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A rapid and sensitive LC–ESI-MS/MS method for detection and quantitation of methylprednisolone and methylprednisolone acetate in rat plasma after intra-articular administration

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ABSTRACT

A rapid, sensitive and specific liquid chromatography–electrospray-tandem mass spectrometric (LC–ESI-MS/MS) method for the simultaneous detection and quantitation of methylprednisolone acetate (MPA) and methylprednisolone (MP) in rat plasma, using a triple-stage quadrupole, has been developed and validated.

MP-D_2 was used as internal standard (IS) and acetonitrile was added to plasma samples for protein precipitation. After extraction with dichloromethane, the analytes were separated on a C-12 reversed-phase column by isocratic elution (6 min at a flow rate 0.2 mL min^{-1}) with water containing 0.01% formic acid (A) and acetonitrile (B) (50:50, v/v). Quantitation was performed in positive ion multiple reaction monitoring (MRM) mode by applying the following precursor-to-product ion transitions: MPA m/z 417 → 135 + 161 + 253; MP m/z 375 → 135 + 161 + 253; IS m/z 377 → 135 + 161 + 253.

The method, validated over the concentration range 6–600 ng mL^{-1}, has been shown to meet the current requirements of bioananalytical validation, providing satisfactory results in terms of linearity, recovery, intra-day and inter-day precision and accuracy. The lower limit of quantitation (LLOQ) was 6 ng mL^{-1} for both the analytes (0.080 and 0.072 pmol injected for MP and MPA, respectively). The method was successfully applied to monitor the plasma levels of MPA and MP following intra-articular (IA) injections of a low MPA (Depo-Medrol®) dose in rats.

1. Introduction

The World Anti-Doping Agency (WADA) has issued a list of prohibited substances including, among others, anabolic agents, hormones, stimulants, and peptide hormones. If the medication that an athlete is required to take to treat an illness or condition falls under the Prohibited List, a Therapeutic Use Exemption (TUE) may give that athlete the authorization to take the needed medicine for therapeutic purposes (TUE Guidelines are a model of best practice developed as part of the WADA Program) [1]. All glucocorticosteroids are prohibited in competition when administered by oral, rectal, intravenous or intramuscular routes, while their administration by non-systemic routes, such as the intra-articular (IA) injection, requires only an abbreviated TUE. IA corticosteroid injections are frequently used in the management of osteoarthritis (OA) and rheumatoid arthritis (RA), or musculoskeletal disorders to reduce pain and inflammation, facilitate motion and function [2,3]. Even though most evidence of efficacy is confined to the knee joint [4], IA corticosteroids have also been evaluated at other joints, including elbow, shoulder, wrist, hip, heel, metacarpophalangeal and metatarsophalangeal [5]. The therapeutic benefits obtained from intra-articular corticosteroid injections for OA, when used in the hip and the carpometacarpal joint of the thumb have been reported [6,7] and also in the wrist of patients with RA [8].

The use of non-systemic administration of corticosteroids for local treatment should ensure low systemic levels with minor side effects. This is the rational the WADA TUE is based on. In addition, the chronic nature of the disease requires the development of drug delivery systems allowing a longer duration of action, since it is desirable to limit the number of IA injections, and to achieve a sustained release over a period of several weeks [9].

For this reason, and in view of both clinical and anti-doping requirements, the technological research is currently focussed on the development of new delivery systems for local treatment. Among corticosteroids suitable for non-systemic administration, methylprednisolone acetate (MPA) is one of the most frequently

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utilised active substances for the treatment of joint pain, and it is commercially available as injectable suspension. Dextran–methylprednisolone succinate (MPS) has been used as a prodrug of methylprednisolone (MP) for the selective delivery of the drug, thus to improve efficacy and to reduce side effects [10]. Biodegradable microspheres for non-systemic administration and for the sustained release of MP have been also proposed [11], with the final aim to minimise the systemic concentration of the active substance and to localise it to the site of action over a prolonged period of time. Suitable analytical methods able to quantify low expected plasma concentrations of MP, following local administration of newly developed formulations, are then mandatory to compare their systemic levels with those attained following administration of conventional formulations. A great number of HPLC [12–14] and LC–MS [15–20] methods for the detection and quantitation of MP and MPA are available in literature, but only LC–MS/MS is adequately specific and sensitive for this purpose. In particular, the use of multiple reaction monitoring (MRM) mode is the most appropriate, due to the specificity and sensitivity attained working with parent-to-product ion transitions. LC–APCI-MS/MS in MRM mode has been applied to human urine for detection and quantitation of MP and other glucocorticosteroids, with a claimed lower limit of quantitation (LLOQ) of 10 ng mL^{-1} [17]. However, the method was not validated at this concentration value, precision and accuracy data being provided only for the 100 ng mL^{-1} concentration level. Recently, two screening methods using ESI ionization in MRM mode for detection of synthetic glucocorticoids, stimulants, anti-oestrogens and synthetic anabolic steroids have been described [21,22]. MP has been detected in human urine in MRM mode attaining a limit of detection (LOD) of 15 ng mL^{-1} [22]. Hence, no studies have been so far proposed for the simultaneous determination of MP and MPA by a suitably validated LC–MS/MS method. On this basis, the objective of this work was to develop and to validate a new LC–ESI-MS/MS method for the simultaneous detection and quantitation of MP and MPA in rat plasma. By using the MRM mode, a low percentage of formic acid in the mobile phase and by selecting three MRM transitions for each analyte, lower LOD values than those found in literature, together with high specificity have been obtained. The method has been then successfully applied for monitoring MP and MPA concentrations in rat plasma after IA injections of a MPA suspension commercially available (Depo-Medrol®).

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents were of analytical-grade and purchased from Sigma–Aldrich Chemical Co. (Milan, 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 water purification system. MPA was purchased from Chemos GmbH (Regenstauf, Germany), and MP from Sigma–Aldrich (Milan, Italy). Deuterated MP (1,4-pregnadien-6α-methyl-11β,17α,21-triol-3,20-dione-21,21-D_2) was purchased from Chemical Research 2000 S.r.l. (Rome, Italy) and Depo-Medrol® from Pfizer.

2.2. LC–MS/MS instrumentation and conditions

The 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 a Surveyor autosampler (200 vials capacity) both with thermostat control, was used for solvent and sample delivery. Separation was performed on a C-12 Sinergy MAX RP column (150 mm, 2 mm i.d.; particle size 4 μm) (Chemtek Analytica, Anzola Emilia, Italy) protected by a C12-RP guard column (4 mm, 2 mm i.d.; 4 μm). The mobile phase (isocratic elution) consisted of 50% water containing 0.01% formic acid (A) and 50% acetonitrile (B) delivered at a flow rate of 0.2 mL min^{-1} (injection volume 5 μL). The A:B was maintained for 6 min, and then the concentration of solvent B linearly increased to 100% B in 7 min, followed by a further 15 min isocratic elution (washing period). The composition of the eluent was then restored to the original conditions A:B (50:50, v/v), and re-equilibrated for 10 min before the following injection. The samples rack and the column compartment were maintained at 4 and 30 °C, respectively.

A TSQ quantum triple-quadrupole mass spectrometer (ThermoFinnigan Italia, Milan, Italy) with electrospray ionization (ESI) source was used for mass detection and analysis. Mass spectrometric analyses were performed in positive ion-mode. ESI interface parameters were set as follows: middle position; capillary temperature 300 °C; spray voltage 5.0 kV. Nitrogen was used as nebulizing gas at the following pressure: sheath gas 40 psi; auxiliary gas 5 a.u. MS conditions and tuning were performed by mixing through a T-connection the mobile phase-diluted stock solutions of analytes (flow rate 5 μL min^{-1}), with the mobile phase maintained at a flow rate of 0.2 mL min^{-1}; the intensities of the $[M+H]^+$ ions were monitored and adjusted to the maximum by using the Quantum Tune Master software.

Ionization efficiency was monitored in MRM mode at 5.00 kV multiplier voltage, by selecting for each analyte the MRM transitions of $[M+H]^+$ precursor ions–product ions and optimising the relative collision energies by the Quantum Tune Master software. The appropriate product ions were selected in infusion experiments. The selected MRM transitions were used for quantitative 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 rate (dwell time), 0.2 s/scan. Data processing was performed by the Xcalibur 2.0 version software.

2.3. Animals treatment, sample collection and preparation

Male Wistar rats (Charles River Laboratories, Calco, Lecco, Italy; initial weight of 250–275 g) were housed in a conditioned environment (22 ± 1 °C, 55 ± 5% relative humidity, 12-h light/12-h dark cycle), with free access to standard laboratory chow and tap water. The animals were maintained in compliance with the policy on animal care expressed in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). A period of at least 5 days was allowed for animals to acclimatise before any experimental manipulations were undertaken.

Rats were anesthetized with 60 mg kg^{-1} i.p. thiopentone sodium before the IA injection of a suitably diluted Depo-Medrol® solution (50 μL/each posterior leg). The dilution was accomplished to inject such a small dose (1 mg MP in 100 μl), comparable to the one that can be delivered by the newly developed formulations (biodegradable PLGA microspheres for the sustained release of MP) [11]. For this purpose, a Depo-Medrol® vial (40 mg of MPA equivalent to 36 mg of MP) was diluted (1:3.6, v/v) by using the supernatant obtained by centrifugation (14,000 rpm, 10 min) of another Depo-Medrol® vial as the diluent. The absence of MPA in the supernatant was checked by LC–MS/MS (in all the prepared samples the MPA signals were below the instrumental LOD).

Blood samples were collected from the cava vein into heparinized-rinsed tubes at $t = 0$ (pre-dose) and 3, 6, 9, 24, 48 and 72 h after administration. Plasma obtained by blood centrifugation

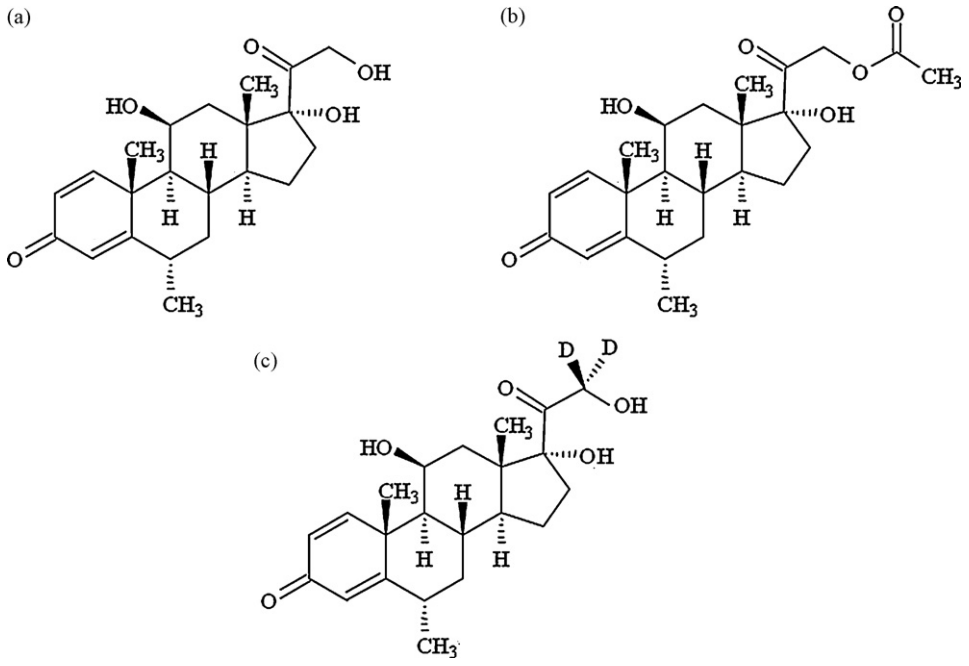

Fig. 1. Chemical structure of (a) MP, (b) MPA and (c) IS.

(3000 rpm for 10 min at 4 °C) was transferred in test-tubes containing 8 mg mL^{-1} NaF. Aliquots of 1 mL were stored at −80 °C until analysis.

To 100 μL plasma aliquots, spiked with IS (10 μL), 600 μL ice-cold acetonitrile was added for protein precipitation. After vigorous vortex-mixing for 5 min, samples were centrifuged at 14,000 rpm for 10 min at 4 °C. Supernatants were extracted with methylene chloride (1 mL) by vortex-mixing for 1 min, and then centrifuged at 2000 rpm for 5 min. The recovered organic phase was evaporated under nitrogen at room temperature and the dried extract reconstituted with 300 μL mobile phase. The reconstituted extracts were vortex-mixed for 30 s, filtered (0.45 μm, Millipore, Milan, Italy) and the filtrates transferred to the autosampler vial insert (5 μL injected) for quantitative analysis.

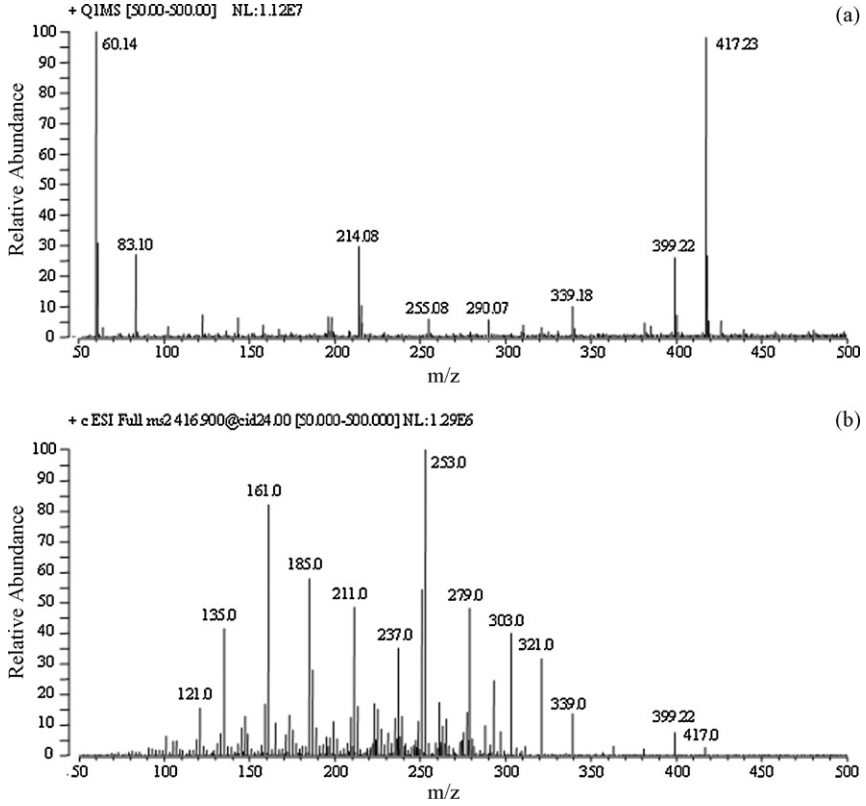

Fig. 2. ESI-MS direct infusion analysis of MPA (positive ion-mode): (a) full MS spectrum (*m*/*z* 50–500). (b) Full MS/MS spectrum of the parent ion at *m*/*z* 417.2 (collision energy 24 V).

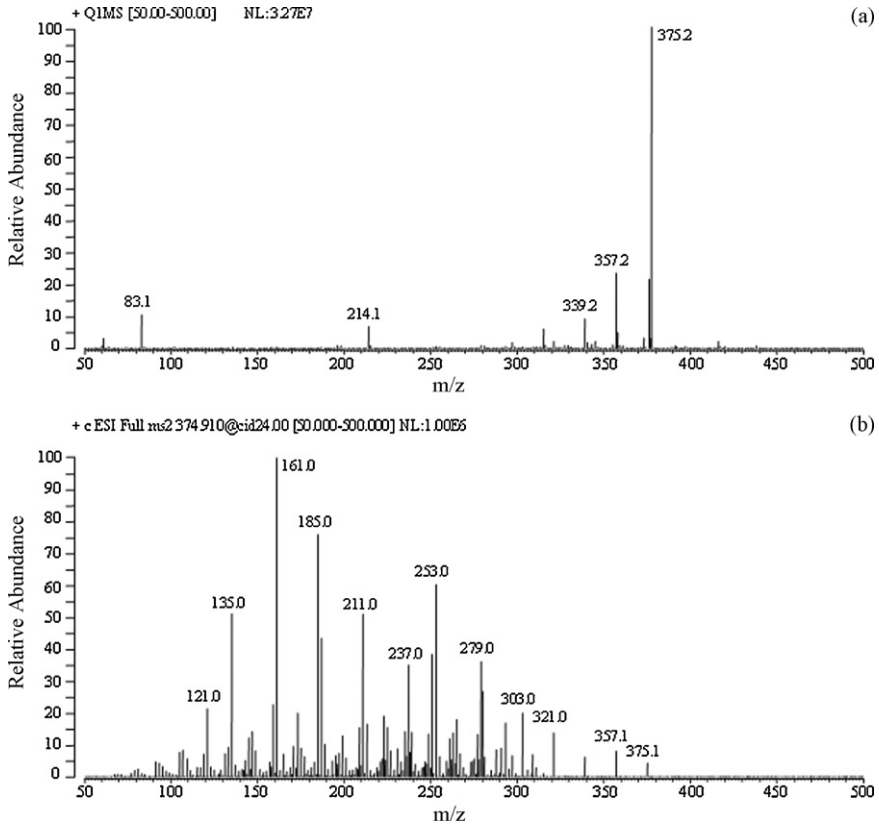

Fig. 3. ESI-MS direct infusion analysis of MP (positive ion-mode): (a) full MS spectrum (*m*/*z* 50–500). (b) Full MS/MS spectrum of the parent ion at *m*/*z* 375.2 (collision energy 24 V).

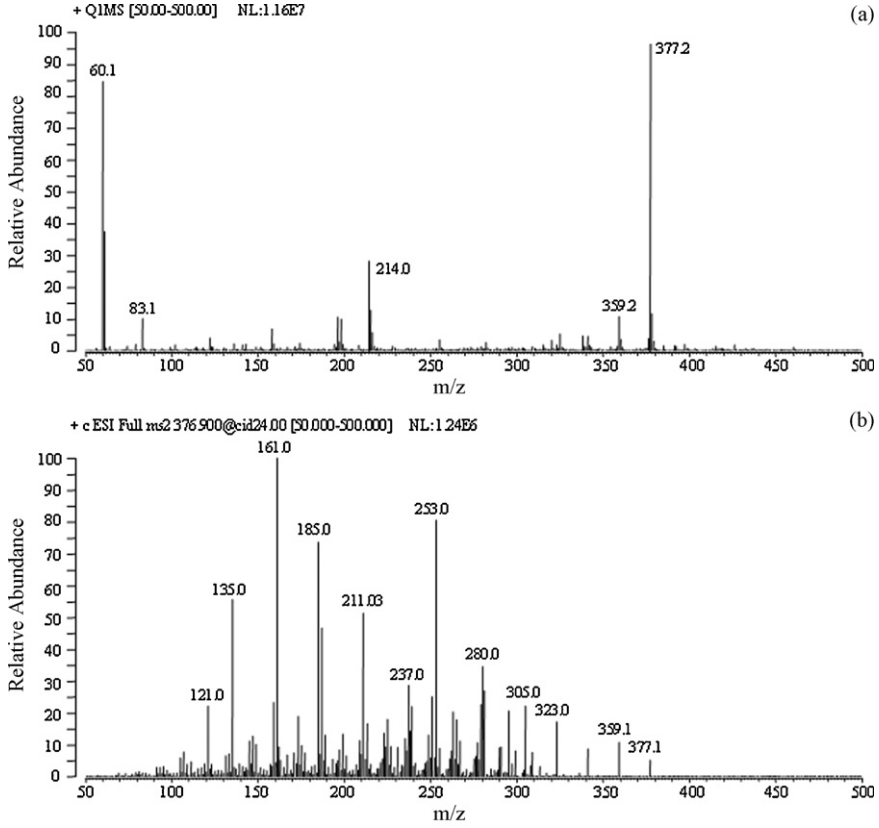

Fig. 4. ESI-MS direct infusion analysis of IS (positive ion-mode): (a) full MS spectrum (*m*/*z* 50–500). (b) Full MS/MS spectrum of the parent ion at *m*/*z* 377.2 (collision energy 24 V).

2.4. Preparation of calibration standards and quality control samples

Primary stock solutions of MP and MPA (1 mg mL^{-1}) and IS (0.2 mg mL^{-1}) were prepared separately in acetonitrile and stored at 4 °C for 1 week. Stock solutions were further diluted with acetonitrile and the solutions of MP and MPA were mixed together to obtain working solutions. Working solutions of 100 ng mL^{-1} were analysed by LC–ESI-MS/MS to ensure that the concentrations of the original solutions were within the limits of the maximum established error (±3%). Calibration samples were prepared by spiking blank rat plasma samples (100 μL aliquots) with working solutions (10 μL) at nine different levels to provide the following final concentrations, always operating at ice-cold temperature: 1.5, 3, 6, 15, 30, 60, 150, 300, 600 ng mL^{-1}. IS (10 μL) was added at the final concentration of 300 ng mL^{-1}.

Quality control (QC) samples at four concentrations (6, 30, 150 and 300 ng mL^{-1}) were prepared in the same way, using blank rat plasma spiked with independently prepared stock standard solutions. Each calibration and QC sample were processed as described above.

2.5. Method validation

Calibration standards were prepared in triplicate and analysed in duplicate in three independent runs. The calibration curves were constructed by equal weighted least-square linear regression analysis of the peak area ratios of each analyte to the IS against nominal analyte concentration. The lower limit of quantitation (LLOQ) was determined as the lowest concentration with values for precision and accuracy within ±20% and a signal-to-noise (S/N) ratio of the peak areas >10.

Intra- and inter-day precisions and accuracies of the method were determined by assaying five replicates of each of the QC samples (four levels in the very low, low, intermediate and high concentration ranges) in three separate analytical runs. Precision and accuracy were determined by calculating the coefficient of variation (CV, %) and the relative error (RE, %), respectively.

The absolute recovery of the analytes, after proteins precipitation and CH_2Cl_2 extraction, was determined by comparing the mass spectrometric response of QC samples in the low, intermediate and high levels ($n = 3$), to that of deproteinized and extracted plasma samples spiked with a corresponding set of concentrations (containing 100% of the theoretical concentration).

The specificity of the assay was evaluated by comparison of LC–ESI-MS/MS chromatograms of the analytes at the LLOQ to those of blank plasma samples in triplicate.

The stability of all the analytes in stock solutions (4 weeks at −20 °C) and in processed samples, including the resident time (24 h) in the autosampler (4 °C), storage temperature (−20 °C), and after freeze–thaw cycles, was determined in triplicate at the concentration of 150 ng mL^{-1}. The results were compared with those for freshly prepared QC samples and the percentage concentration deviation was calculated. The analytes were considered stable when 90–110% of the initial value was found.

3. Results and discussion

3.1. Optimisation of LC and MS conditions

The chemical structures of MP, MPA and IS are shown in Fig. 1. The full mass spectra of MPA, MP and IS obtained in MS infusion experiments showed the protonated molecular ions $[M+H]^+$ at m/z 417.2, 375.2, and 377.2, respectively (Figs. 2a, 3a, 4a). MS/MS experiments of each $[M+H]^+$ were carried out at different collision energies to determine the most stable and abundant product ions

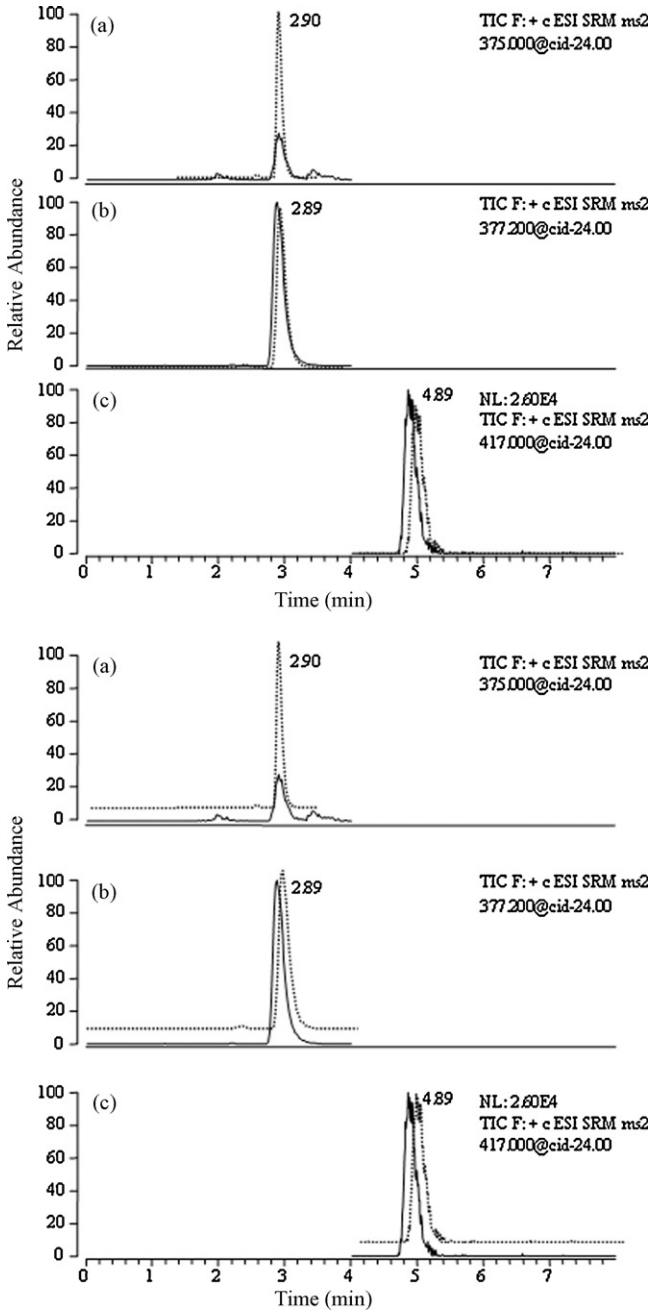

Fig. 5. MRM chromatograms (positive ion-mode) of MP and MPA in rat plasma. Blank plasma samples spiked with 1.5 ng mL^{-1} (LOD level) (bold line); plasma sample from animals IA treated with Depo-Medrol® at 6 h post-administration (dotted line). (a) MP m/z 375.2 → 135.0 + 161.0 + 253.0. (b) IS m/z 377.2 → 135.0 + 161.0 + 253.0. (c) MPA m/z 417.2 → 135.0 + 161.0 + 253.0.

for MRM analysis. A set of preliminary analyses was performed in order to optimise LC separation and the ionization efficiency of the analytes, using formic acid at different percentages in the mobile phase (data not shown). The best results, in terms of peak shape, resolution and signal-to-noise ratio, were obtained working under isocratic conditions using a mobile phase composition A:B (50:50, v/v) in the presence of 0.01% (v/v) formic acid.

Under the optimised LC and MS conditions, no interferences from endogenous compounds were found in blank plasma samples, and the retention times for the analytes were 2.94 ± 0.02, 2.95 ± 0.02 and 4.90 ± 0.03 min for MP, IS and MPA, respectively. Preliminary LC–MS/MS profiles, of blank plasma samples spiked with known amounts of the analytes (data not shown), indicate that

Table 1
Intra- and inter-day accuracy and precision for MP and MPA.

Analyte	Nominal concentration (ng mL^{-1})	Mean measured concentration ± SD (ng mL^{-1})	CV%	Accuracy (RE%)
Intra-day[a]				
MP	6	5.85 ± 0.32	5.43	−2.50
	30	29.78 ± 1.25	4.20	−0.73
	150	151.73 ± 3.61	2.38	1.15
	300	298.26 ± 1.81	0.61	−0.58
MPA	6	6.90 ± 0.38	5.50	15.00
	30	28.53 ± 1.34	4.71	−4.90
	150	148.74 ± 9.57	3.00	−0.84
	300	300.53 ± 1.61	0.54	0.18
Inter-day[b]				
MP	6	4.92 ± 0.55	11.07	−18.00
	30	28.90 ± 0.96	3.31	−3.67
	150	154.73 ± 6.10	3.94	3.15
	300	296.49 ± 10.57	3.56	−1.17
MPA	6	6.77 ± 1.33	19.65	12.83
	30	28.89 ± 1.13	3.91	−3.7
	150	147.44 ± 7.01	4.76	−1.71
	300	313.34 ± 15.03	4.80	4.44

[a] Five replicates at each concentration.
[b] Three runs, five replicates at each concentration over 3 days (n = 15).

they can be selectively detected and quantitated, by the following precursor–product ion transitions:

MPA : m/z 417.2 → 135.0 + 161.0 + 253.0
(collision energy 24 eV; Fig. 2b)

MP : m/z 375.2 → 135.0 + 161.0 + 253.0
(collision energy 24 eV; Fig. 3b)

IS : m/z 377.2 → 135.0 + 161.0 + 253.0
(collision energy 24 eV; Fig. 4b)

As shown in Figs. 2b, 3b and 4b, the product ion at m/z 185.0, common to all the analytes, is more intense than that selected at m/z 135.0, but it was discarded because the significant noise in blank plasma samples.

3.2. Method validation

Standard curves for the analytes, constructed on three different working days, showed good linearity in the 6–600 ng mL^{-1} concentration range. The coefficients of correlation (r^2) were greater than 0.99 for all the curves and the calculated equations were:

$$\text{MP} \rightarrow y = (0.0128 \pm 0.0016)x + (0.0361 \pm 0.0167) \quad (r^2 = 0.9993)$$

$$\text{MPA} \rightarrow y = (0.0221 \pm 0.0047)x + (0.0919 \pm 0.0018) \quad (r^2 = 0.9924)$$

The LLOQ was 6 ng mL^{-1} for both MP and MPA (0.080 and 0.072 pmol injected, respectively), and the LOD was 1.5 ng mL^{-1} for both the analytes (0.020 and 0.018 pmol injected for MP and MPA, respectively). Representative MRM traces relative to MP, MPA and IS obtained from blank plasma spiked with LLOQ concentrations (Fig. 5) indicate that they can be selectively detected and quantitated simultaneously, by the above-reported precursor–product ion combinations.

The intra- and inter-assay precision and accuracy of the method were determined on QC samples by analysing five replicates at four concentration levels (6, 30, 150, 300 ng mL^{-1}) in the same (intra) and three different days (inter). The intra-day precision (CV%) was not higher than 5.50% and accuracy ranged from −4.90 to 1.15% of nominal concentrations; the inter-day precision values were less than 4.80% and accuracy was in the range −3.67% and 4.44%, with the exception of LLOQ (Table 1).

Extraction efficiency was determined by comparing the peak area ratio of QC samples at three concentration levels (low, intermediate, and high) to those of extracted blank plasma spiked with the corresponding concentrations. The mean extraction recovery was satisfactory, ranging from 109% to 91% for both compounds (Table 2).

The stability of the analytes in stock solutions and in processed plasma samples was guaranteed for at least 24 h at 4 °C and no significant differences (t-test) were found between freshly prepared and samples stored at −20 °C (values ranged from 92.54% to 102.01% of the initial value). A significant loss of both MP and MPA was observed only after the second freeze/thaw cycle (28–37% decrease in respect to the nominal value) (data not shown). Stock solutions stored at 4 °C were stable for at least 4 weeks.

3.3. Method application

The fully validated method was finally applied to a pilot animal study aimed at quantifying MP and MPA in rat plasma after IA administration of low MPA doses (diluted Depo-Medrol®, equivalent to 1 mg MP injected). Plasma samples were collected up to 72 h and the plasma concentration–time profiles of the pro- and parent drug are shown in Table 3. MPA was quantifiable only at the first observation time (t = 3 h), while it was always detectable, but below the LLOQ at the following times. On the contrary, plasma concentrations of MP (below the LLOQ at 3 h post-administration time), reached peak levels between 6 and 9 h post-administration times and slowly began to decline thereafter (Fig. 5).

Table 2
Absolute recovery (%) of analytes from plasma samples.

Analyte	Nominal concentration (ng mL^{-1})	% Recovery (mean ± SD)
MP	30	102.29 ± 4.18
	150	96.24 ± 2.01
	300	101.13 ± 3.93
MPA	30	109.49 ± 5.02
	150	91.19 ± 4.37
	300	99.69 ± 3.92

Table 3
Time-course plasma profile of MP and MPA after Depo-Medrol® IA administration.

Time (h)	MP (ng mL^{-1})	MPA (ng mL^{-1})
0 (pre-dose)	bld	bld
3	blq	13.74 ± 0.65
6	16.74 ± 0.72	blq
9	14.22 ± 0.59	blq
24	7.90 ± 0.38	blq
48	6.95 ± 0.27	blq
72	blq	blq

bld: below limit of detection. blq: below limit of quantitation.

4. Conclusion

The newly developed assay here proposed is the first validated method to quantify MP and MPA simultaneously in rat plasma using LC–ESI-MS/MS. It fulfils the current requirements for bio-analytical validation, providing good results in terms of linearity, recovery, accuracy and precision. This method, requiring only 100 μL plasma and a minimal sample manipulation (simple de-proteinization and extraction steps), is simple and fast. Its excellent applicability in PK studies has been demonstrated, although in a preliminary pilot study, by monitoring MPA and MP levels in rat plasma after IA injections of low MPA doses. It has been proved to be highly sensitive, allowing detection and quantitation of the analytes at lower concentrations than those previously reported for MP in urine (LOD = 1.5 ng mL^{-1} instead of 15 ng mL^{-1} [22]; LLOQ = 6 ng mL^{-1} instead of 10 ng mL^{-1} [17]).

Therefore, the high sensitivity of the proposed method makes it suitable to monitor MP and MPA levels in rat plasma following IA administration of new formulations for local treatment.

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