.3 min. This corresponded to hydroxylation and reduction of the parent MP molecule. Similarly to other metabolites (M2, M3, M5, M6, M9, M10, M11 and M12), ionization of M14 was only possible in positive mode suggesting that the reduction took place at C20. At a CE of 20 eV, the product ion spectrum of protonated M14 exhibited five losses of water and two ions related to losses of 30 Da (*m/z* 327 and 309).

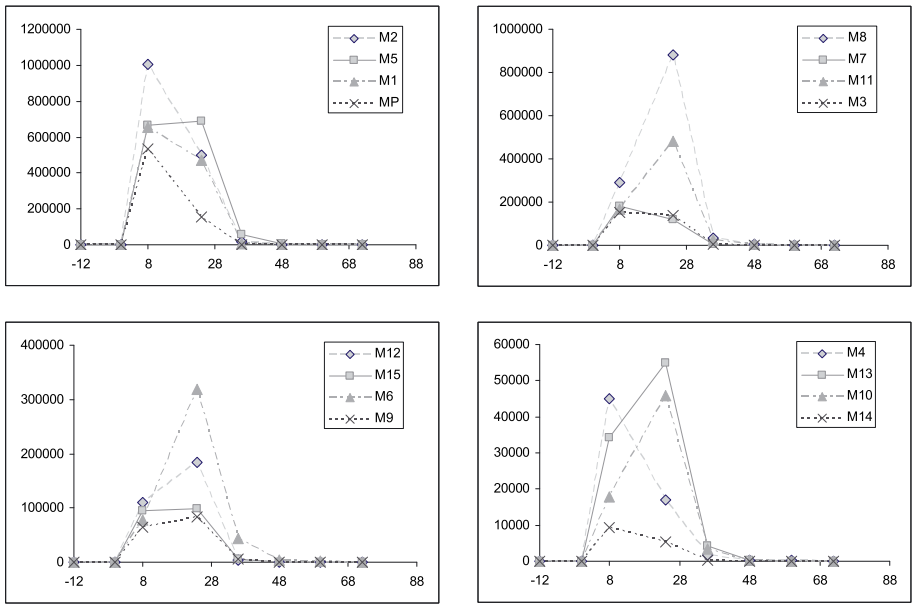
Similar to M13, these losses of 30 Da can be associated with the presence of a hydroxymethyl group in the molecule. In addition, several of the most abundant ions at a CE of 30 eV (*m/z* 171, 147 and 121) corresponded to the steroidal skeleton without a methyl group at C6. Therefore, 11β,17α,20ξ,21-tetrahydroxy-6α-hydroxymethylpregna-1,4-diene-3-one was selected as a feasible structure for M14. In this case, it was not possible to predict the stereochemistry at C20 as only one metabolite was detected. However, as for the other metabolites described here with the C20 hydroxyl group, the α isomer was always detected in higher concentration; this suggests that M14 is the C20α isomer and that the C20β isomer is below the detection limit.

M15

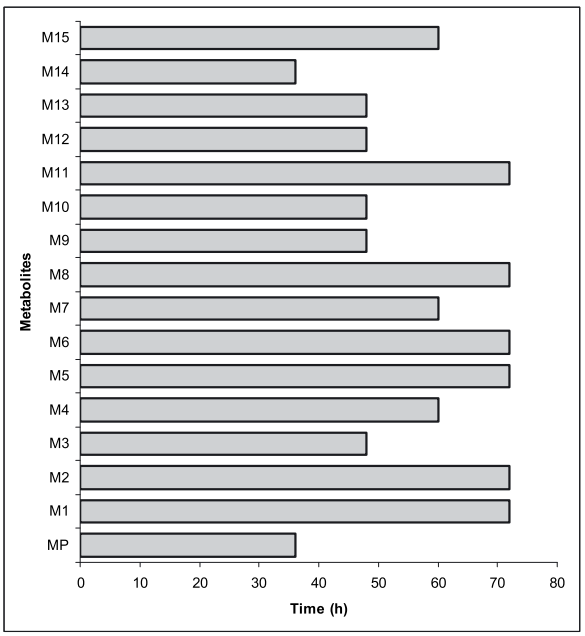
M15 was collected between 5 and 5.5 min during LC fractionation and it was not detected by GC/MS. M15 has a molecular weight of 388 (Table 1) which corresponds to one hydroxylation and one oxidation. Its ionization in both

Table 3. Transitions and collision energies (CE) of the SRM method for the detection of MP metabolites by LC/MS/MS. Cone voltage was set at 25 V for all transitions

Metabolite	Precursor ion (*m/z*)	Product ion (*m/z*)	CE (eV)
MP	375	161	30
M1	373	161	30
M2	377	281	15
M3	377	281	15
M4	373	280	15
M5	375	185	25
M6	375	185	25
M7	391	185	30
M8	389	227	20
M9	393	189	15
M10	393	189	15
M11	375	279	15
M12	375	279	15
M13	391	171	30
M14	393	171	30
M15	389	341	15



(a)



(b)

Figure 5. (a) Excretion rates for methylprednisolone and its metabolites after the oral intake of 40 mg (peak areas obtained by LC/MS/MS for each metabolite with respect to time). (b) Detection of metabolites of MP during time course of excretion.

modes indicated the presence of a carbonyl group at C20 and a hydroxyl group at C21. At a CE of 20 eV, the product ion spectrum of protonated M15 exhibited four losses of water, losses of CO (*m/z* 325 and 307) and losses of 30 Da (*m/z* 341, 323 and 305). These losses suggested the presence of a keto group at C11 (similar to M1, M8, M11 and M12) and a hydroxymethyl group in the C6 position (similar to M13 and M14). This last consideration was supported by the fact that at a CE of 30 eV, the most abundant ion (*m/z* 171) corresponded to a steroidal skeleton without a methyl group in the C6 position. Therefore, 17α,21-dihydroxy-6α-hydroxymethylpregna-1,4-diene-3,11,20-trione was proposed as a feasible structure for M15.

Some of these metabolites were not detected by GC/MS. This could be because the addition of more functional groups, especially alcohol moieties, makes the derivatization step difficult (as in M8, for example). Low concentrations of the metabolites and lower sensitivity in GC/MS than in UPLC/MS/MS (M13 and M14) and a combination of both are other possible explanations.

Analysis of excretion studies

Once all metabolites had been identified, the whole set of samples corresponding to the excretion studies was analyzed. A LC/MS/MS method in SRM mode was developed for this purpose. The optimized SRM method allowed the detection of MP at concentrations of around 1 ng/mL. Table 3 summarizes the most critical parameters for the 15 metabolites. The method was found to be selective for all the selected analytes as no interferences were found in any of the six blank samples analyzed.

Figure 5 shows the time course of excretion. Although the amount excreted dramatically decreased after the first 48 h, it was possible to unequivocally identify six metabolites 72 h after the intake of the drug. When administered topically, none of the reported metabolites was detected in any of the samples.

CONCLUSIONS

A combination of different hyphenated mass spectrometric approaches has been used to shed new light on methylprednisolone metabolism. By combining all the information collected, the overall metabolism for MP has been re-evaluated. Plausible structures for 15 metabolites have been proposed, five of them being novel compounds.

As expected, the activities of the biotransformations of some of the principal corticosteroids were confirmed. It is known that 11β-hydroxysteroid dehydrogenase type 2 present in the kidney and other tissues is responsible for C11 oxidation of cortisol to its inactive metabolite cortisone.[32] Even when this enzymatic activity seems to be diminished, if the cortisol structures is methylated at 2α or 6α (as in methylprednisolone),[33] the excretion of M1 (MW 372) was expected.

Analogously, the unequivocal identification of M2 and M3 (MW 376) confirmed that C20 reduction catalyzed by the two enzymes 20α- and 20β-hydroxysteroid dehydrogenases takes place. A combination of these two basic biotransformations, as occurs in the cortisol metabolism, produced M11 and M12 (MW 374). 6β-Hydroxylation is the origin of M7 (MW 390) as was described by Vree *et al.*[6] The combination of 6β-hydroxylation and C20 reduction produces M9 and M10 (MW 392). According to the data obtained, further unreported hydroxylations may occur at C16α, and C22. We propose that four novel metabolites, M8 (MW 388), M13 (MW 390), M14 (MW 392) and M15 (MW 388), are excretion products generated through these extra hydroxylations.

Finally, in the present work three 6,7-dehydro metabolites (M4 (MW 372), M5 and M6 (MW 374)) have been detected, thus proving that MP undergoes 6,7-dehydration. These metabolites were previously detected only by GC/MS.[5] Their detection by LC/MS/MS leads to the conclusion that they are authentic metabolites and not simply derivatization artefacts.

A method for discriminating between different routes of administration of MP has also been evaluated. However, none of the metabolites was found after topical application of a high dose of the drug. Thus, it seems that establishing cut-off values would suffice for discriminating routes of administration.

Acknowledgements

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REFERENCES

[1] B. Kirby. A review of the rational use of corticosteroids. *J. Int. Med. Res.* **1989**, *17*, 493.
[2] B. J. Parker, I. N. Bruce. High dose methylprednisolone therapy for the treatment of severe systemic lupus erythematosus. *Lupus* **2007**, *16*, 387.
[3] D. Czock, F. Keller, F. M. Rasche, U. Haussler. Pharmacokinetics and pharmacodynamics of systemically administered glucocorticoids. *Clin. Pharmacokinet.* **2005**, *44*, 61.
[4] The World Anti-Doping Code. The 2011 Prohibited List International Standard. World Anti-Doping Agency. Available: http://www.wada-ama.org/en/World-Anti-Doping-Program/Sports-and-Anti-Doping-Organizations/International-Standards/Prohibited-List/The-2011-Prohibited-List/.
[5] G. M. Rodchenkov, V. P. Uralets, V. A. Semenov. Determination of methylprednisolone metabolites in human-urine by gas chromatography–mass spectrometry. *J. Chromatogr.-Biomed.* **1987**, *423*, 15.
[6] T. B. Vree, L. Maljers, N. Van den Borg, N. M. M. Nibbering, C. P. W. G. Verwey-Van Wissen, A. J. Lagerwerf, R. A. A. Maes, P. J. H. Jongen. High-performance liquid-chromatographic-atmospheric-pressure chemical-ionization ion-trap mass-spectrometric identification of isomeric C6-hydroxy and C20-hydroxy metabolites of methylprednisolone in the urine of patients receiving high-dose pulse therapy. *J. Pharm. Pharmacol.* **1999**, *51*, 1155.
[7] A. Panusa, L. Regazzoni, G. Aldini, M. Orioli, A. Giombini, P. Minghetti, C. Tranquilli, M. Carini. Urinary profile of methylprednisolone acetate metabolites in patients following intra-articular and intramuscular administration. *Anal. Bioanal. Chem.* **2011**, *400*, 255.
[8] B. K. Yap, G. A. R. Johnston, R. Kazlauskas. Routine screening and quantitation of urinary corticosteroids using bench-top gas-chromatography mass-selective detection. *J. Chromatogr.-Biomed.* **1992**, *573*, 183.
[9] M. Mazzarino, F. Botre. A fast liquid chromatographic/mass spectrometric screening method for the simultaneous detection of synthetic glucocorticoids, some stimulants, anti-olestrogen drugs and synthetic anabolic steroids. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 3465.
[10] S. J. Park, Y. J. Kim, H. S. Pyo, J. S. Park. Analysis of corticosteroids in urine by HPLC and thermospray LC/MS. *J. Anal. Toxicol.* **1990**, *14*, 102.
[11] O. J. Pozo, K. Deventer, P. Van Eenoo, F. T. Delbeke. Efficient approach for the comprehensive detection of unknown anabolic steroids and metabolites in human urine by liquid chromatography - Electrospray-tandem mass spectrometry. *Anal. Chem.* **2008**, *80*, 1709.
[12] M. Mazzarino, S. Turi, F. Botre. A screening method for the detection of synthetic glucocorticosteroids in human urine by liquid chromatography-mass spectrometry based on class-characteristic fragmentation pathways. *Anal. Bioanal. Chem.* **2008**, *390*, 1389.

[13] M. Thevis, H. Geyer, U. Mareck, W. Schanzer. Screening for unknown synthetic steroids in human urine by liquid chromatography-tandem mass spectrometry. *J. Mass Spectrom.* **2005**, *40*, 955.
[14] O. J. Pozo, P. Van Eenoo, K. Deventer, L. Lootens, S. Grimalt, J. V. Sancho, F. Hernandez, P. Meuleman, G. Leroux-Roels, F. T. Delbeke. Detection and structural investigation of metabolites of stanozolol in human urine by liquid chromatography tandem mass spectrometry. *Steroids* **2009**, *74*, 837.
[15] C. H. L. Shackleton. Profiling steroid-hormones and urinary steroids. *J. Chromatogr.* **1986**, *379*, 91.
[16] O. J. Pozo, R. Ventura, N. Monfort, J. Segura, F. T. Delbeke. Evaluation of different scan methods for the urinary detection of corticosteroid metabolites by liquid chromatography tandem mass spectrometry. *J. Mass Spectrom.* **2009**, *44*, 929.
[17] C. A. Han, C. Monder. Asymmetric reduction of 20-steroidal ketone – synthesis of corticosteroid derivatives containing the 20Alpha-Ol 21-Al side-chain. *J. Org. Chem.* **1982**, *47*, 1580.
[18] C. H. L. Shackleton, J. Marcos, *The Encyclopedia of Mass Spectrometry*, vol. *8*, (Eds: M. Gross, R. Caprioli). Elsevier, Amsterdam, **2006**.
[19] O. J. Pozo, P. Van Eenoo, K. Deventer, F. T. Delbeke. Ionization of anabolic steroids by adduct formation in liquid chromatography electrospray mass spectrometry. *J. Mass Spectrom.* **2007**, *42*, 497.
[20] O. J. Pozo, P. Van Eenoo, K. Deventer, S. Grimalt, J. V. Sancho, F. Hernández, F. T. Delbeke. Collision-induced dissociation of 3-keto anabolic steroids and related compounds after electrospray ionization. Considerations for structural elucidation. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 4009.
[21] O. J. Pozo, P. Van Eenoo, K. Deventer, L. Lootens, W. Van Thuyne, M. K. Parr, W. Schanzer, J. V. Sancho, F. Hernandez, P. Meuleman, G. Leroux-Roels, F. T. Delbeke. Detection and characterization of a new metabolite of 17 alpha-methyltestosterone. *Drug Metab. Dispos.* **2009**, *37*, 2153.
[22] A. Panusa, G. Aldini, M. Orioli, G. Vistoli, G. Rossoni, M. Carini. A sensitive and specific precursor ion scanning approach in liquid chromatography/electrospray ionization tandem mass spectrometry to detect methylprednisolone acetate and its metabolites in rat urine. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1583.
[23] M. E. S. Routhier, P. Bournot, L. C. Ramirez. Aldosterone synthase activity in the Y-1 adrenal-cell line. *J. Steroid Biochem. Mol. Biol.* **1995**, *52*, 581.
[24] G. J. Lawson, J. Chakraborty, M. C. Dumasia, E. M. Baylis. Methylprednisolone hemisuccinate and metabolites in urine from patients receiving high-dose corticosteroid-therapy. *Ther. Drug Monit.* **1992**, *14*, 20.
[25] O. J. Pozo, L. Lootens, P. Van Eenoo, K. Deventer, P. Meuleman, G. Leroux-Roels, M. K. Parr, W. Schanzer, F. T. Delbeke. Combination of liquid-chromatography tandem mass spectrometry in different scan modes with human and chimeric mouse urine for the study of steroid metabolism. *Drug Test. Anal.* **2009**, *1*, 554.
[26] O. J. Pozo, J. Marcos, R. Ventura, A. Fabregat, J. Segura. Testosterone metabolism revisited: discovery of new metabolites. *Anal. Bioanal. Chem.* **2010**, *398*, 1759.
[27] K. D. Gallicano, R. M. Y. Ng, L. M. Young. Identification of metabolites of methylprednisolone in equine urine. *Steroids* **1985**, *46*, 755.
[28] W. Schanzer. Metabolism of anabolic androgenic steroids. *Clin. Chem.* **1996**, *42*, 1001.
[29] O. J. Pozo, P. Van Eenoo, K. Deventer, F. T. Delbeke. Detection and characterization of anabolic steroids in doping analysis by LC-MS. *TrAC, Trends Anal. Chem.* **2008**, *27*, 657.
[30] M. Thevis, W. Schanzer. Mass spectrometry in sports drug testing: Structure characterization and analytical assays. *Mass Spectrom. Rev.* **2007**, *26*, 79.
[31] J. P. Antignac, B. Le Bizec, F. Monteau, F. Poulain, F. Andre. Collision-induced dissociation of corticosteroids in electrospray tandem mass spectrometry and development of a screening method by high performance liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 33.
[32] P. C. White, A. K. Agarwal, B. S. Nunez, G. Giacchetti, F. Mantero, P. M. Stewart. Genotype-phenotype correlations of mutations and polymorphisms in HSD11B2, the gene encoding the kidney isozyme of 11 beta-hydroxysteroid dehydrogenase. *Endocr. Res.* **2000**, *26*, 771.
[33] S. Diederich, E. Eigendorff, P. Burkhardt, M. Quinkler, C. Bumke-Vogt, M. Rochel, D. Seidelmann, P. Esperling, W. Oelkers, V. Bahr. 11 beta-hydroxysteroid dehydrogenase types 1 and 2: An important pharmacokinetic determinant for the activity of synthetic mineralo- and glucocorticoids. *J. Clin. Endocrinol. Metab.* **2002**, *87*, 5695.

