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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

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Abstract

Fourteen metabolites of methylprednisolone have been analysed by gradient-elution high-performance liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS).

The compounds were separated on a Cp Spherisorb 5 μm ODS column connected to a guard column packed with pellicular reversed phase. The mobile phase was an acetonitrile–1·0% aqueous acetic acid gradient at a flow rate of 1·5 mL min^{-1}.

The analysis gave a complete picture of parent drug, prodrugs and metabolites, and the α/β stereochemistry was resolved. The short (1–2 h) elimination half-life of methylprednisolone is explained by extensive metabolism. 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 C21COOH and C20COOH), in competition with or in addition to oxidation at the C6 position.

High-dose pulsed intravenous infusions or oral administration of methylprednisolone hemisuccinate have been used for many years for treatment of acute relapses or progressive worsening of multiple sclerosis (Barnes et al 1985; Milligan et al 1987; Compston 1988; Goodin 1991; Defer et al 1995). Clinical improvement or failure can be correlated with plasma concentrations and, better, brain concentrations of methylprednisolone. A requirement is the availability of validated analytical methodology for analysis of the prodrug and parent drug, including all known metabolites, because the whole spectrum of compounds can enter the brain. Defer et al (1995) elegantly analysed brain concentrations of methylprednisolone after administration of a high intravenous dose and then reconstructed a brain-elimination curve by taking one liquor sample from each patient at different times. After development of an analytical HPLC method for methylprednisolone and its hemisuccinate in plasma and urine, it was noticed that only 5% of a high intravenous dose (1 g) could be recovered from the urine, leaving 95% for further metabolism (Figure 1). Application of a mobile-phase gradient indicated that at least seven main metabolites could be detected and identified after isolation (Vree et al 1999a). Nevertheless, minor metabolites could not be isolated. Liquid chromatography coupled with atmospheric-pressure chemical-ionization mass spectrometry (LC–APCI–MS) could provide molecular-weight data and MS–MS analysis the fragmentation pattern of the ionized molecules.

The aim of this study was to identify the metabolites of methylprednisolone, by means of high-

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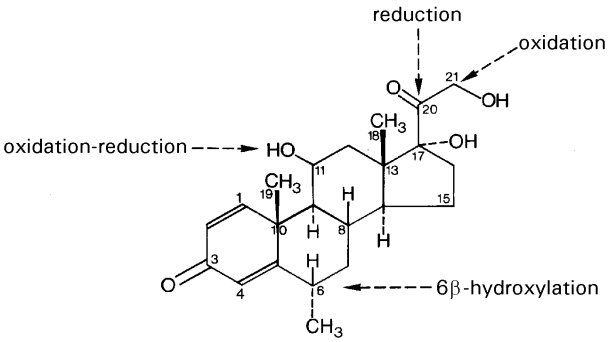

Figure 1. The structure of methylprednisolone and metabolic pathways reported in the literature.

performance liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS), after therapeutic treatment of multiple sclerosis patients by administration of a high intravenous dose.

Materials and Methods

Chemicals

Methylprednisolone (11β,17α,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione, $C_{22}H_{30}O_5$; MW 374; CAS number 83-43-2) and methylprednisolone sodium hemisuccinate (MW 496; Solumedrol) were obtained from Upjohn (Ede, The Netherlands). Other reagents were of analytical quality from Merck (Darmstadt, Germany).

Subjects

Ten patients of the Multiple Sclerosis Centre were therapeutically treated with methylprednisolone hemisuccinate (1 g/day Solumedrol). The study had the approval of the hospital ethics committee and informed consent was obtained from the patients.

Sampling

Urine from each patient was collected for 24 h; the times of voiding were not recorded. Three samples were stored at -20°C pending analysis. The remaining urine from each patient was collected for 24 h in a tank and stored at -20°C, pending isolation.

Structure identification by mass spectrometry

A Finnigan LCQ ion-trap mass spectrometer with an APCI interface (ThermoQuest, Breda, The Netherlands) was used for identification of the minor metabolites of methylprednisolone in urine samples. The interface was a heated capillary at 220°C and at a potential of 13 V. The vaporizer temperature was 450°C and the nitrogen sheath and auxiliary gas flows were 100 and 40 (instrument settings in arbitrary units). For positive ions the corona discharge current was maintained at 100 μA. The analyser temperature was 32°C. The injection volume was 50 μL, the flow rate 3 μL min^{-1} and the volume 250 μL. The API source voltage was 5·00 kV, the source current 100 μA, and the APCI vaporizer temperature 0°C. For positive-ion electrospray ionization the collision energy was 25 V.

Gradient liquid chromatography

HPLC was performed with a Spectra Physics SP 8780 autosampler (Thermo Separation Products, Breda, The Netherlands) and a Spectra Physics SP 8800 ternary HPLC pump. Compounds were separated on a 12·5 cm × 3·0 mm i.d. Cp Spherisorb 5 μm ODS column (Chrompack, Bergen op Zoom, The Netherlands) protected by a 75 mm × 2·1 mm i.d pellicular reversed phase guard column (Chrompack #28653).

The mobile phase gradient was prepared from acetonitrile and 1·0% aqueous acetic acid. At t = 0, the composition was 2 : 98 (v/v) acetonitrile–1% aqueous acetic acid. During the next 45 min the composition was changed linearly to 45 : 55 (v/v) acetonitrile–acid. At 45 min the mobile phase was changed over a period of 10 min to 95% acetonitrile and then to the initial composition, followed by 10 min equilibration. The flow rate was 0·5 mL min^{-1}. The capacity factors of methylprednisolone and the metabolites are given in Table 1.

Results

HPLC

As shown in Figure 2, metabolites of methylprednisolone were observed in the chromatogram obtained from the urine of a patient after a short (0·5 h) infusion of 1 g methylprednisolone hemisuccinate. The identification of seven metabolites isolated from urine samples has been described elsewhere (Vree et al 1999a). The chromatogram revealed more, minor, metabolites of methylprednisolone; these were identified by LC–MS–MS analysis. In total, methylprednisolone and 14 metabolites were distinguished by LC–MS–MS, the results are grouped in the following tables.

Table 1 lists the retention times, capacity factors and molecular weights of all the compounds iden-

Table 1. Retention times (min), capacity factors and molecular weights of methylprednisolone and metabolites as measured by LC–MS.

Compound	Retention time (min)	Capacity factor	Molecular weight
Methylprednisolone 21-hemisuccinate	33·8	27·2	474
Methylprednisolone 17-hemisuccinate	30·7	24·6	474
Methylprednisolone D* C6OHC20COOH	29·0	23·2	360
Methylprednisolone	28·7	22·9	374
Methylprednisolone X C21COOH-20OH	28·1	22·4	390
Methylprednisolone D C20COOH	27·0	21·5	344
MPextra 6β-methyl?	25·7	20·8	374
Methylprednisolone C 20βOH	26·0	20·7	376
Methylprednisolone F glucuronide	25·3	20·1	550
Methylprednisone Ared* C6αOH	22·8	18·0	388
MP21SH?	22·1	17·4	475
MP17SH?	20·1	15·8	475
Methylprednisone Ared C6βOH	17·1	13·3	388
Methylprednisolone A* C6αOH	15·7	12·1	390
Methylprednisolone A C6βOH	14·5	11·1	390
Methylprednisolone E C6βOH20βOH	12·9	9·8	392
Methylpred F* gluc, β form?	12·8	9·7	550
Methylprednisolone B C6βOH20αOH	11·6	8·7	392
Hippuric acid			179
Prednisolone			360
Prednisone			358
t_0	1·2		

C6 oxidation reduces retention times by a factor of 0·48 (A/methylprednisolone = 11·1/22·9 = 0·48). C20 reduction reduces retention times by a factor of 0·90 (C/methylprednisolone = 20·7/22·9 = 0·90). Check: C6C20 reduces the retention time of methylprednisolone from 22·9 × 0·90 × 0·48 = 9·9 (E). B = E (MS, IR, NMR) $\alpha/\beta = 8{\cdot}7/9{\cdot}8 = 0{\cdot}90$. $\alpha/\beta = 0{\cdot}90$ can be found from the ratio B/E = 0·90; A/A* = 0·90; MPextra/methylprednisolone = 0·90. Acyl migration reduces retention times by a factor of 0·90.

tified which are related to methylprednisolone. Metabolism of methylprednisolone results in compounds with a shorter retention time, i.e. more water-soluble compounds, because of the introduction of hydroxyl groups into the molecule.

Methylprednisolone and the hemisuccinates

Table 2 lists the retention times and mass spectra, protonated parent molecules and product ions of methylprednisolone and its precursor methylprednisolone-21-hemisuccinate (MP21S). A second compound with a $[M+H]^+$ ion of m/z 475 was present in the urine sample; this must be methylprednisolone-17-hemisuccinate (MP17S). Methylprednisolone and MP21S eliminate four and three water molecules, respectively; MP17S, in contrast, eliminates only one water molecule. When pure methylprednisolone was subjected to LC–MS analysis a second peak with $[M+H]^+$ of m/z 375 was eluted at a retention time 25·7 min; this could be the isomer 6β-methylprednisolone.

Table 3 lists the retention times and mass spectra, protonated parent molecules and product ions of, presumably, two methylprednisolone congeners with $[M+H]^+$ ions at m/z 476, both of which eliminated four molecules of water. These compounds must be metabolites of methylprednisolone because they are not present in blank urine and are not formed during the LC of methylprednisolone. The molecular weight indicates that methylprednisolone is conjugated with an amino acid.

Oxidation at the C6 position

Table 4 lists the retention time and mass spectra, protonated parent molecules and product ions of three methylprednisolone metabolites with $[M+H]^+$ ions at m/z 391. Methylprednisolone A was identified as 6β-hydroxy-6α-methylprednisolone (Figure 3). Methylprednisolone A* has an apparently similar fragmentation pattern, and might be 6α-hydroxy-6β-methylprednisolone. The third compound, methylprednisolone X, has previously been identified as C21-carboxy-C20hydroxymethylprednisolone; this compound loses only two molecules of water, compared with four water molecules for methylprednisolones A and A*.

Table 5 lists the retention times and mass spectra, protonated parent molecules and product ions, of two methylprednisolone metabolites with $[M+H]^+$ ions at m/z 389. Both compounds lose four molecules of water, though one metabolite (Ared) fragments much more easily than the other (Ared*). The compounds are oxidized at C11, leading to the isomeric compounds A (C6βOH-methylprednisone and A* C6αOH-methylprednisone.

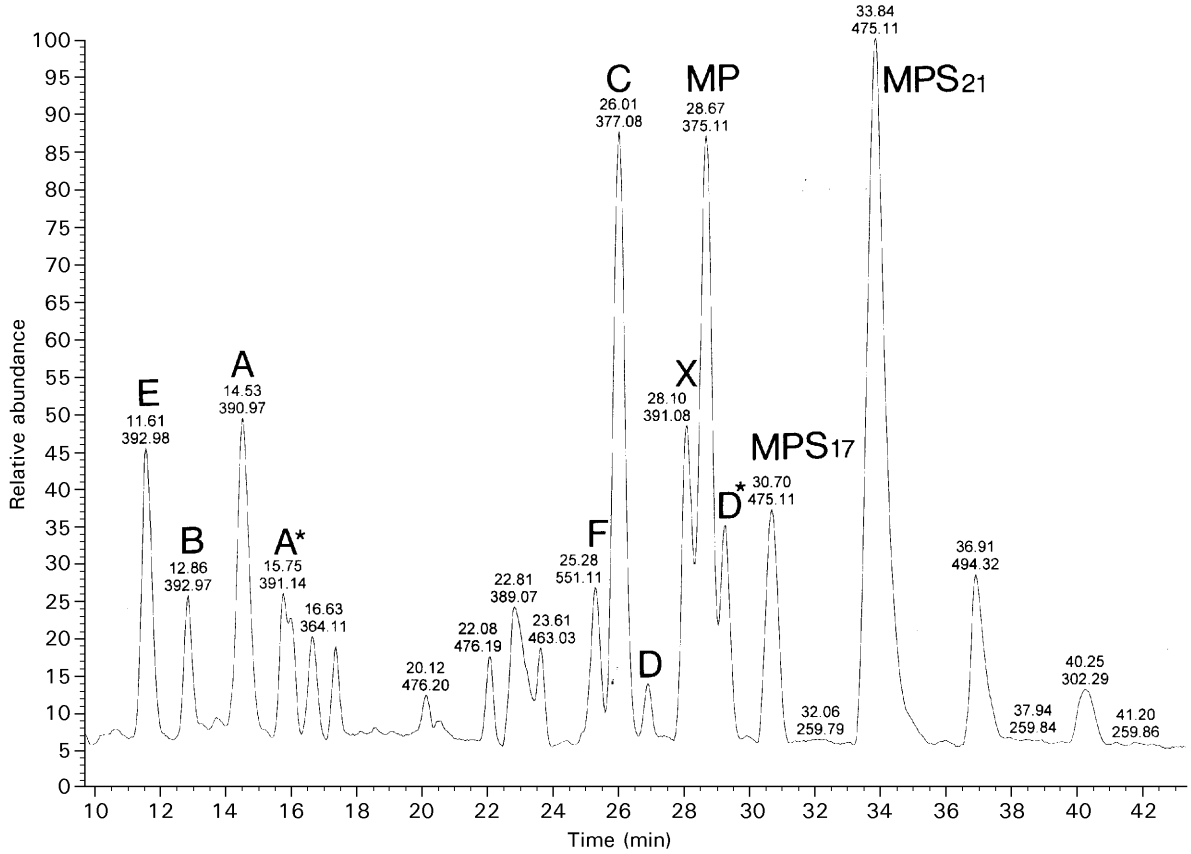

Figure 2. LC–MS chromatogram obtained from urine from man 3 h after infusion of methylprednisolone hemisuccinate sodium (1 g). Methylprednisolone and its main metabolites are present: A, C6β-hydroxy-C6α-methylprednisolone; B, E, C6β-hydroxy-C20(α/β)-hydroxymethylprednisolone; C, C20-hydroxymethylprednisolone; D, C20-carboxymethylprednisolone; F, methylprednisolone glucuronide; X, C21carboxy-C20hydroxymethylprednisolone; MPS, methylprednisolone 21-hemisuccinate (Table 1) (Vree et al 1999a).

Table 2. Mass spectra, MS–MS spectra and retention times of methylprednisolone and the hemisuccinates as measured by LC–MS.

m/z	Fragment	Relative abundance (%)					
		Methyl-prednisolone	Product ion	Methyl-prednisolone-21-hemisuccinate	Product ion	Methyl-prednisolone-17-hemisuccinate	Product ion
478						2	
477				6		10	
476				26	0	24	
475	$[M+H]^+$			100	22	100	10
457	$[M+H-1H_2O]^+$				100		100
439	$[M+H-2H_2O]^+$				26		
421	$[M+H-3H_2O]^+$				2		
377		4					
376		22					
375	$[M+H]^+$	100	0				
357	$[M+H-1H_2O]^+$		100				
339	$[M+H-2H_2O]^+$		38		38		80
321	$[M+H-3H_2O]^+$		28		28		
303	$[M+H-4H_2O]^+$		12		12		
293			18		2		
279			6		2		
Retention time (min)		27·8–28·8		32·8–33·5		30·4–31·0	

Methylprednisolone is 11β,17α,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione.

Table 3. Mass spectra, MS–MS spectra and retention times of metabolites of methylprednisolone hemisuccinates, as measured by LC–MS.

m/z	Fragment	Relative abundance (%)			
		Reduced methylprednisolone 17-hemisuccinate[a]	Product ion	Reduced methylprednisolone 21-hemisuccinate[a]	Product ion
478		2		2	
477		28		24	
476	$[M+H]^+$	100		100	0
458	$[M+H-1H_2O]^+$		84		98
440	$[M+H-2H_2O]^+$		100		100
422	$[M+H-3H_2O]^+$		28		22
404	$[M+H-4H_2O]^+$		4		4
394			12		12
388			6		6
368			10		10
350			4		4
288			6		6
262			12		14
236			12		12
Retention time (min)		19·9–20·1		21·8–22·2	

[a]Minor metabolites (Figure 2), MW 475, containing one nitrogen atom, possibly a conjugate of methylprednisolone or methylprednisolone hemisuccinate.

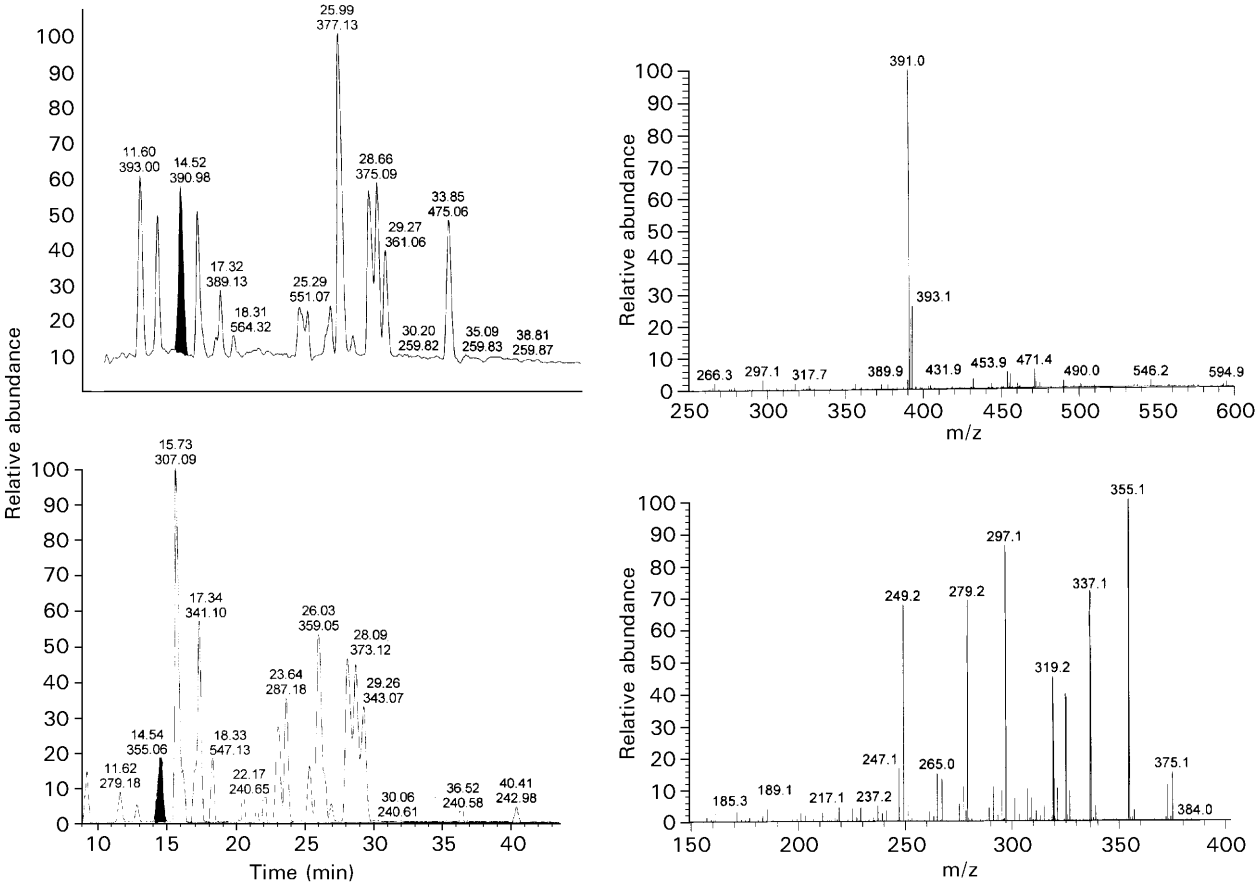

Figure 3. Total ion current (left top) and daughter ion current (left bottom) chromatograms obtained from urine from man (12 h) containing methylprednisolone and its metabolites. The mass spectrum of the metabolite methylprednisolone (A) is shown as an example (full spectrum top right, daughter ions of m/z 391, bottom right).

Table 4. Mass spectra, MS–MS spectra and retention times of metabolites with a molecular weight of 390 as measured by LC–MS.

m/z	Fragment	Relative abundance (%)					
		C6βOH-methyl-prednisolone	Product ion	C6βOH-methyl-prednisolone	Product ion	C21carboxy-methyl-prednisolone	Product ion
393		2		2		2	
392		22		22		22	
391	$[M+H]^+$	100	0	100	0	100	0
373	$[M+H-1H_2O]^+$		12		22		100
355	$[M+H-2H_2O]^+$		100		100		52
344					62		
337	$[M+H-3H_2O]^+$		67		98		4
326			38		38		
319	$[M+H-4H_2O]^+$		42		60		
315	$17C=O^{+a}$		5				6
309							2
307	$[M+H-3H_2O-CH_2O]^+$		10		86		
303					20		
297		5	50				
289					52		
280							10
279			56		8		
277					96		
265			15		10		
263					10		
251					8		
249			70		4		
247			20		4		
Retention time (min)		14·0–14·8		15·5–16·1		27·6–28·6	

C6βOH-methylprednisolone is 6β,11β,17α,21-tetrahydroxy-6α-methylpregna-1,4-diene-3,20-dione, C6αOH-methylprednisolone is 6α,11β,17α,21-tetrahydroxy-6β-methylpregna-1,4-diene-3,20-dione, and C21carboxy-methylprednisolone is 11β,17α-dihydroxy-21carboxy-6α-methylpregna-1,4-diene-3,20-dione. [a]Oxygen substitution at C17.

Oxidation at the C20 position

Table 6 lists the retention time and mass spectra, protonated parent molecules and product ions of two methylprednisolone metabolites with $[M+H]^+$ ions at m/z 345 and 361. Both compounds lose three molecules of water. Methylprednisolone D is the C20-carboxymethyl-prednisolone, the result of oxidation and loss of the C21 atom. The molecular weight of metabolite D* is 16 higher, probably indicative of oxidation at the C6 atom, leading to 6βOH-C20carboxymethylprednisolone.

Reduction

Table 7 lists the retention time and mass spectra, protonated parent molecule and product ions of a methylprednisolone metabolite with an $[M+H]^+$ ion at m/z 377. The compound loses four molecules of water. The compound is reduced at C20, leading to an isomeric compound C, presumably C20βOH-methylprednisolone. No second compound with an $[M+H]^+$ ion of m/z 377 was found.

Oxidation and reduction

Table 8 lists the retention times and mass spectra, protonated parent molecules and product ions of two methylprednisolone metabolites with $[M+H]^+$ ions at m/z 393. Both compounds lose five molecules of water. The compounds are oxidized at C6 and reduced at the C20 atom, leading to isomeric compounds B, C6βOH-C20αOH-methylprednisolone and E, C6βOH-C20βOH-methylprednisolone.

Glucuronidation

Only one glucuronide conjugate (methylprednisolone F) could be identified in the chromatogram; its retention time and mass spectra, protonated parent molecule and product ions are listed in Table 9.

Table 5. Mass spectra, MS–MS spectra and retention times of oxidized metabolites with molecular weight 388 as measured by LC–MS.

m/z	Fragment	Relative abundance (%)			
		6βOH-methyl-prednisone	Product ion	6αOH-methyl-prednisone	Product ion
391		8		2	
390		24		20	
389	$[M+H]^+$	100	0	100	0
371	$[M+H-1H_2O]^+$		5		100
353	$[M+H-2H_2O]^+$		72		10
341	$[M+H-H_2O-CH_2O]^+$		100		0
335	$[M+H-3H_2O]^+$		38		6
323	$[M+H-2H_2O-CH_2O]^+$		10		5
317	$[M+H-4H_2O]^+$		20		2
309			16		2
307			5		4
305	$[M+H-3H_2O-CH_2O]^+$		30		
291			2		2
289			8		1
287			6		
277			20		
275			12		
265			5		
263			3		
251			3		
249			10		
247			20		
Retention time (min)		17·1–17·5		22·7–23·1	

C6βOH-methylprednisone is 6β,11β,17α,21-tetrahydroxy-6α-methylpregna-1,4-diene-3,11,20-trione and C6αOH-methylprednisone is 6α,11β,17α,21-tetrahydroxy-6β-methylpregna-1,4,-diene-3,11,20-trione (prednisone analogues of metabolite A).

Table 6. Mass spectra, MS–MS spectra and retention times of metabolites C20carboxymethylprednisolone and 6βOH-C20carboxymethylprednisolone as measured by LC–MS.

m/z	Fragment	Relative abundance (%)			
		C20carboxy-methyl-prednisolone	Product ion	6βOH-C20carboxy-methylprednisolone	Product ion
362				20	
361	$[M+H]^+$			100	0
347		2			
346		22			
345	$[M+H]^+$	100	0		
343	$[M+H-1H_2O]^+$				100
327	$[M+H-1H_2O]^+$		100		
325	$[M+H-2H_2O]^+$				35
309	$[M+H-2H_2O]^+$		32		
307	$[M+H-3H_2O]^+$				10
303			8		2
297					10
291	$[M+H-3H_2O]^+$		10		
263			6		
187					10
161			16		10
Retention time (min)		27·0–27·9		29·1–29·4	

M is 11β,17α-dihydroxy-20-carboxy-6α-methylpregna-1,4-diene-3-one; *M* is 6β,11β,17α-trihydroxy-20-carboxy-6α-methylpregna-1,4-diene-3-one.

Table 7. Mass spectrum, MS–MS spectrum and retention time of metabolite C20OHmethylprednisolone as measured by LC–MS.

m/z	Fragment	Relative abundance (%)	
		C20OHmethyl-prednisolone	Product ion
379		2	
378		20	
377	$[M+H]^+$	100	0
359	$[M+H-1H_2O]^+$		100
341	$[M+H-2H_2O]^+$		28
323	$[M+H-3H_2O]^+$		12
305	$[M+H-4H_2O]^+$		8
297			2
281			42
185	$[M+H-$ ring C + D$]^+$		6
Retention time (min)		25·1–25·9	

C20OHmethylprednisolone (α/β) is 11β,17α,20,2-tetrahydroxy-6α-methylpregna-1,4-diene-3-one.

When the isolated glucuronide was subjected to LC–MS analysis, a second peak, $[M+H]^+$ of m/z 551, was observed at retention time 12·8 min.

Chromatography

The retention times and capacity factors listed in Table 1 for methylprednisolone and its metabolites give an indication of the contributions of the different structural groups to retention behaviour. C6 oxidation reduces the retention time by a factor of 0·48 (A/methylprednisolone = 11·1/22·9 = 0·48). C20 reduction reduces the retention time by a factor of 0·90 (C/methylprednisolone = 20·7/22·9 = 0·90). As a check, C6 and C20 oxidation reduce the retention time of methylprednisolone from 22·9 to 22·9 × 0·9 × 0·48 = 9·89 (the retention time of E). Compounds B and E are identical (MS, infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy). The ratio of the retention times of the compounds with α and β configurations is $\alpha/\beta = 0{\cdot}90$. Acyl migration from position 21 to position 17 reduces the retention time by a factor of 0·90. Protonation or reduction or conjugation of methylprednisolone hemisuccinate to form MPSH reduce the retention time by a factor of 0·64 (Table 3).

Discussion

The structures proposed for the metabolites of methylprednisolone identified are shown in the metabolic scheme in Figure 1.

Disposition kinetics in plasma and urine of a pulse dose of methylprednisolone in patients with the nephrotic syndrome, showed that only 10% of the dose is excreted unchanged leaving 90% of the dose for metabolism (Assael et al 1982). The metabolism of methylprednisolone has been barely investigated. The large dose and the large concentrations of methylprednisolone and metabolites

Table 8. Mass spectra, MS–MS spectra and retention times of C6βOHC20αOHmethylprednisolone and C6βOHC20βOHmethylprednisolone, metabolites with molecular weight 392 as measured by LC–MS.

m/z	Fragment	Relative abundance (%)			
		C6βOHC20αOH methyl-prednisolone	Product ion	C6βOHC20βOH methyl-prednisolone	Product ion
395		2		2	
394		20		20	
393	$[M+H]^+$	100		100	
392		2		2	
391		2	0		0
375	$[M+H-1H_2O]^+$		6		8
357	$[M+H-2H_2O]^+$		42		70
339	$[M+H-3H_2O]^+$		74		100
321	$[M+H-4H_2O]^+$		52		84
303	$[M+H-5H_2O]^+$		16		22
297			38		34
279	$[M+H-3H_2O-HOCH_2CH_2OH]^+$?		100		55
263			14		12
251			4		2
189			22		12
Retention time (min)		10·5–11·6		12·3–13·0	

C6βOHC20αOHmethylprednisolone is 6β,11β,17α,20α-tetrahydroxy-6α-methylpregna-1,4-diene-3-one and C6βOHC20βOHmethylprednisolone is 6α,11β,17α,20α-tetrahydroxy-6β-methylpregna-1,4-diene-3-one.

Table 9. Mass spectrum, MS–MS spectrum and retention time of metabolite methylprednisolone glucuronide as measured by LC–MS.

m/z	Fragment	Relative abundance (%)	
		Methyl-prednisolone-glucuronide	Product ion
553		4	
552		26	
551	$[M+H]^+$	100	
550		5	
549		16	
533	$[M+H-1H_2O]^+$		100
515	$[M+H-2H_2O]^+$		2
497	$[M+H-3H_2O]^+$		1
375	$[M+H-gluc]^+$		4
357	$[M+H-1H_2O-gluc]^+$		54
339	$[M+H-2H_2O-gluc]^+$		34
321	$[M+H-3H_2O-gluc]^+$		20
293			12
Retention time (min)		24·7–25·6	

in the urine warranted isolation of the metabolites and structure elucidation by MS, NMR, IR and ultraviolet (UV) spectrometry. NMR revealed oxidation at the C6 position, IR revealed the presence of carbonyl and hydroxy groups, and MS with soft ionization (APCI) revealed the molecular weight of the methylprednisolone metabolites (Vree et al 1999a). The NMR spectra of methylprednisolone and its metabolites correspond in general with those of prednisolone analogues (Blunt & Stothers 1977; Kirk et al 1990; Rachwal et al 1996). The rapid metabolism of methylprednisolone results in a short half-life ($t_{\frac{1}{2}}$) for methylprednisolone (1 h) and a longer $t_{\frac{1}{2}}$ of 4 h for the metabolites (Vree et al 1999b).

α or β configuration

With the major metabolites identified, the LC–MS–MS identification of the minor metabolites became possible by comparison of molecular weights, fragmentation patterns, and similarities in the loss of water molecules. The different retention times of compounds of similar molecular weight might reveal the α or β configuration of the group newly introduced into the molecule. From the retention times it is apparent that the more stretched the molecule, the longer the retention time, i.e. retention times decrease in the order MP21S $\gg$ MP17S and MP21SH $\gg$ MP17SH. Oxidation or reduction of methylprednisolone results in metabolites with shorter retention times. With increasing hydrophilic character the compounds with a more folded structure have shorter retention times than compounds with a more stretched structure, i.e. compounds with groups in the plane of the steroid instead of folded over the plane. The C6β hydroxyl group results in loss of water and greater fragmentation than when the OH is in the α position (MPA isomers in Table 10).

Product ions formed by loss of water

Table 10 summarizes losses of water molecules from the protonated parent molecules. The loss of one water molecule is the main fragmentation in eight of fifteen methylprednisolone metabolites, including the 17-, and 21-hemisuccinates. Four out of fifteen compounds lose two water molecules as the main fragmentation, one compound loses three water molecules as the main fragmentation, and two compounds (B and E) fragment even further by eventual loss of five molecules of water. This enables isomeric metabolites to be distinguished from each other. Note also that water is lost more readily when a hydroxyl group is present at the C6 position, as observed for the MPA metabolites and for MPB, MPE, and MPD*. This reaction is possibly promoted by formation in ring B of a double bond which is conjugated with the unsaturated ring A.

However, the detailed sequence and mechanism of the losses of water molecules from the respective groups in the prednisolone skeleton can be elucidated only by use of specific ^{18}O or ^{2}H labelling, or both, and was beyond the scope of this investigation (Vree & Nibbering 1973; Smith et al 1990; Hogenboom et al 1998).

Metabolism of the prodrug

The known metabolic or hydrolytic cleavage is the conversion of the prodrug methylprednisolone 21-hemisuccinate to the parent drug methylprednisolone by serum cholinesterase in man (Myers et al 1982). Acyl migration to position 17 also occurs to form the 17-hemisuccinate (Anderson & Taphouse 1981). Acyl migration occurs in-vitro when urine is kept at room temperature—the 17-hemisuccinate was present in minimal amounts in fresh urine. Two minor metabolites with $[M+H]^+$ of m/z 476 are produced, indicating a molecular mass of 475 and the presence of a nitrogen atom. By analogy with cholesterol, methylprednisolone can be conjugated with an amino acid for excretion in bile and the faeces, a small amount escaping via the urine, as shown by the two isomeric compounds denoted MP21SH and MP17SH.

Table 10. Loss of water from methylprednisolone and its metabolites, as measured by LC–MS.

Fragment	Relative abundance (%)						
	MP	MP21S	MP17S	MP21SH	MP17SH	A	A*
MW	374	474	474	475	475	390	390
Parent							
$[^{13}C_2$ isotope$]^+$	4	6	10	2	2	2	2
$[^{13}C$ isotope$]^+$	22	26	24	24	28	22	22
$[M+H]^+$	100	100	100	100	100	100	100
Products							
$[M+H-1H_2O]^+$	100	100	100	98	84	12	22
$[M+H-2H_2O]^+$	38	26	100	100	100	100	
$[M+H-3H_2O]^+$	28	2	22	28	67	98	
$[M+H-4H_2O]^+$	12	4	4	42	60		

Fragment	Relative abundance (%)							
	Ared	A*red	B	E	C	D	D*	X
MW	388	388	392	392	376	344	361	390
Parent								
$[^{13}C_2$ isotope$]^+$	8	2	2	2	2	2	2	2
$[^{13}C$ isotope$]^+$	24	20	20	20	20	22	20	22
$[M+H]^+$	100	100	100	100	100	100	100	100
Products								
$[M+H-1H_2O]^+$	5	100	6	8	100	100	100	100
$[M+H-2H_2O]^+$	72	10	42	70	28	32	35	52
$[M+H-3H_2O]^+$	38	6	74	100	12	10	10	4
$[M+H-4H_2O]^+$	20	2	52	84	8			
$[M+H-5H_2O]^+$	16	22						
$[M+H-1H_2O-CH_2O]^+$	100	0						
$[M+H-3H_2O-HOCH_2CH_2OH]^+$?	100	55						

$^{13}C_2$,^{13}C and natural isotopes of methylprednisolone C_{22}. MP is methylprednisolone, or 11β,17α,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione; MP21S is methylprednisolone-21-hemisuccinate; MP17S is methylprednisolone-17-hemisuccinate; A is C6βOHmethylprednisolone, or 6β,11β,17α,21-tetrahydroxy-6α-methylpregna-1,4-diene-3,20-dione; A* is C6αOHmethylprednisolone, or 6α,11β,17α,21-tetrahydroxy-6β-methylpregna-1,4-diene-3,20-dione; Ared is C6βOHmethylprednisone, or 6β,11β,17α,21-tetrahydroxy-6α-methylpregna-1,4-diene-3,11,20-trione; A*red is C6αOHmethylprednisone, or 6α,11β,17α,21-tetrahydroxy-6β-methylpregna-1,4-diene-3,11,20-trione; B is C6βOHC20αOHmethylprednisolone, or 6β,11β,17α,20α-tetrahydroxy-6α-methylpregna-1,4-diene-3-one; E is C6βOHC20βOHmethylprednisolone, or 6α,11β,17α,20α-tetrahydroxy-6β-methylpregna-1,4-diene-3-one; C is C20βOHmethylprednisolone, or 11β,17α,20,2-tetrahydroxy-6α-methylpregna-1,4-diene-3-one; D is C20carboxymethylprednisolone, or 11β,17α-dihydroxy-20carboxy-6α-methylpregna-1,4-diene-3-one; D is C6βOHC20carboxymethylprednisolone, or 6β,11β,17α-trihydroxy-20carboxy-6α-methylpregna-1,4-diene-3-one; F is methylprednisolone glucuronide; X is C21COOHmethylprednisolone, or 11β,17α-dihydroxy-21-carboxy-6α-methylpregna-1,4-diene-3,20-dione; MP17SH* and MP21SH* are minor metabolites (Figure 2), of molecular weight 475, containing one nitrogen atom, possibly conjugates of methylprednisolone or MPS.

Isolation and full chemical analysis must be used to identify the structures of these two minor metabolites in the urine.

Literature survey of metabolism

Many attempts have been made to determine the metabolic pathways of methylprednisolone; all have resulted in tentative metabolite identification. GC–MS analysis of methylprednisolone in urine from man revealed unchanged drug and the 11-keto (prednisones) and 20-hydroxy metabolites together with 6 or 7 dehydro analogues of these compounds (Rodchenkov et al 1987). Ebling et al (1984, 1985) were unable to detect methylprednisone in plasma from man. No methylprednisone was shown in their pharmacokinetic curves. They subsequently reported the existence of an oxidation–reduction equilibrium of the 11-keto group in rabbits and man (Ebling et al 1985). The equilibrium also occurs in rats (Haughey & Jusko 1992), as a result of the enzyme 11β-hydroxysteroid dehydrogenase (Bush et al 1968). A similar species-dependent equilibrium exists at the C20-ketohydroxy group (Haughey & Jusko 1992).

Grey et al (1956) and Lawson et al (1992) showed that 20α- and 20β-hydroxymethylpred-nisolones were present in urine from man, in quantities similar to that of methylprednisolone. In equine urine, the metabolites were the 20α- and 20β-hydroxy metabolites of both methylprednisolone and methylprednisone (Gallicano et al 1985).

Metabolism of the synthetic progestin [^{14}C]megestrol acetate resulted in oxidation at

position 2 (2-hydroxymegestrol), oxidation of the 6-methyl group (6-hydroxymethylmegestrol), 2,6-dihydroxymegestrol, and their glucuronide conjugates (Cooper & Kellie 1967). The C2 position was vulnerable to oxidation because there was no unsaturated bond. Oxidation of an unsaturated bond of stanozolol gives 3′-hydroxystanozolol (Schänzer 1996; Schänzer et al 1996).

Prednisone and prednisolone are oxidized (6–10%) at the 6 position to form 6β-hydroxyprednisolone (Frey & Frey 1982, 1983).

Direct measurement of steroid sulphate and glucuronides revealed that glucuronidation and sulphation occur only at a free alcohol group (Cooper & Kellie 1967; Bowers & Borts 1996; Bowers & Sanaullah 1996; Schänzer et al 1996), as in the 17β-glucuronide of testosterone and the 17α-glucuronide of epitestosterone. Cole et al (1987) and Poon et al (1992) reported the glucuronide group of steroids to be at the 3 position. Reduction of the C20 carbonyl group results in the stereoisomers C20α- and C20β-hydroxymethylprednisolones.

Conclusion

In this study metabolites of methylprednisolone were separated and detected by gradient-elution HPLC combined with mass spectrometry. Because of the high pulse dose of 1 g methylprednisolone hemisuccinate, the concentrations in urine were high. We first isolated the compounds present at the highest concentrations and suggested structural formulae. LC–MS analysis furnished the total picture of parent drug, prodrugs and their respective metabolites. 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, oxidation of the C11 hydroxy group to form prednisone derivatives, in competition with or in addition to the oxidation at the C6 position, resulting in rapid elimination and a short $t_{\frac{1}{2}}$ of 1 h.

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