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Isolation and identification of the C_6-hydroxy and C_{20}-hydroxy metabolites and glucuronide conjugate of methylprednisolone by preparative high-performance liquid chromatography from urine of patients receiving high-dose pulse therapy

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Abstract

In the present study metabolites of methylprednisolone were detected using gradient elution high-performance liquid chromatography. Separation was performed by a Cp Spherisorb ODS 5 μm (250 mm×4.6 mm I.D.) column, connected to a guard column, packed with pellicular reversed phase. The mobile phase was a mixture of acetonitrile and 1% acetic acid in water. At $t=0$, this phase consisted of 2% acetonitrile and 98% acetic acid 1% in water (v/v). During the following 35 min the phase changed linearly until it attained a composition of acetonitrile–buffer (50:50, v/v). At 40 min ($t=40$) the mobile phase was changed over 5 min to the initial composition, followed by equilibration during 2 min. The flow-rate was 1.5 ml/min. UV detection was achieved at 248 nm. We have isolated the respective compounds with the most abundant concentration and suggested their chemical structure based on NMR, IR, UV, MS, retention behaviour and melting points. The α/β stereochemistry could not be solved in this study. The overall picture of the metabolic pathways of methylprednisolone is apparently simple: reduction of the C20 carbonyl group and further oxidation of the C20-C21 side chain (into C21-COOH and C20-COOH), in competition with or additional to the oxidation at the C6-position.

Keywords: Steroids; Methylprednisolone

1. Introduction

High dose pulsed intravenous infusions or oral administrations of methylprednisolone hemisuccinate (MPS) have been used for many years as a treatment of acute relapses or progressive worsening of multiple sclerosis [1–5]. Clinical improvement or failure may be correlated with plasma concentrations and even better brain concentrations of methylpred-

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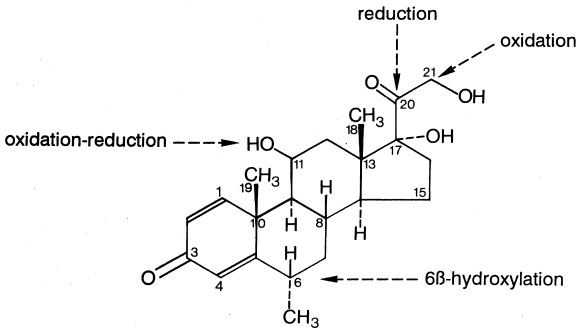

Fig. 1. Structure of methylprednisolone and metabolic pathways as mentioned in the literature.

nisolone (MP). A prerogative is the availability of a validated analytical methodology which is able to measure pro- and parent drug, including all known metabolites, as the whole spectrum of compounds may enter the brain. The analysis of methylprednisolone and its prodrug the hemisuccinate were described frequently in the literature [6–14]. Defer et al. analyzed elegantly the brain concentration of methylprednisolone after a high intravenous dose has been administered and they reconstructed a kind of brain-elimination curve by taking one liquor sample of each patient but at different times [3]. After development of an analytical HPLC method for methylprednisolone and its hemisuccinate in plasma and urine, it was noticed that of a high intravenous dose of 1 g, only 5% of the applied dose could be recovered from the urine, leaving 95% for further metabolism (Fig. 1). Application of a gradient mobile phase indicated that about or at least seven metabolites could be detected.

The aim of this study was to isolate and to identify the metabolites of methylprednisolone after administration of a high intravenous dose for therapeutic treatment in multiple sclerosis patients.

2. Experimental

2.1. Chemicals

Methylprednisolone: ((11β,17α,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione; $C_{22}H_{30}O_5$, M_r 374.5; CAS number 83-43-2) and methylprednisolone sodium hemisuccinate (Solumedrol) were obtained from Upjohn (Ede, The Netherlands). All other reagents were of analytical reagent grade and obtained from Merck (Darmstadt, Germany). β-Glucuronidases were obtained from Sigma (St. Louis, MO, USA). XAD-2 and Celite 545 were obtained from Fluka (Perstorp Analytical, Oud Beyerland, The Netherlands).

2.2. Subjects

Ten patients of the Multiple Sclerosis Centre therapeutically treated with 1 g methylprednisolone hemisuccinate/day (Solumedrol, Upjohn, Ede, The Netherlands) collected one total urine portion. The study had the approval of the hospital ethics committee and informed consent was obtained from the patients.

2.3. Sampling

Urine was collected upon untimed voiding. The total time of sample collection was 24 h. Three samples of each void were stored at −20°C pending analysis. The remainder of the urine void was collected for 24 h in a tank, stored at −20°C, pending isolation.

2.4. Isolation of metabolites

2.4.1. Column chromatography

The collected urine (2 l, 24 h, pH 9.3) were brought to pH 5.0 by adding dropwise concentrated HCl and allowing to stand overnight for precipitation of endogenous urates. Celite 545 (20 g) was added to the urine and the suspension filtered. The pH of the supernatant was adjusted to 5.0 with concentrated acetic acid.

A preparative column (40×6 cm, packed with 1 kg XAD-2) was rinsed successively with 2×1 l of methanol, 2×1 l of water and once with 1 l of 0.2 *M* KH_2PO_4 buffer, pH 5.0. Thereafter 2 l of urine were added to the column, followed by 2× 1 l of the 2 m*M* KH_2PO_4 buffer, pH 5.0, for 100× diluted. The column was dried by air suction for 10 min.

Elution of the column was carried out by a mixture of methanol–water according to the following scheme: first 500 ml CH_3OH–H_2O (20:80, v/v), followed by eight portions of 250 ml consisting of a

methanol concentration increased by steps of 10% to a final composition of 100% methanol.

The effluent fractions 3–5 (CH_3OH–H_2O, 40:60–60:40, respectively, v/v) contained the metabolites of methylprednisolone. In total 14 l of urine were used.

2.4.2. Preparative high-performance liquid chromatography

The volumes of each fraction containing the methylprednisolone metabolites isolated from the preparative column were reduced to 500 ml, of a 'black' liquid by reduced pressure (Rotavapor), filtered and used for further fine purification by preparative HPLC.

The preparative Gilson HPLC consisted of a Gilson 302 sample pump (Gilson, Meyvis, Bergen op Zoom, The Netherlands), two 305 Gilson gradient pumps, a 811 B Dynamic mixer, a Kratos 757 UV detector (Separations, Hendrik Ido Ambacht, The Netherlands), an LKB 2211 superrac (LKB, Woerden, The Netherlands), and a BD7 recorder (Kipp and Zonen, Delft, The Netherlands). The column was a C_8 8 μm, 250 mm×10 mm I.D., Rainin Dynamax 60 C column (Meyvis).

The mobile phase consisted of 1% acetic acid in water and acetonitrile. At $t=0$ it contained 2% acetonitrile which changed linearly to 70% acetonitrile at $t=35$ min (v/v). The flow-rate was 4.7 ml/min, UV detection was achieved at 248 nm.

Concentration of the trapped samples was carried out by a IKA rotavapor (Janke & Kunkel, Staufen, Germany) equipped with a Trivac vacuum pump (Leybold-Heraeus, Woerden, The Netherlands). Seven crude samples containing the seven metabolites were collected and labelled MPS A-G.

2.4.3. Fine purification by preparative HPLC

2.4.3.1. MPS-A. Isolation was carried out in two steps with different mobile phases: (1) methanol–1% acetic acid in water (30:70, v/v) and (2) acetonitrile–water (25:75, v/v).

The collected eluate was concentrated under reduced pressure using the rotavapor to 20 ml, thereafter in a smaller flask to 0.5 ml. The metabolite already crystallizes in water. The crystals were transferred into a small tube for freeze drying.

2.4.3.2. MPS-B. Isolation was carried out in three steps with different mobile phases: (1) acetonitrile–water (15:85, v/v), (2) methanol–water (30:70, v/v), and (3) tetrahydrofuran–water (10:90, v/v).

The collected eluate was concentrated under reduced pressure using the rotavapor to 20 ml, thereafter in a smaller flask to 0.5 ml. The final volume of 0.5 ml was transferred into a small tube for freeze drying.

2.4.3.3. MPS-C. Isolation was carried out in two steps with different mobile phases: (1) methanol–1% acetic acid in water (60:40, v/v) and (2) acetonitrile–water (25:75, v/v).

The collected eluate was concentrated under reduced pressure using the rotavapor till 20 ml, thereafter in a smaller flask to 0.5 ml. The final volume of 0.5 ml was transferred into a small tube for freeze drying.

2.4.3.4. MPS-D. Isolation was carried out in three steps with different mobile phases: (1) tetrahydrofuran–water (40:60, v/v), (2) acetonitrile–1% acetic acid in water (45:55, v/v) and (3) tetrahydrofuran–1% acetic acid in water (40:60, v/v).

The collected eluate was concentrated under reduced pressure using the rotavapor till 20 ml, thereafter in a smaller flask to 0.5 ml. The final volume of 0.5 ml was transferred into a small tube for freeze drying.

2.4.3.5. MPS-E. Isolation was carried out in two steps with different mobile phases: (1) methanol–water (30:70, v/v) and (2) acetonitrile–water (15:85, v/v).

The collected eluate was concentrated to 5 ml under reduced pressure using the rotavapor The compound crystallised, was centrifuged and collected, has been washed two consecutive times with water and transferred into a small tube for freeze drying.

2.4.3.6. MPS-F. Isolation was carried out in three steps with different mobile phases: (1) tetrahydrofuran–1% acetic acid in water (27:73,

v/v), (2) methanol–1% acetic acid in water (65:35, v/v) and (3) acetonitrile–water (30:70, v/v).

The collected eluate was concentrated under reduced pressure using the rotavapor till 20 ml, thereafter in a smaller flask to 0.5 ml. The final volume of 0.5 ml was transferred into a small tube for freeze drying.

2.4.3.7. MPS-X. Isolation was carried out in a single step: the mobile phase was methanol–1% acetic acid in water (70:30, v/v).

The collected eluate was concentrated until crystallization under reduced pressure using the rotavapor. The crystals were collected, washed two consecutive times with water, and transferred into a small tube for freeze drying.

2.5. Deconjugation

Deconjugation reactions with β-glucuronidase (24 h, 37°C) and 5 *M* HCl (90°C, 1 h) were carried out.

Four different β-glucuronidase enzymes (A–D) were tested:

1. 100 000 U/ml β-glucuronidase type B1 (bovine liver, Sigma, St. Louis, MO, USA, cat. No. G-0251) and phosphate buffer pH 5.0.
2. 107 200 U/ml β-glucuronidase type H2 (*Helix pomatia*, Sigma, cat. No. G-0876) and phosphate buffer pH 5.0.
3. 100 000 U/ml β-glucuronidase type LII (lyophilized powder from limpets *Patella vulgata*, Sigma, Cat. No. G-8132) and phosphate buffer pH 3.8.
4. 20 000 U/ml β-glucuronidase type VIIA (*Escherichia coli*, Sigma, cat. No. G-7646) and phosphate buffer, pH 6.8.

2.6. Structure identification

2.6.1. Mass spectrometry

The LC–MS analyses were carried out with a VG Platform bench top LC–MS plus Mass Lynx Data system (VGbiotech, Fisons, Tudor Road, Altrincham, UK). The inlet was regulated by a loop injection (100 μl), showing a direct flow of 900 μl/min acetonitrile–water (1:1, v/v).

The instrument settings were as follows: (a) Corona 3.3 kV, cone voltage +10 V. Temperature probe 500°C (b) Corona 3.3 kV, cone voltage +5 V. Temperature probe 400°C. Multiplier 650 V, Source temperature 150°C, Calibration was performed with NaJ. Standard operating conditions were used for working with the atmospheric pressure chemical ionization mode (API).

A Finnigan TSQ45 MS–MS mass spectrometer was used in the positive chemical ionisation mode (Finnigan, Thermoquest, Breda, Netherlands) with methane 0.40 mTorr. The emission current was 0.20 mA, and the multiplier voltage 2400 V. In the ion product mode argon 2.00 mTorr was used as collision gas. Sample inlet was performed via a probe at 30°C, ramped with 30°/min to 280°C.

2.6.2. Nuclear magnetic resonance

^{1}H-NMR spectra were recorded on a Bruker AM 400 spectrometer (400 MHz,FT) (Bruker, Wormer, The Netherlands) in solutions of $C^2H_3O^2H/C^2HCl_3$ (3:1, v/v) (I.S. Me_4Si). ^{13}C-NMR spectra were recorded with a Bruker AM 400 spectrometer operating at 100.6 MHz in solutions of $C^2H_3O^2H$–C^2HCl_3 (3:1, v/v) (I.S. Me_4Si). Chemical shift values are reported as δ-values relative to tetramethylsilane (TMS) as I.S.; deuteromethanol and deuterochloroform were used as solvents.

2.6.3. Infrared spectrometry

Infrared spectra were recorded on a Perkin Elmer FT-IR spectrometer Spectrum 2000 (Perkin Elmer, Nieuwerkerk aan de IJssel, The Netherlands).

2.6.4. UV–Vis spectrometry

UV spectra were recorded on a Perkin-Elmer PE Lambda2 spectrometer (Perkin Elmer).

2.6.5. Melting points

Melting points were determined using a Büchi melting point apparatus (Dr. Tottoli), Büchs (Flawil, Switzerland).

2.7. Gradient analytical HPLC analysis

The HPLC system consisted of a Spectra Physics SP 8780 autosampler (Thermo Separation Products, Breda, The Netherlands), a Spectra Physics SP 8800 ternary HPLC pump, a Spectraflow UV 2000 detector, and a Spectra Physics SP 4290 integrator.

The column was Cp Spherisorb ODS 5 μm, (250

Table 1
Retention times, capacity factors and molecular mass of methylprednisolone and metabolites[a]

	t_R (min)	k'	M_r
Methylprednisolone 21hemisuccinate	32.9	26.4	474
Methylprednisolone	29.0	23.2	374
Methylprednisolone X C21COOH–20OH	28.0	22.3	390
Methylprednisolone C 20βOH	26.2	20.8	376
Methylprednisolone F -gluc	25.2	20.0	548
Methylprednisolone D C20COOH	24.3	19.3	344
Methylprednisolone 5 C6αOH?	23.4	18.5	
Methylprednisolone 4 20αOH?	20.1	15.8	
Methylprednisolone A C6βOH	18.6	14.5	390
Methylprednisolone E C6βOH20βOH	16.5	12.8	392
Methylprednisolone B C6βOH20αOH	15.8	12.2	392
Hippuric acid	13.0	9.83	179
Prednisolone	24.0	19.0	360
Prednisone	24.2	19.2	358
t_0	1.2		

[a] C6 oxidation reduces the retention times by 0.63 (A/MP=14.5/23.2=0.63). C20 reduction reduces the retention times by 0.90 (C/MP=20.8/23.2=0.90). Check: C6C20 reduces the retention time of MP from 23.2×0.90×0.63=13.2 (B=E) B=E (MS,IR,NMR) $\alpha/\beta = 12.2/12.8 = 0.95$. The $\alpha/\beta = 0.95$ can be found in the following ratio B/E=0.95; A/4=0.92; 5/D=0.96.

mm×4.6 mm I.D.) (Chrompack, Bergen op Zoom, The Netherlands) with a guard column (75 mm×2.1 mm), packed with pellicular reversed phase (Chrompack cat. no. 28653).

The mobile phase was a mixture of acetonitrile and 1.0% acetic acid in water. At $t=0$, this mobile phase consisted of 2% acetonitrile and 98% acetic acid 1% in water (v/v). During the following 35 min the mobile phase changed linearly until it attained a composition of 50% acetonitrile and 50% buffer (v/v). At 40 min ($t=40$) the mobile phase was changed over 5 min to the initial composition, followed by equilibration during 2 min. The flow-rate was 1.5 ml/min. UV detection was achieved at 248 nm.

The capacity factors of methylprednisolone and the metabolites are given in Table 1.

3. Results

As shown in Figs. 2 and 3, the metabolites of methylprednisolone were noticed in the chromatogram of a urine sample of a patient after an oral intake of 1 g of methylprednisolone hemisuccinate.

Seven metabolites of methylprednisolone were isolated from the urine sample and labeled: A–X. The isolated compounds were identified as follows:

3.1. Methylprednisolone

3.1.1. NMR spectrum

This shows that the C27–CH_3 at the C6 position is a doublet at 1.12 and 1.14 ppm, thus the C6 proton is present, located at 2.69–2.73 ppm as a sextet. The C21 protons are located at 4.58–4.63 and at 4.22–4.27 ppm, each proton as a doublet. The C11 proton is located at 4.38–4.41 ppm as a quartet. C19–CH_3 is positioned at 1.48 ppm and the C18 CH_3 group at 0.92 ppm. In the A ring the C1 proton is at 6.24–6.27 as quartet, C4 proton singlet at 5.99 ppm, and C2 proton as a doublet at 7.45–7.48 ppm. The ^{13}C spectrum shows that 3×CH_3 groups, 5×CH groups and 5×CH_2 groups are present, with 3×CH bonds in the A ring.

3.1.2. Mass spectrometry

Cone voltage 10 V (*m/z*, relative abundance %) *m/z* 375 (M^++H; 40%), 356 (M^+-H_2O; 55%), 345 (15%), 339 (25%), 327 (15%), 315 (100%; base peak, M^+-COCH_2OH), 297 (50%; $M^+-COCH_2OH-H_2O$).

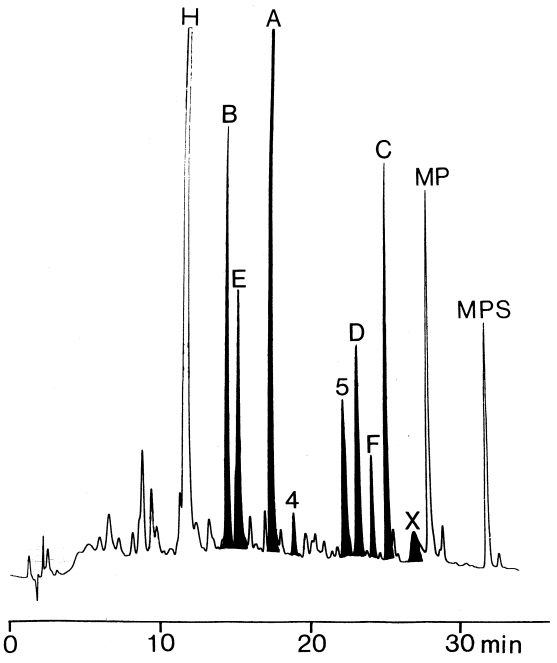

Fig. 2. Chromatogram of partly purified human urine containing methylprednisolone (MP) and its metabolites. A = 6βhydroxy,6αmethylprednisolone, B = E = 6βhydroxy,C20 (α/β)hydroxymethylprednisolone, C = C20hydroxymethylprednisolone, D = C20carboxymethylprednisolone, F = methylprednisoloneglucuronide, X = C21carboxy–C20hydroxymethylprednisolone, MPS = methylprednisolone 21-hemisuccinate, H = hippuric acid. 4,5 = unidentified metabolites (α/β isomers?).

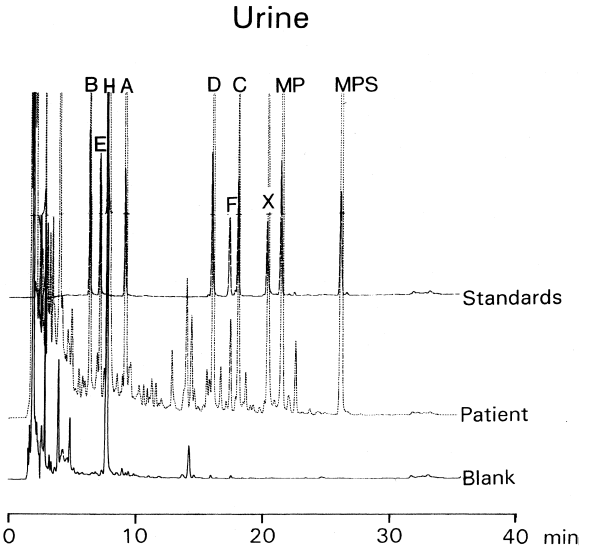

Fig. 3. Chromatograms of an actual human urine (12 h) containing methylprednisolone (MP) and its metabolites, and a blank urine. Legend metabolite identification as in Fig. 2.

3.1.3. Infrared spectrometry

1594, 1651, 1716 cm^{-1} (C20 carbonyl group) 3333, 3056, 2977, 2945, 2922, 2867, 2850 cm^{-1}.

The UV_{max} was at 243 nm. Melting points: methylprednisolone-hemisuccinate 221–223°C, melting under decomposition; methylprednisolone 240–243°C, melting with decomposition.

3.2. Metabolite A, 6β-hydroxy-6αmethylprednisolone

3.2.1. NMR spectrum

This shows that the C6–CH_3 has lost its doublet (singlet at 1.44 ppm), thus the C6–H has disappeared. In the A ring there are still three protons, the C4 proton moved into the region of C1. The C22 protons are identical with those in methylprednisolone. The C19 CH_3 at 1.68 ppm and the C18 CH_3 group at 0.93 ppm are similar to those in methylprednisolone. The ^{13}C spectrum shows that 3×CH_3 groups, 4×CH groups and 5xCH_2 groups were present, with 3×CH bonds in the A ring. Extra present, in comparison to methylprednisolone itself is a quaternary carbon atom.

There is a 6β-hydroxy group present at the C6 position, giving rise to the singlet CH_3 group at C6.

3.2.2. Mass spectrometry

Cone voltage 10 V: *m/z* 391 (10%; M^++H), 377 (45%), 372 (15%; M^+-H_2O), 359 (35%), 356 (20%), 341 (25%), 331 (100%; base peak, M^+-COCH_2OH), 313 (90%; $M^+-COCH_2OH-H_2O$), 296 (65%), 295 (70%; $M^+-COCH_2OH-2H_2O$), 281 (15%), 205 (60%), 187 (15%). Cone voltage 5 V: *m/z* 432 (21%; M^++H+AcCN), 414 (15%; M^++H+Na); 391 (98%), 372 (100%), 362 (45%; M^+-CO), 355 (35%), 343 (25%), 331 (95%), 313 (45%), 295 (10%), 205 (15%), 187 (5%).

The rapid loss of water may indicate that the C6OH group is out the plane of the skeleton, β position.

3.2.3. Infrared spectrometry

1594, 1651, 1716 cm^{-1} (C20 carbonyl group); 3510, 3464, 3381, 3300 cm^{-1} (broad peak).

The UV_{max} absorption was at 244 nm. Melting points: 196–205°C yellow colour, 222–224°C melting under decomposition.

3.3. Metabolite B, 6-βhydroxy–C20αhydroxy-6αmethylprednisolone

3.3.1. NMR spectrum

This shows that the C6–CH_3 has lost its doublet (singlet at 1.44 ppm), thus the C6–H has disappeared. The C4 proton coincides with the C2 proton at 6.20–6.23 ppm (2 H). There are still three protons, the C4–H moved into the region of C2. The C21 protons are not identical with those of methylprednisolone, they are located at 4.58–4.63 and at 4.22–4.27 ppm, each proton as a doublet. The C20 doublet at 76.45 ppm in ^{13}C-NMR, shows reduction of the C20 carbonyl group. C19 CH_3 at 1.68 ppm and the C18 CH_3 group at 1.09 ppm.

There is a 6β-hydroxy group present at the C6 position, giving rise to the singlet CH_3 group at C6, while also the C20 apparently is reduced to an alcohol.

3.3.2. Mass spectrometry

(Identical with E) Cone voltage 10 V: *m/z* 415 (5%, M^+ + Na), 393 (65%, M^++H), 375 (40%, M^++H−H_2O), 357 (65%, M^++H−2H_2O), 345 (25%), 339 (90%), 327 (30%), 321 (40%), 315 (90%; M^+−$COCH_2OH$−H_2O), 309 (70%), 297 (100%, base peak, M^+−$COCH_2OH$−2H_2O), 279 (95%; M^+−$COCH_2OH$−3H_2O), 189 (85%). Cone voltage 5 V: *m/z* 434 (15%; M^++H+AcCN), 393 (100%), 375 (25%), 357 (18%), 339 (15%), 331 (30%), 315 (10%), 309 (10%), 297 (15%), 189 (15%).

3.3.3. Infrared spectrometry

In the IR spectrum the carbonyl band at 1720 cm^{-1} has disappeared 1602, 1654 cm^{-1} (C20 carbonyl group reduced); 3402 (one broad absorption), 2936 cm^{-1}.

The UV_{max} was at 245 nm. Melting points: 149°C shrinking, 160°C water loss, 180°C melting.

3.4. Metabolite C, C20hydroxy-6αmethyl-prednisolone

3.4.1. NMR spectrum

This shows that the C6–CH_3 is a doublet at 1.12 and 1.14 ppm, thus the C6 proton is present, located at 2.70–2.76 ppm as a sextet. There are three C21 protons, located at 3.62–3.68 as a quartet and at 3.74–3.78 ppm as a triplet. The C11 proton is located at 4.35–4.36 ppm as a doublet. The C19 CH_3 at 1.49 ppm and the C18 CH_3 group at 1.07 ppm are similar to those of methylprednisolone.

The ^{13}C spectrum shows that 3×CH_3 groups, 8×CH groups and 6×CH_2 groups are present, with 3×CH bonds in the A ring.

The C6 position is similar to that of methylprednisolone, the C20 position is at 76.48 ppm (d) instead of 211.70(s), thus reduced.

The metabolite shows an alcohol group at C20 and is C20-hydroxymethylprednisolone.

3.4.2. Mass spectrometry

Cone voltage 10 V: *m/z* 399 (5%; M^++Na), 377 (M^++H; 100%, base peak), 359 (60%; M^++H−H_2O), 341 (55%; M^++H−2H_2O), 323 (15%; M^++H−3H_2O), 315 (15%, M^+−$CH_2OHCHOH$), 297 (12%; M^+−$CH_2OHCHOH$−H_2O), 281 (50%; M^+−$CH_2OHCHOH$−2H_2O). Cone voltage 5 V: *m/z* 418 (35%; M^++H+AcCN), 377 (100%), 375 (40%), 359 (20%), 341 (50%), 323 (15%), 315 (30%), 297 (2%), 281 (40%).

3.4.3. Infrared spectrometry

In the IR spectrum the carbonyl band at 1720 cm^{-1} has been disappeared 1655, 1599 cm^{-1} (C20 carbonyl group reduced) 3370 (one broad absorption), 2919 cm^{-1}.

The UV_{max} was at 243 nm. Melting points: 140°C water loss, 210°C decomposition, 222°C melting.

3.5. Metabolite D, 20carboxy-6αmethyl-prednisolone

3.5.1. NMR spectrum

This shows that the C6–CH_3 is a doublet at 1.12 and 1.14 ppm, thus the C6 proton is present, located at 2.61–2.74 ppm as a sextet. C11 proton is located at 4.38–4.39 ppm as a doublet. The C19 CH_3 is

located at 1.49 ppm and the C18 CH_3 group at 1.00 ppm. The C21 protons have been disappeared or shifted to a lower field.

The ^{13}C spectrum shows that $3\times CH_3$ groups, $8\times CH$ groups and $5\times CH_2$ groups are present, with $3\times CH$ bonds in the A ring. The C20 and C21 in the ^{13}C-NMR are missing. In the A ring the C1 proton is at 6.24–6.26 as a quartet, C4 proton is singlet at 5.99 ppm, and C2 proton is a doublet at 7.46–7.49 ppm.

In this metabolite the C20–C21 side chain is missing or not visible.

3.5.2. Mass spectrometry

Cone voltage 10 V *m/z* 812 (10%), 797 (30%; $2(M^+ + H) + Na$), 775 (20%; $2(M^+ + 1)$), 468 (5%), 387 (100%; base peak, $M^+ + H + AcCN$), 359 (5%), 343 (25%). Cone voltage 5 V *m/z* 386 (45%; $M^+ + H + AcCN$), 345 (100%; $M^+ + H$), 343 (50%), 327 (25%; $M^+ + H - H_2 0$). This can be a COOH at C20.

Mass spectrometry of the *m/z* ions (Finnigan Mass-mass spectrometer): when isolated compound D is directly inserted into the source, heating the probe showed that two compounds entered the source. The first evaporating compound showed a *m/z* 297 ($M^+ + Na$), 267 (5%; $M^+ + 1$), 255 (10%) 227(100%). The second compound showed *m/z* 367 (5%; $M^+ + Na$), 345 (55%; $M^+ + H$), 327 (100%; $M^+ + H - H_2O$), 309 (30%). Daughter ions of *m/z* 345 *m/z* 345 (5%; $M^+ + H$), 327 (15%; $M^+ + H - H_2O$), 309 (10%; $M^+ + H - 2H_2O$), 187 (35%), 161 (100%), 135 (50%). Daughter ions of *m/z* 327: *m/z* 327 (15%; $M^+ + H$), 309 (10%; $M^+ + H - H_2O$), 187 (30%), 185 (25%), 161 (100%), 135 (50%). Daughter ions of *m/z* 309: *m/z* 309 (50%; $M^+ + H$), 291 (25%, $M^+ + H - H_2 0$), 187 (20%), 185 (60%), 161 (100%), 135 (50%).

3.5.3. Infrared spectrometry

1594, 1654, 1719 cm^{-1} (C20 acid carbonyl group). 3449 cm^{-1} (one broad peak), 2931 cm^{-1}.

The UV_{max} was at 241 nm. Melting points: 90°C water loss, 125°C yellow colour, 139°C melting.

3.6. Metabolite E, 6βhydroxy-C20βhydroxy–6αmethylprednisolone

The NMR spectrum shows that the C6–CH_3 has lost its doublet (singlet at 1.44 ppm), thus the C6–H has disappeared. The C4 proton coincides with the C2 proton at 6.20–6.23 ppm (2 H). The C21 protons are not identical with methylprednisolone, there are three protons, one quartet at 3.57–3.67 and one triplet at 3.77–3.80 shifted to lower field. The C19 CH_3 is located at 1.69 ppm, the C18 CH_3 group at 1.15 ppm, and the C11 proton at 4.391–4.398 (doublet).

There is a 6β-hydroxy group present at the C6 position, giving rise to the singlet CH_3 group at C6, but also the C20 apparently is reduced. The proposed structure is 6βhydroxy-C20βhydroxy-methylprednisolone (=isomer of metabolite B).

3.6.1. Mass spectrometry

Cone voltage 10 V *m/z* 415 (5%; $M^+ + Na$), 393 (60%; $M^+ + H$), 377 (60%), 375 (55%; $M^+ + H - H_2O$), 357 (65%, $M^+ + H - 2H_2O$), 345 (30%), 339 (100%, base peak, $M^+ + H - 3H_2OH$), 331 (40%), 321 (40%), 315 (50%), 313 (45%), 309 (70%), 297 (65%), 281 (45%), 279 (40%), 189 (65%). Cone voltage 5 V *m/z* 434 (15%; $M^+ + H + AcCN$), 393 (100%; $M^+ + H$, base peak), 375 (55%), 357 (75%), 339 (90%), 331 (15%), 321 (50%), 313 (15%), 309 (35%), 297 (40%), 279 (35%), 189 (55%).

Loss of water is the main fragmentation, C20–OH is in the same direction as the 17βOH, meaning $\beta C_{20}OH$.

3.6.2. Infrared spectrometry

1652, 1600 cm^{-1} (the carbonyl band at 1720 cm^{-1} has been disappeared) 3400 (one strong broad peak), 2932 cm^{-1}.

The UV_{max} was at 244 nm. Melting points: 160°C water loss, 198–210°C decomposition, 220–223°C melting.

3.7. Metabolite F, the glucuronide of methylprednisolone

The NMR spectrum shows that the C6–CH_3 is a doublet at 1.12 and 1.14 ppm, thus the C6 proton is present, located at 2.69–2.73 ppm as a sextet. The C21 protons are located at 4.58–4.63 and at 4.22–4.27 ppm, each proton as a doublet. The C11 proton is located at 4.33–4.44 ppm as a quartet. A doublet is located at the same position 4.410–4.417 ppm.

The C19 CH_3 is located at 1.48 ppm and the C18 CH_3 group at 0.92 ppm.

In the A ring C1 the proton is at 6.24–6.27 as quartet, C4 proton is a singlet at 5.99 ppm, and C2 proton is a doublet at 7.48–7.50 ppm.

The ^{13}C spectrum shows that 3×CH_3 groups, 11×CH groups and 3×CH_2 groups are present, with 3×CH bonds in the A ring. An additional doublet of the glucuronide group is present .

3.7.1. Mass spectrum

Cone voltage 10 V *m/z* 1098 (5%; $2M^+$), 571 (5%; M^++Na), 548 (100%; base peak, cluster of peaks 547–549 M^++H;M^+−H), 355 (12%; M^+-glucuronide). Cone voltage 5 V *m/z*, 550 (30%; M^++H), 375 (30%; M^++H−glucuronide), 357 (75%; M^++H−glucuronide−H_2O), 345 (80%), 343 (100%; base peak), 342 (60%), 315 (30%).

3.7.2. Infrared spectrometry

1598, 1653, 1700 cm^{-1}, 3420 cm^{-1}, (one strongest peak), 2907 cm^{-1}.

The UV_{max} was at 242 nm. Melting points: 170–175°C yellow/brown, decomposition only glucuronide group?

Glucuronidase (system D) hydrolyses compound D in methylprednisolone.

3.8. Metabolite X, 21carboxy-20hydroxy-methylprednisolone

The NMR spectrum shows that the C6–CH_3 is a doublet at 1.12 and 1.14 ppm, thus the C6 proton is present, located at 2.71–2.73 ppm as a triplet/sextet. The C11 proton is located at 4.33–4.44 ppm as a quartet. A doublet is located at the same position 4.410–4.417 ppm, the C19 CH_3 is at 1.48 ppm and the C18 CH_3 group at 1.17 ppm (shifted to higher field).

In the A ring C1 proton is at 6.24–6.27 as quartet, C4 proton is a singlet at 5.99 ppm, and C2 proton is a doublet at 7.48–7.50 ppm.

The ^{13}C spectrum shows that 3×CH_3 groups, 5×CH groups and 4×CH_2 groups are present, with 3×CH bonds in the A ring.

3.8.1. Mass spectrum

Cone voltage 10 V *m/z* 391 (30%; M^++H), 373 (45%; M^++H−H_2O), 370 (15%), 355 (100%; base peak, M^++H−2H_2O), 329 (55%), 315 (50%), 311 (50%), 297 (55%), 293 (20%), 281 (45%), 279 (15%). Cone voltage 5 V *m/z* 432 (15%; M^++H+AcCN), 391 (100%), 373 (30%), 355 (40%), 329 (70%), 315 (100%).

3.8.2. Infrared spectrometry

1596, 1654, 1720 cm^{-1} 3216 cm^{-1} (strong peak+shoulder), 2907, 2929 cm^{-1}.

The UV_{max} was at 243 nm. Melting points: 145°C decomposition, 154°C melting.

3.9. Chromatography

Table 1 shows the retention times, capacity factors of methylprednisolone and metabolites and group contributions to the retention behaviour. C6 oxidation reduces the retention time by a factor 0.64 (A/MP=14.5/23.2=0.63). C20 reduction reduces the retention time by a factor 0.90 (C/MP=20.8/23.2=0.90); Check: C6OHC20OH reduces the retention time of MP from 23.2×0.90×0.63=13.15 (=E). B=E (MS, IR, NMR). The ratio in retention times of the α/β configuration is α/β=12.2/12.8=0.95. Fig. 3 shows the chromatogram of an actual 24 h urine of a patient to demonstrate that the chromatograms of preparative and analytical columns are similar.

4. Discussion

The proposed structures of the isolated metabolites of methylprednisolone are shown in Fig. 4 and the metabolic scheme in Fig. 5.

Disposition kinetics in plasma and urine of pulse dose methylprednisolone in patients with the nephrotic syndrome, demonstrated that only 10% of the dose is excreted unchanged leaving 90% of the dose for metabolism [16]. The metabolism of methylprednisolone is barely investigated. The large dose, the large concentrations of MP and metabolites in the urine merited isolation of the metabolites and structure elucidation by MS, NMR, IR and UV spectrometry. NMR revealed oxidation at the C6 posi-

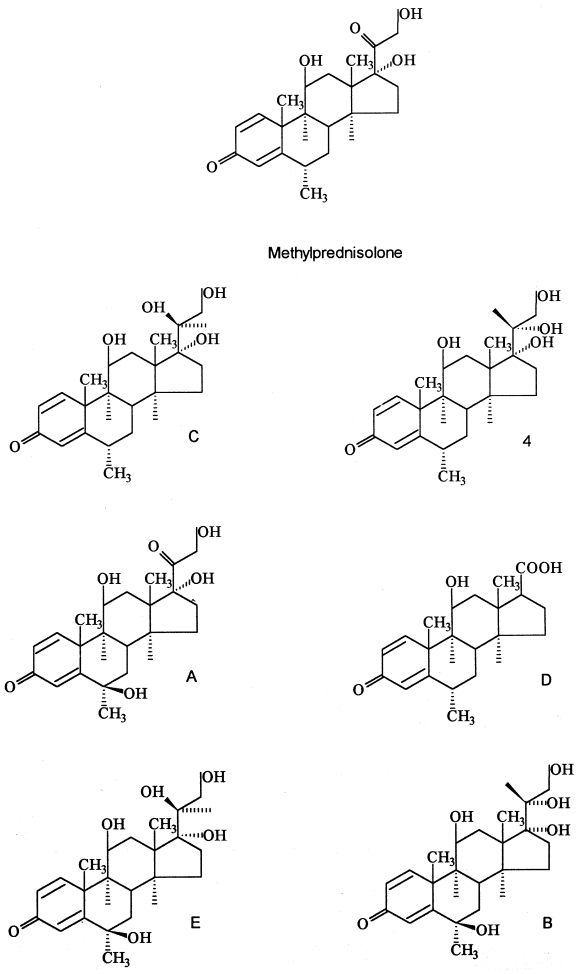

Fig. 4. Structure of methylprednisolone and metabolites.

tion, IR revealed the presence of carbonyl and hydroxy groups, and MS in the soft ionisation mode revealed the molecular mass. The cone energy of 10V for expelling the ionised molecule from the orifice into the mass spectrometer, rendered the molecule an internal energy high enough for fragmentation of the C17 side chain which resulted in low abundances of the molecular ion ($M^+ + H$). Therefore also the lower cone energy of 5V was applied, resulting in higher abundance of the $M^+ + H$ mass fragment, and a lower fragmentation (Platform mass spectrometer). With mass spectrometry of the parent and daughter ions of compound D (TSQ mass spectrometer), the molecular mass and fragmentation was checked. Compound A was oxidised at the C6 atom, compound C was reduced at the C20 atom. Compounds B and E were oxidised and reduced, had almost similar mass spectra at 10V cone voltage, and were identified as α/β isomers at the C20 atom. The spectra were different in fragmentation intensity at 5V cone voltage; E loses water (3×), while compound B stayed almost intact. E was labeled C20βhydroxy - 6βhydroxy - 6αmethylprednisolone. With existing C20α/β isomery in B and E then also a similar isomery must exist with compound C and its isomer (compound no 5?, ratio k' C/5=1.12). Depending on the mechanism of C6 oxidation, the α/β configuration is retained or inversed (compound no 4?, ratio k' 4/A=1.08). Also compounds 4/5 can be C6 isomers of B and E. Compounds 4 and 5 in the chromatogram in Fig. 2 and 3 were not yet isolated and identified.

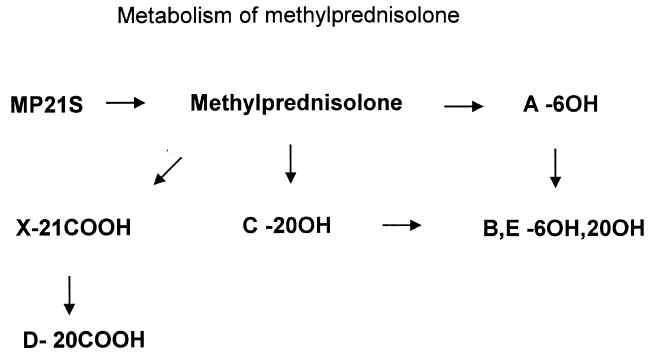

Fig. 5. Metabolic scheme of methylprednisolone.

Compound X had molecular mass of 390, indicating oxidation, but not at the C6 atom, because NMR analysis showed the doublet at C6 to remain intact. This means that C21 is oxidised to the C21 carboxy group, because this group was left as the only available group for oxidation. This process results in a metabolite with a molecular mass of 388, so at the same time reduction at C20 must have been carried out. When the C21 carboxy group is further oxidised, compound D is formed. The high concentration of D and the low concentration of X indicates that the oxidation of the C21 atom is a rapid process. The tentative metabolic scheme, shown in Figs. 1 and 5, was in part seen and reported in the literature.

Many attempts to solve the metabolic pathways of methylprednisolone were carried out, all resulting to a tentative metabolite. The known metabolic or hydrolytic cleavage is the conversion of the prodrug methylprednisolone 21-hemisuccinate to the parent drug methylprednisolone by human serum cholinesterase [15]. Also there exists an acyl migration to position 17 to form the 17-hemisuccinate [7].

GC–MS analysis of methylprednisolone in human urine revealed unchanged drug and the 11-keto (prednisones) and 20-hydroxy metabolites together with 6–7 dehydro analogues of these compounds [17]. Ebling et al. were unable to detect methylprednisone in human plasma [9,18]. No methylprednisone was shown in their pharmacokinetic curves. They reported the existence of an oxidation–reduction equilibrium of the 11-keto group in rabbits and humans later on [18]. The equilibrium also exists in rats [11] and is performed by the 11β-hydroxy steroid dehydrogenase [19]. A similar species dependent equilibrium exists at the C20-keto-hydroxy group [11].

Gray et al. [20] and Lawson et al. [21] demonstrated that 20-hydroxymethylprednisolone was present in human urine in the 20α- and 20β-configuration in quantities similar to those of methylprednisolone. In equine urine, the metabolites were the 20α,β hydroxy metabolites of both methylpredisolone and methylprednisone [22].

The metabolism of the synthetic progestin ^{14}C-megestrol acetate showed the oxidation at position 2 (2-hydroxymegestrol) and oxidation of the 6-methyl group (6-hydroxymethyl-megestrol), the 2,6 dihydroxymegestrol, together with their glucuronide conjugates [23]. The C2 position was vulnerable for oxidation because there was no unsaturated bond. Oxidation of a unsaturated bond takes place in stanozolol to 3′-hydroxystanozolol [24,25].

Prednisone and prednisolone are oxidized (6–10%) at the 6 position to form 6β-hydroxyprednisolone [10,26].

The IR spectrum of the 2-hydroxymegestrol metabolite showed three carbonyl absorptions at 1739 (2-hydroxy), 1722 (20-ketone), and 1677 cm^{-1} (3-keto) respectively [22].

Direct measurement of steroid sulfate and glucuronides revealed that glucuronidation and sulfation takes only place at a free alcoholic group [23,24,27,28], like the 17β-glucuronide of testosterone and the 17α-glucuronide of epitestosterone. Cole et al. positioned the glucuronide group of steroids at the 3-position [29,30].

Reduction of the C20 carbonyl group results in the respective stereoisomers C20-αhydroxy, and C20-βhydroxymethylprednisolone.

5. Conclusion

In the present study metabolites of methylprednisolone were detected using a gradient elution HPLC. Due to the high pulse dose of 1 g methylprednisolone hemisuccinate, the concentrations in the urine were high. We have isolated the compounds with the most abundant concentration and suggested structural formulas. The NMR spectra of methylprednisolone and its metabolites correspond in general with those of prednisolone analogs [31–33]. There exist indications that there was α/β stereochemistry present which could not be solved in this study. The overall picture of the metabolic pathways of methylprednisolone is apparently simple: reduction of the C20 carbonyl group and further oxidation of the C20–C21 side chain, in competition with or additional to the oxidation at the C6-position as shown in Fig. 5.

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