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ORIGINAL PAPER

Urinary profile of methylprednisolone acetate metabolites in patients following intra-articular and intramuscular administration

Alessia Panusa · Luca Regazzoni · Giancarlo Aldini · Marica Orioli · Arrigo Giombini · Paola Minghetti · Carlo Tranquilli · Marina Carini

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Abstract A study on urinary metabolites of methylprednisolone acetate (MPA) has been performed by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) in precursor ion scanning (PIS) and neutral loss (NL) modes. Patients suffering from joint inflammation have been treated with Depo-Medrol® (MPA marketed suspension, 40 mg) intra-articularly (IA) and after a wash-out period, intramuscularly (IM) at the same dose. Urine samples have been collected after both the administration routes. Metabolites were identified in PIS mode by setting the fragment ion at *m/z* 161 which is specific for MPA, methylprednisolone (MP), methylprednisolone hemisuccinate, and in NL mode by selecting the losses of 54, 72, 176 and 194 Da. The MP-related structure of each target ion detected in both the MS modes was then confirmed by MS/MS acquisitions, and by accurate mass experiments. By using this approach, 13 MPA metabolites (M1–M13) have been identified, nine already reported in the literature and four unknown and for which the chemical structures have been proposed. No differences in the metabolic pattern of MPA when administered IM or IA were observed. The relative abundances of metabolites compared with the internal standard (MP-D2) were monitored by multiple reaction monitoring analysis for 19 days after both the administration routes.

Keywords Methylprednisolone acetate · Metabolites · Intra-articular · Intramuscular · LC–ESI–MS/MS

Abbreviations

MP	Methylprednisolone
MPA	Methylprednisolone acetate
LC–ESI–MS/MS	Liquid chromatography–electrospray ionization–tandem mass spectrometry
LC–APCI–MS/MS	Liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry
PIS	Precursor ion scanning
NL	Neutral loss
MRM	Multiple reaction monitoring

Introduction

Methylprednisolone (MP) and its related pro-drugs are largely utilised to relieve inflammation (swelling, heat, redness and pain) and for the treatment of certain forms of arthritis, skin, blood, kidney, eye, thyroid, intestinal disorders (e.g. colitis), severe allergies and asthma. MP and its pro-drugs are commercially available for oral (OS) and parenteral use, in particular, methylprednisolone acetate

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A. Panusa (✉)
Dipartimento del Farmaco, Istituto Superiore di Sanità,
Viale Regina Elena 299,
00161 Rome, Italy
e-mail: alessia.panusa@iss.it

L. Regazzoni · G. Aldini · M. Orioli · P. Minghetti · M. Carini
Dipartimento di Scienze Farmaceutiche "P. Pratesi",
Universita' degli Studi di Milano,
20133 Milan, Italy

A. Giombini · C. Tranquilli
Istituto di Medicina e Scienza dello Sport del CONI,
Largo Piero Gabrielli 1,
00197 Rome, Italy

(MPA) is utilised in intra-articular (IA) and intramuscular (IM) administrations for the treatment of joint inflammation, while methylprednisolone hemisuccinate (MPHS) is mainly administered intravenously (IV).

In the "2010 Prohibited List" issued by the World Anti-Doping Agency [1], glucocorticosteroids are classified as substances prohibited in competition. In particular, they are prohibited when administered by OS, IV, IM or rectal routes, and their use requires a therapeutic use exemption (TUE) approval, i.e. a permission to use, for therapeutic purposes, the substance otherwise prohibited. On the contrary, a simple declaration of use must be completed by athletes if glucocorticosteroids are administered by IA, periarticular, peritendinous, epidural, intradermal and inhalation routes.

Topical preparations when used for auricular, buccal, dermatological (including iontophoresis/phonophoresis), gingival, nasal, ophthalmic and perianal disorders are not prohibited and require neither a TUE nor a declaration of use.

The different rules and restriction of glucocorticoid administration clearly depend on the systemic adsorption and distribution which is related to the administrations routes. However, in some cases, glucocorticoids also when administered locally can be adsorbed systemically thus to give false positive in the anti-doping control.

Hence, it is of great importance to identify metabolites whose concentrations are characteristic of one administration route, like the IA or the IM, thus to help in distinguishing the forbidden from the allowed use in anti-doping controls. For this purpose identification and characterization of metabolites related to different uses is mandatory.

Studies on the identification of MP metabolites after OS and IV administration routes in humans are available in literature. Metabolites of MP in human urine (0–6 h) have been previously detected by GC–MS after OS administration of MP [2] and by high-performance liquid chromatography (HPLC) after IV administration of MPHS [3, 4]. Later, a LC–APCI–MS/MS study on patients receiving a high-dose pulse therapy of MPHS (IV administration) has been performed and 14 urine metabolites in the 0–24 h have been identified [5, 6]. Only more recently, a LC–ESI–MS/MS method with a precursor ion scanning (PIS) approach was applied to the analysis of urine of a healthy volunteer. Urine were collected up to 72 h after the OS administration of MP and the metabolites were identified by selecting the fragment ions at *m/z* 237 and 91 and by the neutral loss (NL), of 76 Da [7]. The unchanged drug, together with four metabolites already described in literature, has been detected.

PIS approach in liquid chromatography–electrospray ionization–tandem mass spectrometry is particularly useful for investigating groups of compounds which generate common product ions after fragmentation. For this reason, it is valuable for the rapid confirmation of targeted compounds or for the non-targeted detection of compounds with a common moiety [8–10] in complex matrices. Also the NL mode is valuable for the discovery of unknown substances and their metabolites [11]. Only few studies have been published about the LC–MS/MS analysis of corticosteroids and their metabolites by using these approaches [7, 12, 13], and a new and sensitive PIS method specific for the detection of MPA, MP and their metabolites, utilising the *m/z* 161 as diagnostic product ion, has been recently proposed [14].

The goal of this study was to identify MPA metabolites in human urine after MPA administration by IA and IM routes in order to investigate the presence of a metabolic marker specifically linked to one administration route. Metabolites identification was carried out by LC–ESI–MS/MS in both PIS and NL modes. Moreover, the relative abundance of MPA and its metabolites compared to an internal standard was monitored in the 19 days following the administrations.

Materials and methods

Chemicals and reagents

MP (CAS 83-43-2, ≥98%), MPHS (CAS 2375-03-3, ≥98%) and all the other reagents were of analytical grade and were purchased by Sigma-Aldrich Chemical Co. (Milan, Italy). MPA (CAS 53-36-1, ≥97%) was purchased from Chemos GmbH (Regenstauf, Germany). Deuterated MP (1,4-pregnadien-6α-methyl-11β,17,21-triol-3,20-dione-21,21-D_2) (CAS 83-43-2, 99%, internal standard (IS)) was purchased from Chemical Research 2000 S.r.l. (Rome, Italy) (Fig. 1). Depo-Medrol® was from Pfizer (Borgo S. Michele, Latina Italy). HPLC-grade and analytical-grade organic solvents were also purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade water was prepared with a Milli-Q (Millipore, Vimodrone, Italy) water purification system.

LC–ESI–MS/MS instrumental conditions

A HPLC system (Surveyor, ThermoFinnigan Italia, Milan, Italy), equipped with a quaternary pump, a surveyor UV–vis diode array programmable detector 6000 LP, a vacuum degasser, a column compartment and an autosampler, both with thermostat control, was used for solvent and sample delivery. Separations were carried out on a C-12 Sinergy MAX RP column (150×2 mm I.D., particle size 4 μm; Chemtek Analytica, Anzola Emilia, Italy) protected by a C12-RP guard column (4×2 mm I.D., particle size 4 μm).

The mobile phase consisted of water containing 0.01% of formic acid (*A*) and acetonitrile containing 0.01% formic

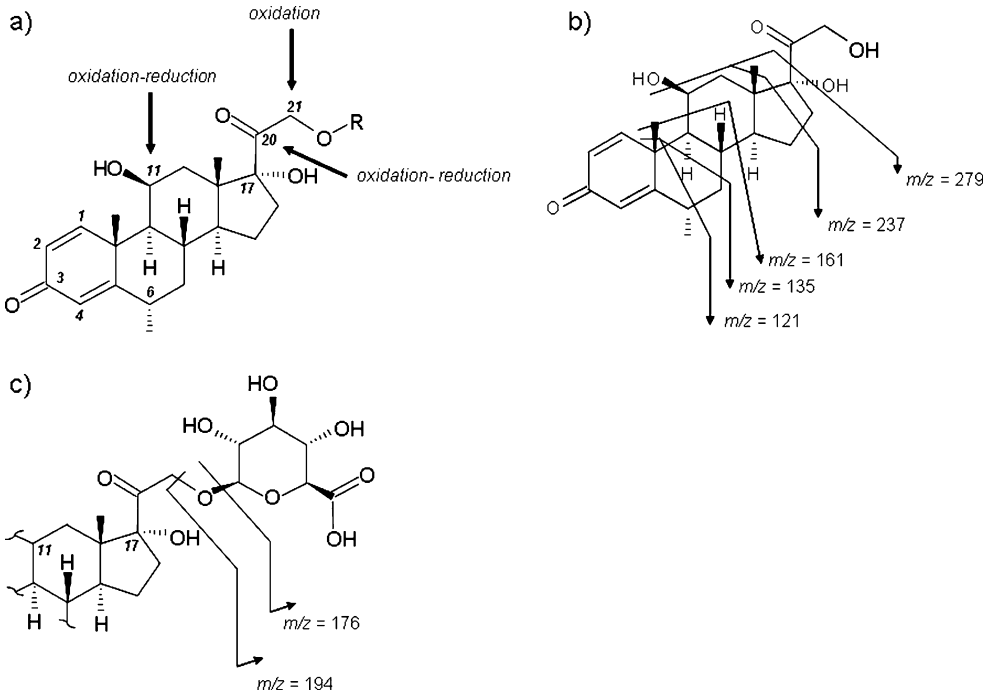

Fig. 1 Molecular structures of: **a** methylprednisolone $[MP+H]^+$ *m/z*=375.2 (R=H); methylprednisolone acetate $[MPA+H]^+$ *m/z*=417.2 ($R=C(O)CH_3$); methylprednisolone hemisuccinate $[MPHS+H]^+$ *m/z*=475.2 ($R=C(O)CH_2CH_2CO_2H$) and main metabolic sites; fragment ions used in **b** PIS and **c** NL analysis and structural assignment

acid (*B*), delivered at a flow rate of 0.2 mL min^{-1}. The elution gradient was from *A*/*B* (95:5 *v*/*v*) to *A*/*B* (35:65 *v*/*v*) in 60 min. Then the proportion of *B* was linearly increased to 100% in 5 min, followed by a further 5 min isocratic elution (washing period). The eluents were then restored to their original proportions *A*/*B* (95:5 *v*/*v*), and re-equilibrated for 10 min before the next injection (injection volume, 20 μL). The samples rack and the column compartment were maintained at 4 and 30 °C, respectively.

A TSQ Quantum triple-quadrupole mass spectrometer and a LTQ Orbitrap™ XL mass spectrometer, for accurate mass measurement (Thermo Scientific, Milan, Italy) both with an electrospray ionization (ESI) source were used for mass detection and analysis. Mass spectrometric analyses were carried out in positive ionization mode. ESI interface parameters were set as follows: probe middle (*B*) position; capillary temperature 300 °C; spray voltage 5.0 kV. Nitrogen was used as nebulising gas at the following pressure: sheath gas 40 and 36 psi for TSQ and LTQ, respectively; auxiliary gas 5 arbitrary units (a.u.); sweep 3 a.u. To optimise the MS conditions and for tuning, the mobile phase and diluted stock solutions of analytes (flow rate, 5 μL min^{-1}) were mixed through a T-connection, with the mobile phase flow rate kept at 0.2 mL min^{-1}; the intensities of the $[M+H]^+$ protonated molecules were monitored and adjusted and maximized using the Quantum Tune Master software.

Infusion analyses of standards were carried out with the *A*/*B* (50:50 *v*/*v*) mobile phase composition, maintained at a flow rate of 0.2 mL min^{-1}. Standard solutions (10 μg mL^{-1}) were infused post-column through a T-connection (flow rate, 5 μL min^{-1}).

PIS analyses with triple-quadrupole mass spectrometer were based on the product ion at *m/z* 161.0. The Q1 quadrupole was scanned from *m/z* 100 to 600.

NL analyses were based on the loss of 194, 176, 54 and 72 Da. The Q1 quadrupole was scanned from *m/z* 200 to 600 for NL 54 and 72, from 230 to 600 for NL 194 and from 220 to 600 for NL 176; for both PIS and NL analysis scan time 1 s with resolution of 0.70 *m/z* units; Q3 was set to transmit only ions above mentioned with resolution 0.70 *m/z*; spectra were acquired using the optimised collision energies of 25 and 20 eV for PIS and NL analysis, respectively.

In product ion scan analysis, MS/MS acquisitions on the ions of interest were done at collision energies from 5 to 30 eV. The Q1 quadrupole was set to transmit only the ions of interest, with resolution of 0.70 *m/z*; Q3 was scanned from *m/z* 100 to the mass of interest in 0.8 s (scan time) with a resolution of *m/z* 0.70 units.

For multiple reaction monitoring (MRM) analysis the appropriate product ions were selected in product ion analysis. The parameters influencing these transitions were optimised as follows: argon gas pressure in the collision Q2, 1.0 mbar; peak full width at half-maximum (FWHM), 0.70 *m/z* at Q1 and Q3; scan width for all MRM channels, 1 *m/z*; scan time 0.2 s$scan^{-1}$.

The following transitions were selected for quantitative analysis at 25 eV collision energy if not differently specified:

M1 *m/z* 373.2→184.9+251.2
M2 *m/z* 375.2→279.0+161.0 (collision energy, 22 eV)
M3 *m/z* 377.1→135.0+161.0+253.0

IS *m/z* 377.2→359.2+253.1 (collision energy: 20 eV)
M4 *m/z* 389.2→227.1+212.0
M5 *m/z* 389.2→161.0+185.0 (collision energy: 23 eV)
M6, M7, M8 *m/z* 391.2→149.0+280.0
M9 *m/z* 549.2→355.2+327.1
M10, M11, M12 *m/z* 551.2→279.0+357.1
M13 *m/z* 553.2→281.2+341.1

No interferences from endogenous compounds were found in blank urine samples.

Accurate mass measurements were performed with the data-dependent scan mode enabled.

$[M+H]^+$ molecular ions, from 14 phthalates and siloxanes including dibutylphthalate (plasticizer, *m/z* 279.159086); bis (2-ethylhexyl)phthalate (*m/z* 391.284286) and dodecamethylcyclohexasiloxane (*m/z* 445.120025) were used for real-time internal mass calibration.

Tandem mass spectra were recorded in profile mode for the three most intense ions in the following conditions: isolation width 3 *m/z*; normalized collision energy 25 a.u. HCD (higher energy collisional dissociation); minimum signal threshold 5×10^4; scan range, *m/z* 100–600 in full-scan mode with full-scan injection waveforms enabled; AGC target setting, 2×10^5; maximum inject time, 1,000 ms; scan time, 0.35 s $scan^{-1}$ (7,500 resolving power at *m/z* 400, FWHM). Dynamic exclusion was enabled (repeat count, three; repeat duration, 30 s; exclusion-list size, 50; exclusion duration, 45 s; relative exclusion mass width, 5 ppm). Charge-state screening and monoisotopic precursor selection were enabled; multi-charged and unassigned charged ions were rejected.

Data processing was performed by the Xcalibur 2.0.7 version software.

Patients treatment

Exclusion criteria included previous total knee arthroplasty, history of cardiovascular disease, uncontrolled hypertension, rheumatologic or neurological disease, surgery and/or physiotherapy to this joint in the past 6 months. Inclusion criteria included patients with history of pain in one knee lasting no more than 1 year with independent ambulation. Three patients between 44 and 60 years of age, complaining of knee pain, who did not take corticosteroids in the previous 6 months, were firstly treated with Depo-Medrol® (MPA suspension, 40 mg) IA, and after a wash-out of 3–5 months, with the same dose of Depo-Medrol® IM. Informed consent was obtained from patients before treatment. The investigation was conformed to the principles outlined in the Declaration of Helsinki.

First urine samples in the morning (40 mL) were collected in conical tubes before and 1, 2, 3, 6, 7, 9, 11, 13, 15, 17, 19 and 21 days after the administration, and stored at −20 °C until assayed.

Sample preparation

Four different sample preparation methods were tested and in all cases urine were preventively centrifuged at 13,150 g for 10 min. In the first method (P1), 400 μL of urine were simply filtered on Microcon YM30 centrifugal filter devices (Millipore, Milan, Italy) and an aliquot of 20 μL was injected into the column. In the second method (P2), acetonitrile (1,200 μL) was added to 400 μL of urine and kept on ice for 1 h. After centrifugation at 13,150 g for 10 min, the supernatant was evaporated under nitrogen at room temperature. In the other two methods a preconcentration step was accomplished; in the third method (P3) urine samples (2 mL) were liophylised and reconstituted with 200 μL of water. In the last method (P4), urine samples (1 mL) were dried in a speedVac system, and then reconstituted with 200 μL of water. Then acetonitrile (600 μL) was added (P3 and P4), and samples kept on ice for 1 h. After treatment, samples were centrifuged at 13,150 g for 10 min and the supernatant was evaporated under nitrogen at room temperature (P3) or in a SpeedVac system (P4). In P2, P3, P4 samples were then reconstituted with 200 μL of water/acetonitrile/formic acid (95:5:0.01 *v/v*), vortex-mixed for 30 s filtered, (0.45 μL, Millipore, Milan, Italy) and transferred to vials.

For quantitative analysis in MRM mode IS (10 μL) was added at the concentration of 100 ng mL^{-1} and urine samples prepared as in P4 method. An aliquot of 20 μL was injected into the column.

Results and discussion

Optimization of LC and MS conditions

The LC–ESI–MS/MS conditions previously reported to detect MPA, and its metabolites in rat urine [14] have been applied in the present study. The retention times (RTs) of MP and MPA (standards), spiked in the blank matrix were 31.8 and 40.9 min, respectively. The full-scan mass spectra of MP and MPA (*m/z* 50–500) in MS infusion experiments, showed the protonated molecular ions $[M+H]^+$ as base peaks at *m/z* 375.2 and 417.2, together with the corresponding $[M+H+CH_3CN]^+$ adducts at *m/z* 416.2 and 458.2 respectively (data not shown).

In previous MS/MS experiments [14], the $[M+H]^+$ ions of MP and MPA were fragmented at different collision energies (from 5 to 40 eV, 5-eV steps) to determine the most abundant product ion(s) suitable for PIS analysis. The product ion at *m/z* 161 was selected as the most abundant

for both MP and MPA at the collision energy of 25 eV. A similar MS/MS fragmentation study was also performed in the present study for MPHS ($[M+H]^+$ *m/z*=475.2) which is a pro-drug of MP, and the results confirmed the *m/z* 161 product ion as the base peak from collision energy of 25 eV. The *m/z* 161 product ion was previously [15] assigned to a portion of the molecule bearing the methyl group on C6, thus involving the *A* and *B* rings of the molecule (Fig. 1b). MS/MS product ions and their relative abundances at different collision energies are summarized in ESM1 in the Electronic supplementary material and the chemical structures of MP, MPA and MPHS are displayed in Fig. 1a.

NL of 54 and 72 Da correspond to the loss of three and four molecules of water, respectively, and are characteristic of MPA, MP and of their metabolites [5]. The glucuronic metabolites of MP show a characteristic NL of 176 and 194 Da, corresponding to the loss of [Gluc] and [Gluc+H_2O], respectively [5]. Optimisation of the collision energy for NL was then performed at different collision energies (from 5 to 40 eV, 5-eV steps), and the value of 20 eV was set in all the subsequent NL experiments.

Matrix effect on different sample preparation

PIS and NL analyses were then carried out to compare four different sample preparation methods in terms of peak sensitivity (matrix effect). Between P1 and P2, the latter was found more sensitive, due to the sample concentration (1:2) not carried out in P1. Between P3 and P4, despite the sample concentration was higher in P3, the best sensitivity, evaluated on the basis of total ion current (TIC), was attained in P4 (increase of 10%) and this was clearly due to a reduced matrix effect. Also P4 was more sensitive than P2 showing an average increase in the TIC of 30%. As a result, the P4 method was utilised in the following analyses.

Under the optimised LC and MS conditions, the method was found to be specific since no interferences from endogenous compounds were observed in human urine, like in rat urine, as demonstrated by the representative PIS and NL traces relative to blank urine and blank human urine spiked with 10 ng mL^{-1} concentration of MP and MPA (data not shown). As previously determined in rat urine, a LOD of 5 ng mL^{-1} and a LLOQ of 10 ng mL^{-1} were found also in human urine.

Method application to the detection of metabolites: precursor ion scan profiling

PIS analyses were performed on urine of patients collected before (0 h) and after the IA and the IM administration of Depo-Medrol®.

The LC–ESI–MS/MS chromatograms (PIS mode) of urine collected from patients treated IA and IM were characterized by several peaks absent in the urine samples collected before the drug administration. The MW of the detected species were then confirmed by setting the extracted ion currents corresponding to the identified precursor ions and are following reported: *m/z* 373 (RT= 32.0 min), *m/z* 375 (RT=28.0 min), *m/z* 377 (RT= 27.5 min), *m/z* 389 (RT=18.9, 40.5 min), *m/z* 391 (RT= 15.5, 16.9, 31.0 min), *m/z* 549 (RT=30.1 min), *m/z* 551 (RT=25.9, 27.3, 30.6 min), *m/z* 553 (RT=27.8 min) (Fig. 2).

As shown in Fig. 2. other peaks, like the ones at RT= 41 min (extracted ions at *m/z* 375, 377 and 391), which were absent in the urine samples collected before the drug administration were detected. They were then discarded on the basis of subsequent MS/MS experiments which did not show fragmentation pattern characteristic of MP metabolites.

Neutral loss profiling

All the peaks detected in PIS mode were then confirmed by NL scan as shown in Table 1. Also in this case, the MW of the detected species were confirmed by setting the extracted ion currents of the previously identified precursor ions. In particular, by setting the NL at 54 and 72, eight of 13 metabolites were identified. In the sequential loss of four molecules of water, the three hydroxyl groups of the molecules are involved (on C11, C17 and C21; Fig. 1) together with the carbonyl group on C20.

The remaining metabolites were detected by setting the NL at 176 and 194, to search for the presence of a glucuronic moiety in the metabolites. In this case only M9, M10, M11, M12 and M13 (with $[M+H]^+$ at *m/z*=549, 551, 551, 551 and 513, respectively), already detected in PIS mode, were confirmed by NL. An example of the peak matching between PIS an NL modes, is shown in ESM2 in the Electronic supplementary material, where the extracted ions of the glucuronic metabolites at *m/z* 551, 549 and 553, recorded in PIS and NL modes are displayed.

Characterization of metabolites

Each metabolite was characterized on the basis of its retention time, molecular weight, and chemical formula. With the use of high resolution mass spectrometry it was possible to unequivocally determine the molecular weight and the elementary composition of each metabolite and the results are displayed in Table 2. With MS/MS experiments in product ion scanning mode it was possible to confirm the MP-related structure of all metabolites by the presence of the *m/z* 161 fragment ion and on the basis of the fragmentation pattern. All MS/MS acquisitions in positive ion mode were taken at a collision energy of 25 eV.

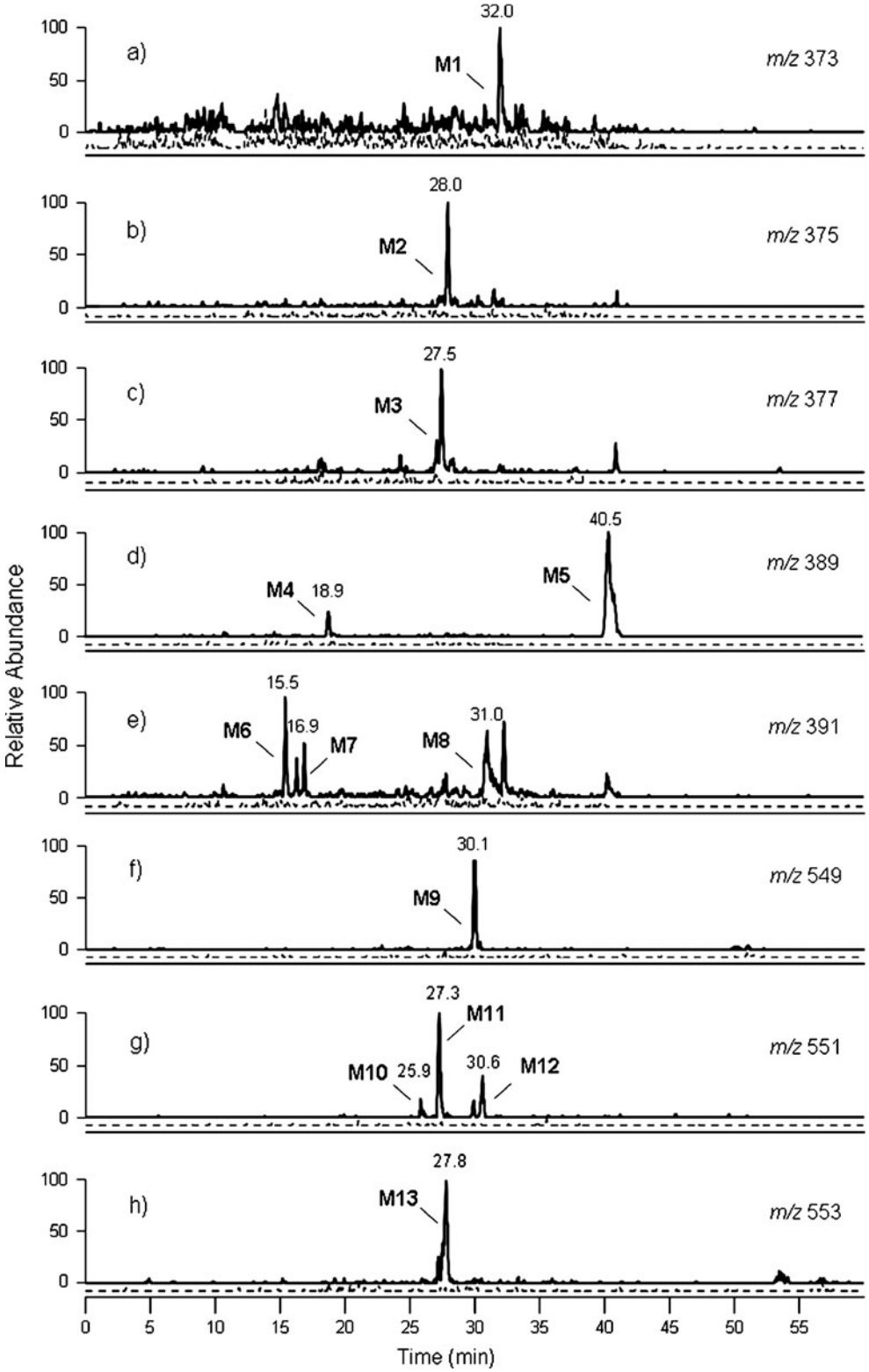

Fig. 2 LC-ESI-MS/MS chromatograms of patients before (*dotted lines*) and after (1st day, *thick lines*), the IM administration of Depo-Medrol®; PIS *m/z* 161 traces at 25 eV collision energy. Extracted ion chromatogram: *m/z* 373 (**a**); *m/z* 375 (**b**); *m/z* 377 (**c**); *m/z* 389 (**d**); *m/z* 391 (**e**); *m/z* 549 (**f**); *m/z* 551 (**g**); *m/z* 553 (**h**)

Free metabolites (M1–M8)

Characteristic fragment ions of MP at *m/z*=121, 135, 161, 237 and 279, and common to its metabolites, had been previously assigned [12, 15] (Fig. 1b). Their simultaneous presence in MS/MS spectra permitted the unequivocal identification of MP metabolites. Also the sequential loss of three or four molecules of water was diagnostic of a MP-related structure. The relative abundance of product ions depends on collision energy utilised in MS/MS

Table 1 *m/z* of protonated metabolites, retention time (RT) and mode of detection

Metabolite	*m/z*	RT (min)	PIS	NL 54	NL 72	NL 176	NL 194
M1	373	32.0	Y	Y	N	–	–
M2	375	28.0	Y	Y	N	–	–
M3	377	27.5	Y	Y	Y	–	–
M4	389	18.9	Y	Y	N	–	–
M5	389	40.5	Y	Y	N	–	–
M6	391	15.5	Y	Y	N	–	–
M7	391	16.9	Y	Y	Y	–	–
M8	391	31.0	Y	Y	N	–	–
M9	549	30.1	Y	–	–	Y	Y
M10	551	25.9	Y	–	–	Y	Y
M11	551	27.3	Y	–	–	Y	Y
M12	551	30.6	Y	–	–	Y	Y
M13	553	27.8	Y	–	–	Y	Y
MP	375	31.8	Y	Y	Y	–	–

experiment. The following reported are fragmentation pattern at 25 eV.

M1 is assigned to the oxidized form of MP (methylprednisone, Fig. 3a) where the oxidation of the hydroxyl group in C11 has taken place. This structure has previously been suggested [2, 7, 15], and it is confirmed by the MS/MS spectrum (Fig. 4a), showing diagnostic product ions, like the loss of 46 Da [$CO+H_2O$] (*m/z*=327) which is also observed in prednisone (characterized by the same structure of methylprenisone except the methyl group at C6) but not in MP nor in prednisolone. The sequential loss of three molecules of water (at *m/z*=355, 337 and 319, respectively) is also visible in the spectrum. This metabolite was not detected in rat urine [14].

M2 (Fig. 4b) is isobaric to MP but it is characterized by a different RT (Table 1) and a different relative abundance of the product ions compared to MP standard (ESM3 in the Electronic supplementary material). In fact, the base peak in MP spectra at 25 eV (*m/z*=161) is different from the base peak in M2 (*m/z*=279). In addition, the fragmentation pattern of the MS/MS spectrum of M2 and of the isobaric metabolite observed in rat urine, are identical. As previously observed in rat urine, M2 can be assigned to the reduced-oxidized form of MP, where the hydroxyl group on C11 has been oxidized to a carbonyl group, and the carbonyl group on C20 has been reduced to a hydroxyl group, with the OH group in C20 in the S or in the R configurations (Fig. 3b, c). Losses of two and three

Table 2 Molecular formula and accurate mass of protonated M1–M13

Metabolite	Precursor ion	Molecular formula	Measured mass	Theoretical mass	Δppm
M1	$[M+H]^+$	$C_{22}H_{29}O_5$	373.19940	373.2009505	−4.1
M2	$[M+H]^+$	$C_{22}H_{31}O_5$	375.21637	375.2166005	−0.6
M3	$[M+H]^+$	$C_{22}H_{33}O_5$	377.23225	377.2322506	0.0
M4	$[M+H]^+$	$C_{22}H_{29}O_6$	389.19635	389.1958651	1.2
M5	$[M+H]^+$	$C_{22}H_{29}O_6$	389.19534	389.1958651	−1.3
M6	$[M+H]^+$	$C_{22}H_{31}O_6$	391.21158	391.2115151	0.2
M7	$[M+H]^+$	$C_{22}H_{31}O_6$	391.21170	391.2115151	0.5
M8	$[M+H]^+$	$C_{22}H_{31}O_6$	391.21152	391.2115151	0.0
M9	$[M+H]^+$	$C_{28}H_{37}O_{11}$	549.23328	549.2330383	−0.2
M10	$[M+H]^+$	$C_{28}H_{39}O_{11}$	551.24691	551.2486884	−3.2
M11	$[M+H]^+$	$C_{28}H_{39}O_{11}$	551.24615	551.2486884	−4.6
M12	$[M+H]^+$	$C_{28}H_{39}O_{11}$	551.25037	551.2486884	3.0
M13	$[M+H]^+$	$C_{28}H_{41}O_{11}$	553.26434	553.2643384	0.0
MP	$[M+H]^+$	$C_{22}H_{31}O_5$	375.21708	375.2166005	1.3

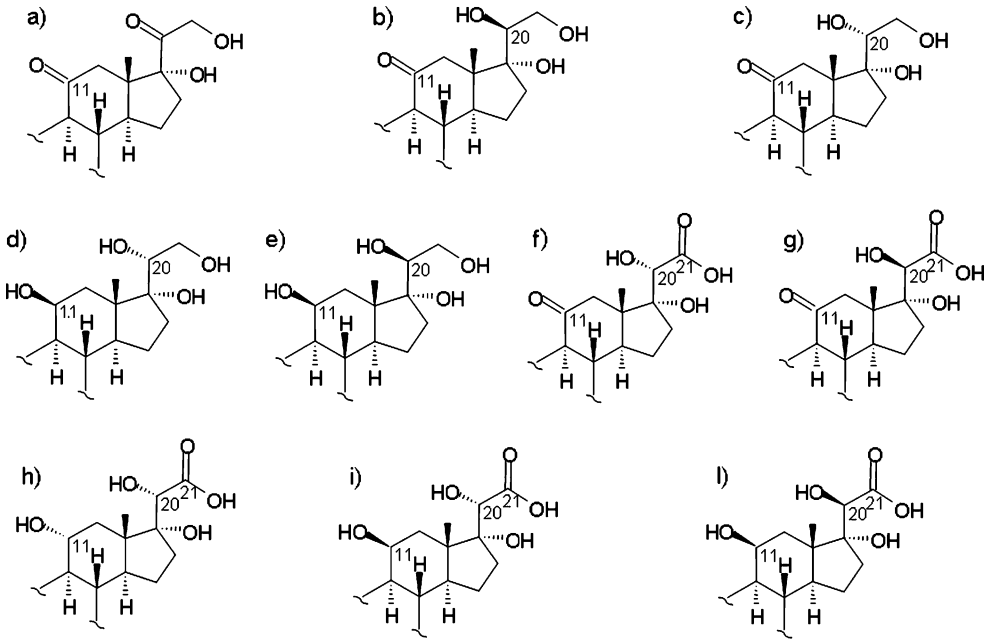

Fig. 3 Chemical structures assigned to M1 (**a**), M2 (**b**, **c**), M3 (**d**, **e**), M4 (**f**), M5 (**g**), M6 and M7 (**h**, **i**) and M8 (**l**)

molecules of water at *m/z*=339 and 321 are present with very low abundances at 25 eV.

M3 was attributed to the reduced form of MP (C20-OH in S or in R configuration) (Fig. 3d, e) as previously suggested [5, 7]. The MS/MS spectrum is shown in Fig. 4c. Losses of water are evident at *m/z*=359, 341, 323 and 305. In this case a diagnostic fragment ion of M3 at *m/z*=281 is present with a higher relative abundance than the fragment ion at *m/z*=279. Also in this case the RT and the fragmentation pattern were the same of the isobaric metabolite observed in rat urine [14].

Both the isobaric ions at *m/z* 389 eluting at 18.9 and 40.5 min were confirmed as MP metabolites (M4 and M5) by MS/MS spectra (Fig. 4d, e) by the presence of main diagnostic fragment ions which differ in relative abundance. In particular, while M5 shows *m/z*=161 as base peak, like MP, M4 shows the *m/z*=227 fragment ion. In addition, sequential losses of water are less evident than other metabolites at 25 eV. In a recent paper [7], only one metabolite at *m/z* 389 was detected. Taking into account the retention time of the metabolite detected in that study, it can be assumed it corresponds to M4. The lack of the second (M5) can be due to the different administration route of MP (OS) or to the different sensitivity of the two methods. In another study [5], although the administration route (IV), the chromatographic conditions and the sample preparation were different, also two metabolites at *m/z* 389 were observed and probably they correspond to M4 and M5. The chemical structures already proposed bear a hydroxyl function in C6 in both α and β positions and the oxidation of the hydroxyl group on C11 to the corresponding carbonyl function. As already explained [14], this attribution is questionable, because these proposed metabolites would not have been detected by PIS because the *m/z* 161 product ion is characteristic of a portion of the molecule bearing the methyl group on C6, thus involving the *A* and *B* rings of the molecule. We propose that the two metabolites are characterized by a carboxyl function in C21, a hydroxyl function on C20 and a carbonyl function on C11 (Fig. 3f, g). The significant difference between the RT of the two C20-hydroxy isomers, can be due to the strong H-bond that only the more hydrophobic isomer can stabilize between the C20 (R) hydroxyl group and the C21-carboxyl group. M4 and M5 were not observed in rats.

Three species characterized by $[M+H]^+$ at *m/z* 391 (M6, M7 and M8) were detected. In all MS/MS spectra (Fig. 4f–h), the sequential losses of water are evident at *m/z*=373, 355, 337 and 319. M7 shows the fragment ion $[M+H-4H_2O]^+$ at *m/z*=319 with a relative abundance higher than 50% which permitted the identification also with NL 72, like M3. Only one metabolite with a MW of 390 Da was detected by Pozo et al. [7] while Vree et al. [5] also detected three isobaric species with MW 390. For one metabolite, it was assigned [5] a structure arising from: (a) reduction of the carbonyl function at C20 and simultaneous oxidation of the hydroxyl function on C21 to the corresponding carboxyl group; while to the other two metabolites the (b) oxidation of C6 by the addition of a hydroxyl group, in both α and β positions. Pozo et al. [7] proposed that hydroxylation can take place elsewhere in the molecule, maybe on C16.

Based on the reason above explained, the structure of metabolites detected by PIS *m/z* 161 cannot be explained on the basis of pathway b) but are consistent with pathways a). Moreover, M6 and M7 have RTs (15.5 and 16.9 min, respectively) which are significantly different from M8 (RT,

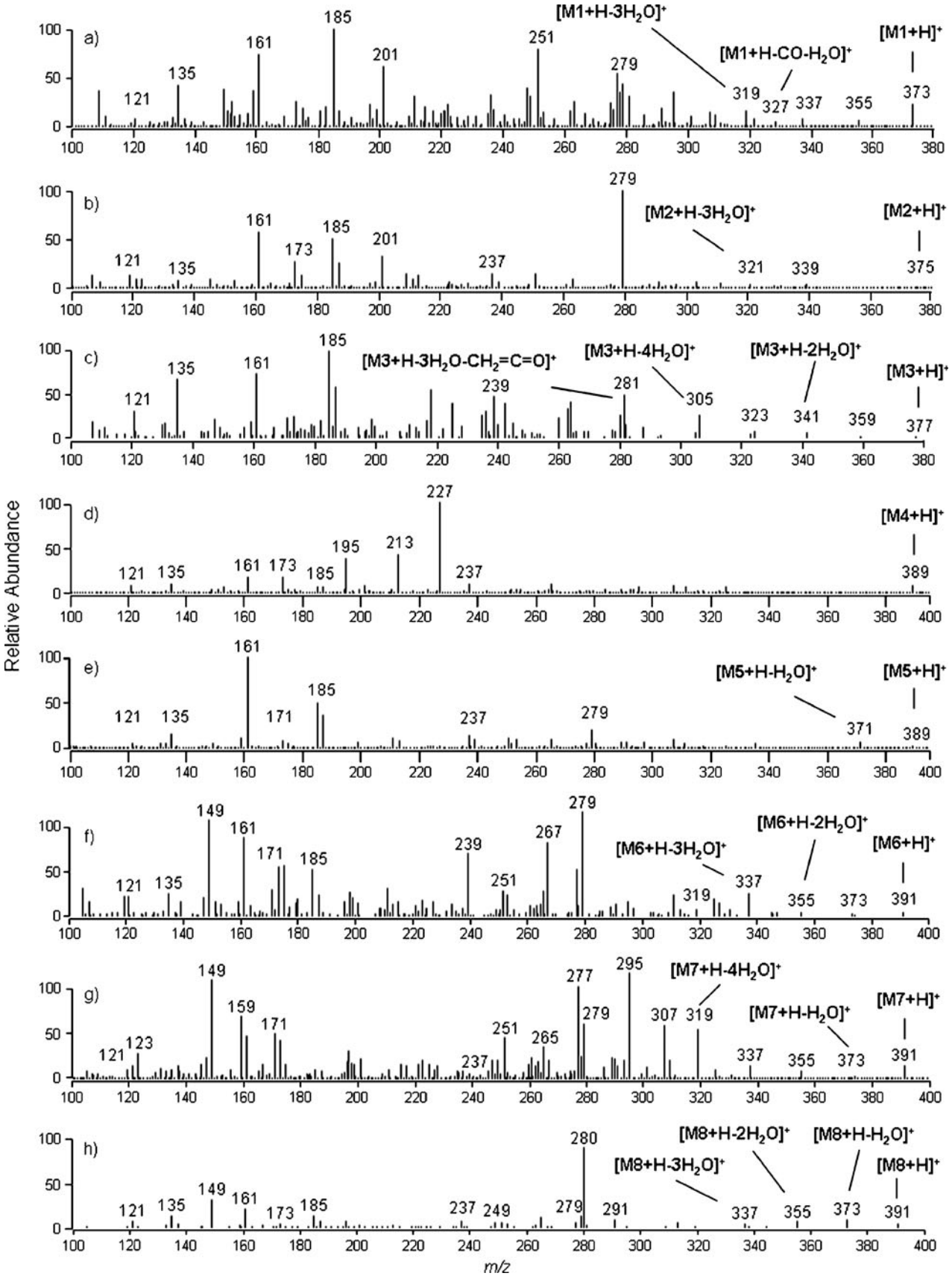

Fig. 4 LC-ESI-MS/MS full spectra (*m/z* 100–400), positive ion mode at collision energy 25 eV of protonated M1–M8

31.0 min) and this can be explained by a significant different lipophylicity. As above mentioned for M4 and M5, the difference in RT between the two C20OH isomers is probably due to the strong H-bond that only the more hydrophobic isomer can stabilize between the C20(R)OH group and the carboxyl group. For this reason, to M8 can be assigned the structure with the C20OH group in R configuration that stabilizes both the hydroxyl group and the C21 carboxyl group (Fig. 3l), while to M6 and M7 the structure with C20(S)OH. M6 and M7 are diasterisomers with C11OH in α and β position (Fig. 3h, i). MS/MS spectra of M6, M7 and M8 are displayed in Fig. 4f–h. These metabolites have the same MW of two metabolites detected in rat urine [14]. Also, the difference between RT of M6, M7 and M8 is comparable to those observed in rats.

Conjugated metabolites (M9–M13)

The detection of MP and other corticosteroids in biological matrix is usually performed after an enzymatic hydrolysis to deconjugate glucuronic metabolites, and for this reason only limited information on MP glucoronic metabolites are reported in the literature. To our knowledge, only one paper reports the detection of a single MP glucuronide by LC–APCI–MS/MS after an IV high-dose (1 g/day) of MPHS [5]. Antignac et al. [16] detected a glucuronide of dexamethasone in cow urine in PIS mode based on fragment ions characteristic of the glucuronic moiety (*m/z* 75, 85, 113, 175). By using the PIS and NL methods here proposed, five different MP glucuronic metabolites were detected which were then confirmed by accurate mass experiments and MS/MS analyses and in particular: three isobaric metabolites at *m/z* 551 (M10–M12) and other two at *m/z* 549 (M9) and 553 (M13), respectively. The MS/MS spectra of the glucuronic species are displayed in Fig. 5 and are characterized by the diagnostic fragment ions of MP and of the glucuronic moiety. In particular, the MS/MS spectra of all the four metabolites are characterized by the protonated molecular ion $[M+H]^+$, and by fragments arising from the loss of glucuronic acid $[M+H\text{-}Gluc]^+$, the loss of glucuronic acid and water, and by the sequential losses of four molecules of water. The fragment ion of the glucuronic moiety $[Gluc+H–2H_2O–CO]^+$ at *m/z* 113 is present for M11 at 25 eV collision energy. The base peak for M10 and M11 is *m/z* 279, while for M12 and M13 are *m/z* 375 and 281, respectively.

The three conjugated species with $[M+H]^+$ at *m/z* 551 can reasonably be the glucuronides of M2 or glucuronides of methylprednisolone.

The chemical structure of MP glucuronide detected by LC–APCI–MS/MS [5] and investigated by IR and NMR spectrometry [17] was not assigned. Also Antignac et al. [16] could not assign the structure of the dexamethasone glucuronide without uncertainty. If the C21 position appears the more probable glucuronidation site from a sterical point of view, also the other two sites in the molecule are available for this phase II metabolic pathway (C11OH and C17OH).

The species showing the $[M+H]^+$ at *m/z* 549 (M9) is probably the C21 glucuronide of M1. As shown in Fig. 5a, also for this metabolite the MS/MS spectrum is diagnostic since characterized by the protonated molecular ion $[M9+H]^+$, and by the fragment ions arising from the loss of glucuronic acid moiety $[M9+H–Gluc]^+$ at *m/z*=373, as well as by the loss of glucuronic acid and two molecules of water $[M9+H–Gluc–2H_2O]^+$ at *m/z*=337 and the characteristic fragment ion of M1 at *m/z*=327.

M13 can be assigned to the C21 glucuronide of M3. Also in this case, the MS/MS spectrum is diagnostic since characterized by the molecular ion $[M13+H]^+$ at *m/z*=553, and fragments arising from the loss of glucuronic acid $[M13+H–Gluc]^+$ at *m/z*=377, the loss of glucuronic acid and two molecules of water $[M13+H–Gluc–2H_2O]^+$ and the characteristic fragment ion of M3 at *m/z*=281(Fig. 5e).

The qualitative profile obtained on urine samples at the following observation times after the IM and the IA administrations were substantially the same as no differences in number of metabolites was observed between the IA and IM use. In conclusion, no difference in metabolic pathway was observed.

Relative abundance of metabolites

MRM analyses of each detected metabolite were carried out by selecting the most suitable transition ions on the basis of the MS/MS spectra as above reported.

Absolute quantification of MP metabolites was not carried out as synthetic standards and the corresponding deuterated standards were not available.

Therefore, a relative quantification was determined for each metabolite by calculating the peak area ratio to the IS. The relative abundance of each metabolite was monitored for 19 days following both administrations.

As expected, for each metabolite, higher values of area ratios were observed in the IM than in IA use although in the days following the fifth, the situation was in most cases reversed; it can be easily explained by a higher clearance in the IM use and a longer residence time of the drug in the IA administration.

The only exceptions of this behaviour were M3 and M5 which showed always higher or equal values of area ratio in the IA than IM use.

In the IM administration and for all the patients, the highest relative abundance was observed for M11 (the main metabolite) reaching 1.9 as mean maximum value of area

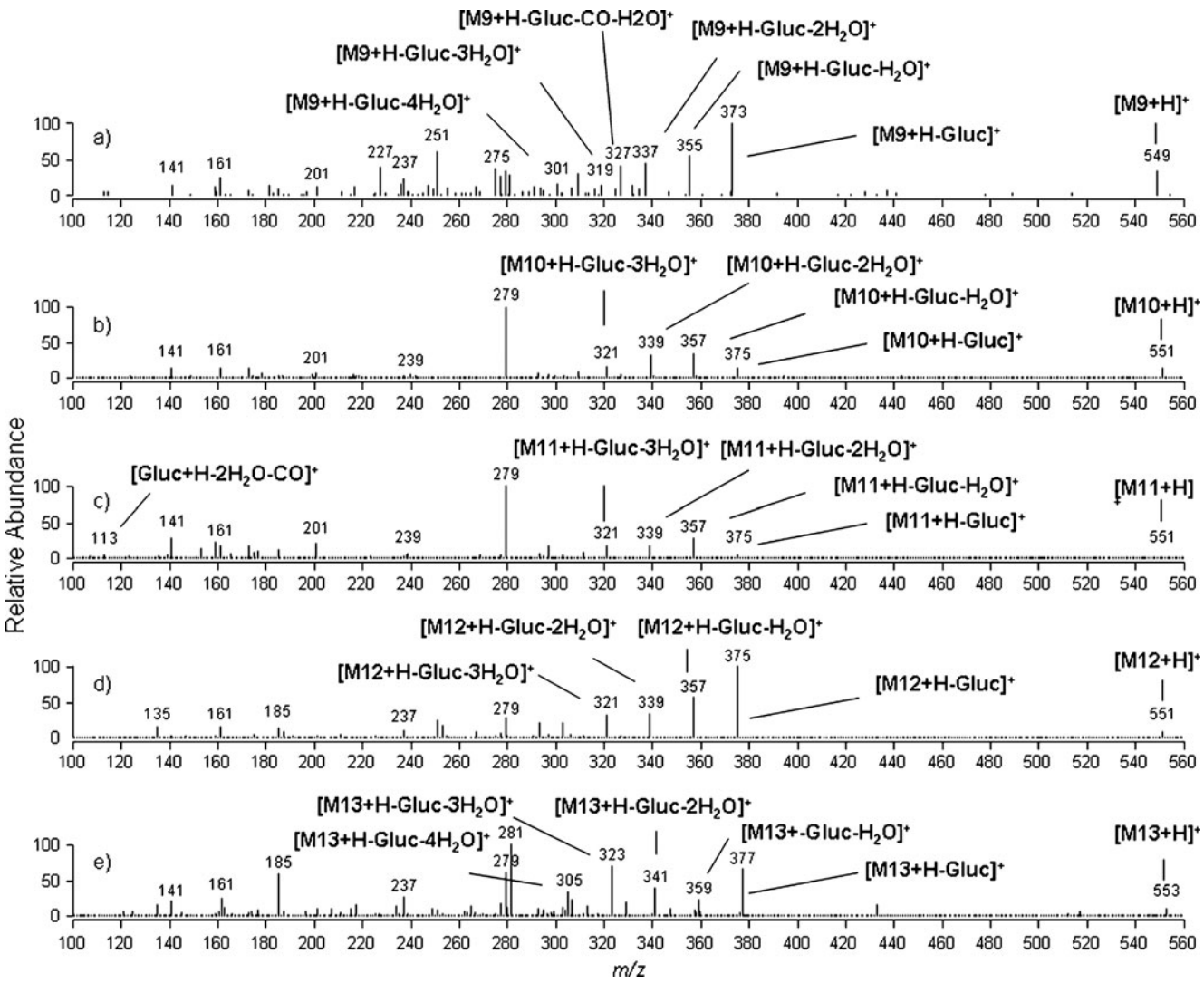

Fig. 5 LC-ESI-MS/MS full spectra (*m/z* 100–560), positive ion mode at collision energy 25 eV of glucuronic metabolites with $[M+H]^+$ at *m/z* 549 (**a**); *m/z* 551 (**b**–**d**); *m/z* 553 (**e**)

ratio, while in the IA administration M5 was the main metabolite, reaching the maximum mean value of 0.96. The mean maximum values for all metabolites are shown in Table 3.

Although M2 and MP have different molecular structures, they are isobaric and we could estimate the urine concentration of M2 on the basis of the peak area ratio of MP standard to IS.

For M2, after the IM use, maximum concentration levels were observed between the first and the ninth day after the administration for the three patients. The mean maximum value (Table 3) corresponds to 50.5±3.5 ng mL^{-1}. After the IA use maximum levels were observed between the third and the fifth day after the administration. The mean maximum level (Table 3) corresponds to 36.9± 23.9 ng mL^{-1}. In this case, a higher variability among patients was observed.

On the basis of these preliminary results, the concentration of M2 in urine cannot be utilised to differentiate the IA from the IM use. In fact, although the concentration of M2 after the IM use is always higher than IA the first day after administration, the situation can be reversed in the following days and IA concentrations as high as IM concentrations have been observed.

As previously stated, no significant differences in the quantitative profile of metabolites were observed, and consequently it was not possible to find a marker specific for the IA or the IM use.

In another attempt to find a way to discriminate the two routes of administration, we started a study intended to identify whether the abundances of selected couples of metabolites is reversed in the two administration routes at all the time points. We found that for a same patient the area ratio of M5 to M11 at the same data time was >1 after

Table 3 Mean values (three patients) of maximum ratio values for M1–M13

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13
IM	0.19±0.03	0.48±0.03	0.35±0.02	0.35±0.09	0.73±0.15	0.14±0.03	0.05±0.01	0.11±0.02	0.65±0.14	0.36±0.04	1.90±0.70	0.55±0.12	0.48±0.16
IA	0.19±0.02	0.38±0.20	0.55±0.13	0.19±0.03	0.96±0.31	0.07±0.02	0.01±0.02	0.10±0.01	0.46±0.11	0.32±0.05	0.80±0.66	0.26±0.07	0.09±0.02

the IA use and <1 after IM use. If this result was confirmed for all patients it could be selected as a marker to distinguish the two administration routes and it can be utilised for anti-doping purpose.

Unfortunately these preliminary interesting results were observed only for two of the three patients. Consequently, a greater number of patients needs to be monitored before drawing any conclusion.

Conclusions

LC–ESI–MS/MS methods in PIS and NL modes have been applied to detect metabolites of MPA in urine of patients suffering from joint inflammation treated with Depo-Medrol® (a marketed MPA formulation) intra-articularly and intramuscularly.

The combined PIS/NL approach allowed to unequivocally detect 13 (M1–M13) different metabolites some of them not previously reported. For some others, new chemical structures were proposed. Among these an isobaric metabolite of MP (M2), previously observed in rat urine, was detected for the first time in human urine. Five different glucuronic species (only one had been identified by LC–MS/MS before) have also been detected. Three different isobaric (MW=550) species can reasonably arise from conjugation of each of the three hydroxyl groups present on the M2 molecule or they can be glucuronides of methylprednisolone. The other two, reasonably involving conjugation of the metabolite M1 and M3, have never been reported before. The molecular masses of all metabolites were confirmed by accurate mass measurements and MS/MS experiments. All metabolites were monitored up to 19 days after both the administrations and their relative abundance (peak area relative to IS) was calculated. Although the successful results obtained with these methods it was not possible to distinguish metabolites whose concentrations can be specifically linked to one of the two administration routes, i.e. it was not possible to find a marker specific for the IA or the IM use. For this reason, a study intended to identify whether the abundances of selected couples of metabolites is reversed in the two administration routes at all the time points has been initiated. It could be chosen as a marker able to distinguish the two administration routes and it could be utilised for anti-doping purpose.

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