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Mass spectrometric and high-performance liquid chromatographic studies of medroxyprogesterone acetate metabolites in human plasma

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ABSTRACT

Medroxyprogesterone acetate (MPA) treatment has been shown to exert several beneficial effects in cancer patients. It has been suggested that such effects are due in part to the metabolites derived from MPA *in vivo*. The first results are reported on the identification of 2α-hydroxy- and 21-hydroxy-MPA, 20-dihydro-MPA, 17α-acetoxy-2α,3β-dihydroxy-6α-methylpregn-1,4-dien-20-one and two X,21-dihydroxy-MPAs, one of them presumably being 6α-hydroxymethyl-21-hydroxy-MPA, in patient's plasma by high-performance liquid chromatographic (HPLC), gas chromatographic–mass spectrometric and NMR methods. Additionally, the presence of other metabolites such as di- and tetrahydro-MPAs and 6,21-dihydroxy-MPA, found in urine and other samples, was demonstrated in plasma. For routine clinical examinations an HPLC method is described for determination of, *e.g.*, the unreduced MPA metabolite group in Sep-Pak-ODS column extracts of patients' plasma.

INTRODUCTION

Medroxyprogesterone acetate (MPA) (17α-acetoxy-6α-methylpregn-4-ene-3,20-dione, Fig. 1) was first synthesized in 1958 [1,2] and subsequently used in fertility control and, in low doses, in hormonal cancer therapy [3]. Since 1978, high-dose MPA therapy (1–5 g/day) has been shown to increase its effectiveness in the management of metastatic carcinoma [4], especially in cases where plasma MPA levels exceeded 100 ng/ml [5]. The observed relatively low MPA levels in patients' plasma may, in general, be due to extensive metabolism of MPA. How-

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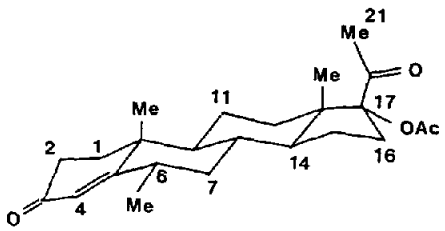

Fig. 1. Stereochemical structure of MPA. Me = Methyl.

ever, the individual variations in the steady-state MPA levels may be assumed to be due to differing enzymatic conversion capacities of the patients. Further, it is not yet clear whether the observed endocrine, cytotoxic, myeloprotective, anabolic, anxiolytic and analgetic effects [5,6] of the drug can really be attributed to the action of MPA itself or to that of MPA metabolites.

MPA metabolite studies date back to the early 1960s: 6β,21-dihydroxy-MPA and its 21-acetate isomer were isolated from urine [7,8], the latter being an artifact of the isolation procedure [9]. Ring A reduction was reported to occur by the action of human faecal and rat caecal bacteria [10] and some di- and tetrahydro metabolites and 17-deacetylated 6β,21-dihydroxy-MPA were isolated from urine [9].

As regards their presence in plasma, apart from a few suggestions, to our knowledge there are no published reports available. It was therefore considered of interest to develop some methods capable of detecting such substances in plasma. In earlier studies, direct radioimmunoassay (RIA) of MPA gave an overestimation of MPA concentrations by about 10-fold in plasma compared with isotope dilution mass spectrometry (IDMS) [11], demonstrating the presence of interfering substances such as MPA glucuronides in addition to unconjugated metabolites [12].

Although glucuronides represent the major part of the MPA metabolites, being the end products of metabolism, biologically they are of minor significance. We therefore focused our attention on analysing the unconjugated metabolites.

This paper reports on the identification of MPA metabolites in plasma, using gas chromatographic (GC) or high-performance liquid chromatographic (HPLC) separation and structural analysis by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectrometry, and the development of an HPLC method for the determination, in plasma, of selected, potentially bioactive metabolites of MPA, using UV detection.

EXPERIMENTAL

Plasma and urine samples from long-term, high-dose MPA-treated patients

were the source material. Plasma samples (0.1–1 ml) were extracted twice with 40 volumes of diethyl ether; the organic solvent was evaporated and the residue dissolved in 80% methanol in water (100 μl); 30-μl aliquots were used for analytical HPLC.

Derivatization for MS studies

Methoximes (MO) were formed by adding 30 μl of 2% methoxylammonium chloride in pyridine (MOX; Pierce, Rockford, IL, U.S.A.) to the dried extract, the reaction time being 3 h at 56°C. Silylation was achieved by adding 30 μl of N-methyl-N-trimethylsilyltrifluoroacetamide containing 1% of trimethylchlorosilane (Macherey, Nagel & Co., Düren, F.R.G.) to the dried extract or methoximation product and reaction for 8–10 h at room temperature.

Gas chromatography–mass spectrometry

MS measurements were done using a Kratos MS 25 S instrument with a General Data DS 55 system and a Perkin-Elmer Sigma-3 gas chromatograph, equipped with a Grob-type solid-phase injector and a CP-Sil 5 column (20 m × 0.32 mm I.D.; WCOT-fused silica, film thickness 0.13 mm) (Chrompack, Middelburg, The Netherlands) directly connected to the ion source. The GC column was programmed from 160 to 275°C in 30 min. The MS conditions were 40 kPa helium, ion source temperature 220°C and electron energy 50 eV.

NMR spectrometry

For the stereochemical evaluation of the structures, UV and especially NMR spectra (in $CDCl_3$, 0.02–0.1 *M*) of some isolated metabolites were obtained using a Beckman DB spectrophotometer and a Jeol GX-400 NMR spectrometer, respectively. The structural changes in the metabolites were defined by comparison with the well known spectra of MPA [13].

HPLC

HPLC was carried out using a Beckman Gold system consisting of a Model 126 gradient pump and a Model 168 diode-array detector, spectral range 235–245 and 275–285 nm; routine detection at 238 (280) nm; Beckman RP-ODS (5 μm) columns (25 cm × 4.6 mm I.D. and 25 cm × 10 mm I.D.) were used, the eluent being aqueous acetonitrile with a 40–70% gradient in 20 min and the total elution time being 35 min.

Determination of MPA metabolites in plasma samples

The samples were extracted either with diethyl ether or using Sep-Pak-ODS cartridges (Waters Assoc., Milford, MA, U.S.A.). In the latter instance methanol was used as the eluent. As internal standards, independently measured MPA and, in some instances, for checking, additionally megestrol acetate (MA; 17α-acetoxy-6α-methylpregn-4,6-diene-3,20-dione) was added to the samples. In HPLC

the substances were detected at 238 nm (MPA and unreduced metabolites) and when MA was added as an additional standard they were also detected at 280 nm (MA and reduced MPA metabolites). Calculation was done by dividing the area of the unknown peak by the area of the standard (MPA or MA) peak and multiplying by a factor *F*. *F* takes into account the differences in molar absorptivity and the yield of the individual substances and was determined in a separate experiment, in which known amounts of previously purified metabolites at three levels (1/5, 1 and 5 ×) *versus* MPA were added to normal plasma, equilibrated for 1 h and then extracted by Sep-Pak columns. HPLC analysis was done as described above.

RESULTS

Fig. 2 shows the peaks of some metabolites identified in a typical GC–MS total ion current measurement on a plasma sample extract after methoximation and silylation. A typical HPLC run of the diethyl ether extract of a plasma sample is shown in Fig. 3. The positions of peaks of so-far identified steroids are indicated by numbers, the compounds being listed in Table I.

The characteristics of the UV spectrum and HPLC retention times (RT), the kind of derivative formation, the molecular ion and specific MS fragmentation patterns (see Table I) revealed the final or preliminary structures of the metabolites. The assessment of the structure in some instances was done by NMR after

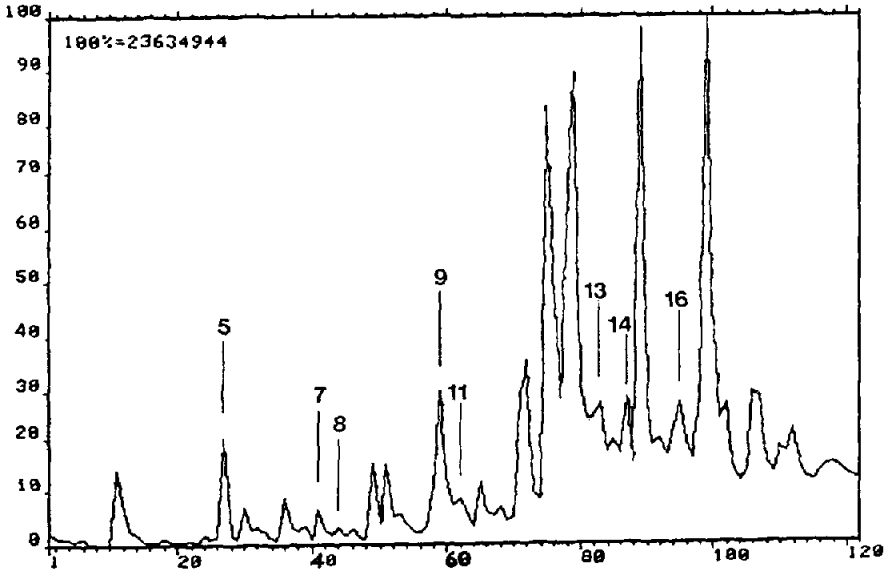

Fig. 2. GC, total ion current of a plasma extract after MO and TMSi derivatization. CP-Sil 5 column (20 m × 0.32 mm I.D.), programmed from 160 to 275°C in 30 min. Horizontal axis: time in min.

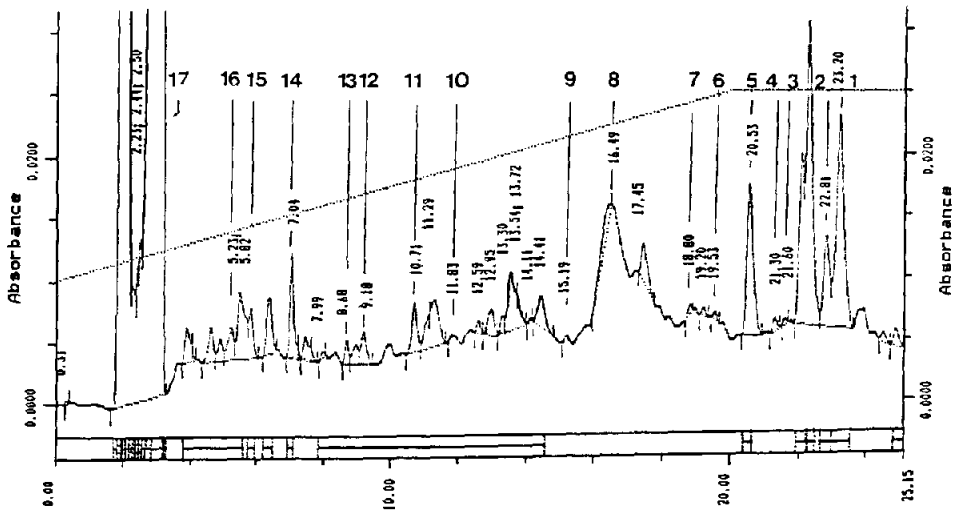

Fig. 3. HPLC profile of a plasma extract, recorded at 238 nm. RP-C_{18} column; elution with a gradient from 40 to 70% acetonitrile in water, in 20 min. Horizontal axis: time in min.

isolation of sufficient material using preparative HPLC of a plasma pool extract or of urine preparation [14].

Fig. 4a and b show the mass spectra of the identified 2α- and 21-hydroxy-MPAs: 17α-acetoxy-21-hydroxy-6α-methylpregn-4-ene-3,20-dione (21-hydroxy-MPA) as the 3-MO-21-OTMSi derivative with a molecular peak *m/z* 503 is characterized by the loss of the C_{20}–C_{21} side-chain and acetic acid [10,15], leading to the base peak at *m/z* 312 ($M^+ - 191$: TMSi–O–CH_2–CO plus CH_3COOH).

In 17α-acetoxy-2α-hydroxy-6α-methylpregn-4-ene-3,20-dione (2α-hydroxy-MPA) as the MO-OTMSi derivative the ring-system hydroxylation is demonstrated by the strong peaks at *m/z* 488 ($M^+ - CH_3$) and 400 ($M^+ - 103$; loss of the unchanged side-chains at C-17) and the base peak at *m/z* 310 {$M^+ - 103 - 90$ [$Si(CH_3)_3OH$]}. When compared with the parent substance MPA in the NMR spectrum, the position of the 2β-proton at 2.45 ppm is shifted to 3.2 ppm (d) and the coupling pattern is altered correspondingly.

Similarly, 17α-acetoxy-6β-hydroxy-6α-methylpregn-4-ene-3,20-dione (6β-hydroxy-MPA, M^+ 503 as MO-TMSi derivative) in NMR characteristically exhibits loss of the 6β-proton interaction (m) with the 6α-methyl group and the protons at C-7, as compared with MPA.

Fig. 4c shows the mass spectrum of 17α-acetoxy-2α,3β-dihydroxy-6α-methylpregn-4-en-20-one as the di-TMSi derivative with M^+ 548. Characteristic ions are at *m/z* 458 ($M - 90$) and 368, representing the loss of one and two silanols, respectively, and *m/z* 445 ($M^+ - 103$), showing the loss of the unchanged C_{20}–C_{21} side-chain together with the acetic acid. The proposed structure was further as-

TABLE I

METABOLITES OF MPA IDENTIFIED IN PLASMA

RP-C_{18} column; for conditions see Fig. 3. Absorption at 238 nm; X (ξ), (steric) structure as yet undefined.

No.	Metabolite	Retention time (min)	HPLC (238 nm)	Derivative		Mass spectrometry (*m/z*)	
				MO	TMSi	M^+	Base peak, fragment ions
1	Tetrahydro-MPA (3α,5β)	23.2	−	−	+	462	269($M^+ - 103 - 90$), 359($M^+ - 103$)
2	Tetrahydro-MPA (ξ)	22.5	−	−	+	462	269($M^+ - 103 - 90$), 359($M^+ - 103$)
3	Δ^4-Dihydro-MPA	21.6	−	+	−	417	314($M^+ - 103$), 402($M^+ - 60$)
4	3β-Dihydroxydihydro-MPA	21.0	−	−	+	460	370($M^+ - 90$), 357($M^+ - 103$)
5	MPA	20.5	+ +	+	−	415	415(M^+), 312($M^+ - 103$)
6	3α-Dihydroxydihydro-MPA	19.5	(−)	−	+	460	370($M^+ - 90$), 357($M^+ - 103$)
7	Tetrahydro-MPA (ξ)	18.8	−	−	+	462	269($M^+ - 103 - 90$), 359($M^+ - 103$)
8	Medroxyprogesterone (MP)	16.3	+ +	+ +		402	371($M^+ - 31$), 316($M^+ - 86$)
9	20ξ-Dihydro-MPA	15.2	+	+	+	489	312($M^+ - 177$)
10	17α-Acetoxy-2α,3β-dihydroxy-6α-methylpregn-4-en-20-one	11.8	(+)	−	+ +	548	458($M^+ - 90$), 445 ($M^+ - 86$)
11	6β-Hydroxy-MPA	10.7	+ +	+	+	503	310($M^+ - 103 - 90$), 400($M^+ - 90$)
12	17α-Acetoxy-X(6β?),20-dihydroxy-6α-methylpregn-1,4-dien-3-one	9.2	+ +	+	+	503	308($M^+ - 103 - 90$), 398($M^+ - 90$)
13	21-Hydroxy-MPA	8.7	+ +	+	+ +	503	312($M^+ - 191$), 488($M^+ - 15$)
14	6β,21-Dihydroxy-MPA	7.0	+ +	+	+ +	591	310($M^+ - 191$), 501($M^+ - 90$)
15	X,21-Dihydroxy-MPA	5.8	+	+	+ +	591	310($M^+ - 191$), 501($M^+ - 90$)
16	X,21-Dihydroxy-MPA (6β-CH_2OH?)	5.2	+ +	+	+ +	591	310($M^+ - 191$), 488($M^+ - 103$)

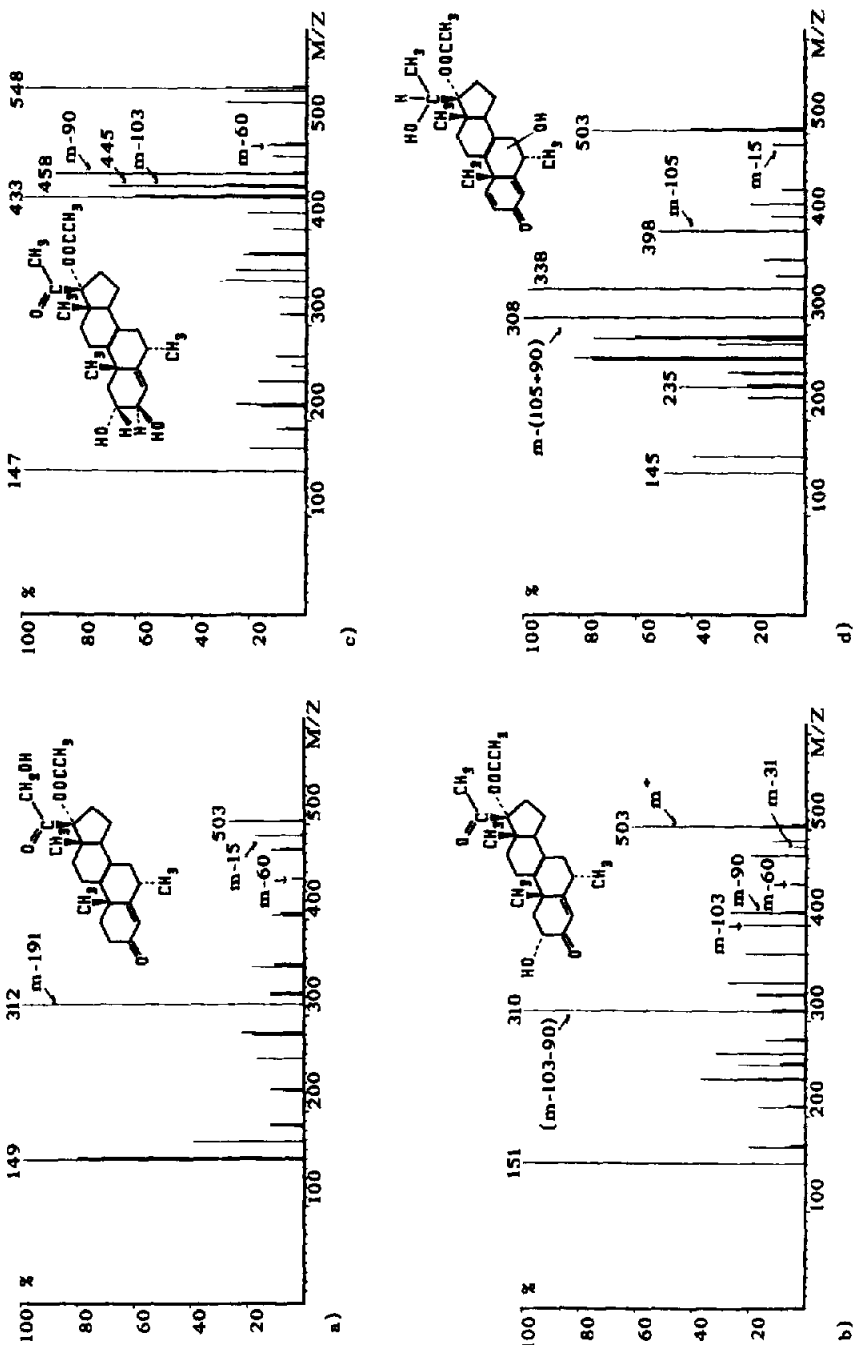

Fig. 4. Mass spectra (50 eV) of MPA metabolites: (a) 21-hydroxy-MPA × 1 MO × 1 TMSi; (b) 2α-hydroxy-MPA × 1 MO × 1 TMSi; (c) 17-acetoxy-2α,3β-dihydroxy-6α-methylpregn-4-en-20-one × 2 TMSi; (d) 17-acetoxy-20, X(6β-methyl?)-dihydroxy-6α-methylpregn-1,4-dien-3-one × 1 MO × 2 TMSi.

sessed by NMR, which exhibits the 2β-proton as a septet at 3.6 ppm, J (1α,2β) = 3.0 Hz, J (1β,2β) = 15.4 Hz and J (2β,3α) = 7.3 Hz.

Fig. 4d shows the mass spectrum of 17α-acetoxy-20ξ,X-dihydroxy-6α-methyl-pregn-1,4-dien-3-one. This mass spectrum is very similar to that of 6α-hydroxy-MPA with the same M^+ 503. However, there is no $M^+ - 103$ ion, but in its stead an equally intense $M^+ - 105$ ion (m/z 398) is seen. This indicates the presence of a reduced keto group of the 17β-side-chain, and the 20-hydroxy group remains unsilylated. The same M^+ 503 needs the presence of an additional double bond in the ring system. This can be assumed to be in conjugation with the 3-carbonyl group (MO-formation!) in the 1- or 6-position. The UV-spectrum, showing a strong absorption maximum at 243 nm, further confirms the 1,4-dien-3-one structure [16] as against the expected 288 nm for a 4,6-dien-3-one metabolite [15–17]. The base peak at m/z 308 ($M^+ - 105 - 90$) demonstrates further the presence of a silylated hydroxyl group in the ring system. It can only be assumed that the hydroxyl group is attached to the 6β-position and hence the great similarity with the spectrum of 6β-hydroxy-MPA. The final proof of this structure, however, has still to be examined by NMR.

The presence of at least three tetrahydro-MPAs, metabolites 1, 2 and 7 (see Fig. 3), was demonstrated using HPLC and MS. Metabolite 1, its structure being elucidated as the 3β,5α-isomer by NMR, in plasma is the predominant tetrahydro-MPA. The mass spectra of all isomers are closely related and differ only slightly in their fragment ion intensities.

The metabolite 6 reveals the 3β-hydroxy reduced MPA, its mass spectrum being in accordance with the literature [9]. The other dihydro-MPA (3) may be attributed to a Δ^4-reduced MPA, because the 3-keto group can still be derivatized to the methoxime.

In addition to ring A reduction, side-chain reduction is a further metabolic pathway, as evidenced by the presence of a 20-dihydro-MPA (metabolite 9) in plasma, demonstrated by the mass spectral peaks M^+ 489 and m/z 312, obtained from the 3-MO-20-OTMSi derivative by loss of the silylated side-chain and acetic acid ($M^+ - 177$).

The presence of at least three dihydroxy-MPAs could be demonstrated, metabolite 14 being identical with 6β,21-dihydroxy-MPA, known from the literature [8,9]. Both metabolites 15 and 16 in MS exhibit the same molecular peak at m/z 591 and the base peaks at m/z 310; this corresponds to the loss of the 21-hydroxy-TMSi side-chain and acetic acid. While substance 15 shows the loss of TMSi-OH (m/z 501), characteristic for a ring hydroxyl group (probably OH in the 2-position), metabolite 16 could be attributed to the 6α-hydroxymethyl-MPA derivative, as evidenced by the ion at m/z 488, showing the loss of a silylated hydroxymethyl group ($M^+ - 103$), similar to the observations of Adlercreutz *et al.* [15] for 6-hydroxymethyl-MA. To elucidate the final structure, however, it would be necessary to isolate ample amounts of the metabolites in question for NMR characterization.

Only some of these metabolites could be isolated in larger amounts for use as standards in the standardized HPLC method for setting up a profile of selected and possibly bioactive metabolites in plasma from individual patients. Plasma MPA levels were independently determined by radioimmunoassay (RIA) and HPLC methods [11,12] and served as internal standards for the HPLC run for calculating the metabolite levels on the basis of their peak areas; factors *F* were elaborated taking into account differences in their molar absorptivities at the recording wavelength of 238 nm. The *F* values were MPA 1, MP 0.62, 6β-hydroxy-MPA 4.2, 21-hydroxy-MPA 1.2, 6β,21-dihydroxy-MPA 4.0 and X,21-dihydroxy-MPA 2.1. Repeated measurements on standard preparations gave deviations of less than 5%, thus showing the method to be satisfactory.

As an example, Table II gives the profiles of the metabolites in plasma from a patient with progressive breast carcinoma during the course of high-dose MPA treatment. As can be seen, the plasma levels of MPA never exceeded 45 ng/ml, whereas the metabolites themselves showed a dramatic increase.

DISCUSSION

It is believed that extensive metabolism may be the reason for the low levels of MPA measured in plasma from cancer patients under chronic treatment with very high doses of MPA. Fukushima *et al.* [9] demonstrated that about 20% of MPA is excreted as 6α, 21-dihydroxy-MPA and hydrated MPA mainly as glucuronides. As regards the MPA metabolite levels in plasma, little is known from the literature. In this paper, we have described an HPLC method for the separation of unconjugated fractions of the MPA metabolites in diethyl ether- or Sep-Pak-extracted plasma samples. Using the Sep-Pak method, although glucuronides were also extracted, under the conditions used for HPLC, they could be eliminated as highly polar unbound fractions, and thus did not interfere with the resolution of other metabolites. The individual substances were identified, in part, by MPA RIA, which shows differential cross-reactivity to MPA metabolites but not to natural hormones [18]. Further identification was carried out by MS following derivatization of the individual substances. For the components which could not be identified by HPLC–RIA criteria, GC–MS using multiple ion detection proved to be an effective method.The nature of MPA metabolites could be distinguished by their kind of derivatization as methoximes and/or as trimethylsilyl ether (see Table I). For some components, because of biological matrix effects, this derivatization appeared to be inadequate. This problem could be overcome by increasing the actual mass of the ions by forming *tert.*-butylsilyl or heptafluorobutyl derivatives.

Using MS, MPA metabolites could be identified by the characteristic loss of the 17α- and 17β-side-chains [10]. The possible interference of endogenous hormones of adrenal origin in the identification of metabolites is excluded on the basis that both the molecular ions and the remaining fragment ions differ from

the natural hormones by the presence of the additional methyl group in the C-6 position and because it has been shown that MPA treatment effectively suppressed adrenal function [5,12]. Depending on their MS fragmentation pattern, the functional groups of the metabolites could be assigned either to the side-chain or to the ring system. Further assessment of the position of the functional groups could be made by NMR measurements.

This study has demonstrated that MPA is metabolized in various ways. The main routes seem to be ring A and/or side-chain reduction, loss of the acetyl group, hydroxylation preferentially in the 2-, 6- and 21-positions or a combination, resulting in numerous derivatives. Identification of metabolites 9, 10 and 12 suggests, for example, that hydroxylation in the 2-position can be followed either by a reduction of the 3-keto group giving rise to metabolite 10 and related compounds, or by dehydration leading to the 1,4-diene-3-keto structure of metabolite 12. This pattern can be further complicated by conjugation, predominantly with glucuronic acid [12,19].

Detection of metabolites at 238 nm in HPLC, described here, is limited to those with an unchanged Δ^4-3-keto structure and 1,4-diene compounds such as metabolite 12 (see Table I). Therefore, in some instances, detection was done additionally at 280 nm, which also allowed the measurement of internal standard MA.

The di- and tetrahydro components being the degradation products and the glucuronides being the end-products, one may assume that only the unconjugated metabolites identified and determined by the HPLC methods are of biological significance. The observed beneficial effects such as corticoid activity, weight gain

TABLE II

LEVELS OF MPA AND SOME METABOLITES IN PLASMA FROM A PATIENT AS DETERMINED BY HPLC

H_4-MPA = tetrahydro-MPA; MPA-Gluc = more polar metabolites and glucuronides.

Compound	Substance No.	Concentration (ng/ml)					
		Day 1	Day 3	Day 8	Day 30	Day 60	Day 97
MPA	5	2.2	30.7	43.7	42.5	15.7	18.1
MP	8	7	89	107	85	70	70
6α-OH-MPA	11	2	7	25	60	191	140
21-OH-MPA	13	12	23	18	60	107	94
6,21-Di-OH-MPA	14	11	33	91	103	252	170
X,21-Di-OH-MPA	16	–	13	38	88	151	180
H_4-MPA	1[a]	7	27	42	40	73	35
MPA-Gluc	17[b]	37	647	763	1015	1710	1200

[a] Determined by RIA.

[b] Determined by RIA as ng MPA equiv.

and pain relief [5,6,20] may be attributed to metabolites with a 21-hydroxy structure, which are more similar to endogenous cortisol. It should be noted, however, that 21-hydroxylation of MPA in this instance characteristically occurs in the liver, as shown in adrenalectomized, ovariectomized patients [8,21], and not necessarily in steroid-producing tissues. Further, hydration of ring A is slowed compared with the degradation of the natural hormones, because of the 6α-methyl group in MPA [22]. The extent of this reaction may be patient-dependent.

The described HPLC method enables one to draw a profile of selected metabolites during the course of treatment and to correlate it with patients' responses. In Table II, a non-responder to MPA is shown as an example. Whereas the plasma MPA levels were very low, the measured metabolites rose dramatically and the hydrated products and glucuronides as measured by RIA appeared to be predominant. In MPA responders, on the other hand, preliminary studies suggested that the low hydration and high hydroxylation reactions may correlate with high MPA levels (unpublished observations).

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