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Journal of Chromatography B, 859 (2007) 42–51
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous determination of cortisol, dexamethasone, methylprednisolone, prednisone, prednisolone, mycophenolic acid and mycophenolic acid glucuronide in human plasma utilizing liquid chromatography–tandem mass spectrometry

Robin DiFrancesco, Valerie Frerichs, Julie Donnelly, Colleen Hagler, Jill Hochreiter, Kathleen M. Tornatore *

School of Pharmacy & Pharmaceutical Sciences, University at Buffalo, Buffalo, NY, United States

Received 5 April 2007; accepted 2 September 2007
Available online 14 September 2007

Abstract

Chronic combination immunosuppressive regimens are commonly prescribed to renal transplant recipients. To develop an assay method for pharmacokinetic studies and therapeutic drug monitoring of multiple immunosuppressives, a liquid chromatography–tandem mass spectrometry (LC/MS/MS) approach for the simultaneous analysis of several glucocorticoids, mycophenolic acid (MPA) and mycophenolic acid glucuronide (MPAG) was investigated. The resultant method utilized a gradient reverse phase separation over a Symmetry C18 column using an ammonium acetate–methanol mobile phase at pH 3.5. The analytes were detected by coupling the chromatography system via electrospray to a triple quadrupole mass spectrometer. Multiple-reaction monitoring in the negative mode ion (MH-/product) was employed selecting MPA at 319.1/190.9, MPAG at 495.1/191.0, dexamethasone at 391.0/361.0, hydrocortisone at 361.1/331.1, methylprednisolone at 373.1/343.1, prednisone at 357.1/327.2, and prednisolone at 359.1/329.1. The calibration curve concentrations ranged from 3.60 ng/mL to 50 μg/mL with the lowest limit of quantitation for corticosteroids being 3.60–7.20 ng/mL and 0.656–6.75 μg/mL for MPA and MPAG, respectively. The relative standard deviation for quality control intraday variation and interday variation was between 0.76% and 9.57% for all analytes. This assay offers a versatile, unique method for multi-analyte immunosuppressive determinations during combination immunosuppression.

Keywords: Immunosuppressives; Mycophenolic acid; Glucocorticoids; Assay; LC/MS/MS

1. Introduction

Combination immunosuppressive regimens are commonly prescribed to renal transplant recipients requiring chronic administration. The pro-drug, mycophenolate mofetil (MMF) is converted to the active moiety, mycophenolic acid (MPA), and is one component in combination immunosuppressive regimens in renal transplant recipients that has resulted in improved graft survival [1–5]. *Myfortic*, a new formulation of MPA is also prescribed in a fixed-dosing regimen and has recently been approved [6]. However, notable interpatient and intrapatient variability in pharmacokinetics and pharmacologic response has been described with both formulations of MPA which suggests the need for individualized drug therapy [3,4]. Determination of MPA area under the concentration versus time curve (AUC) are correlated with the risk of rejection and drug toxicities [3,4]. In addition, accumulation of the major MPA metabolite, mycophenolic acid glucuronide (MPAG) increases as renal function declines [3,4,7]. This metabolite undergoes enterohepatic recycling to MPA over the dosing interval to a variable extent based on which calcineurin inhibitor the patient is receiving [8,9]. Therefore, TDM of MPA and MPAG may provide consid-

Abbreviations: LC/MS/MS, liquid chromatography–tandem mass spectrometry; MPA, mycophenolic acid; MPAG, mycophenolic acid glucuronide

* Corresponding author at: Pharmacotherapy Research Center, Core Analytical Laboratory, Department of Pharmacy Practice, 319 Cooke Hall, School of Pharmacy & Pharmaceutical Sciences, University at Buffalo, Buffalo, NY 14260-1200, United States. Tel.: +1 716 645 2828x231; fax: +1 716 645 2001.
E-mail address: tornator@buffalo.edu (K.M. Tornatore).

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doi:10.1016/j.jchromb.2007.09.003

erable insight into the individualization of dosing regimens for transplant recipients receiving MPA.

As our understanding of MPA and MPAG pharmacokinetics and the resulting pharmacodynamic responses has increased, the clinical application of TDM has been suggested to guide chronic dosing. Since MPA is often prescribed with low-dose prednisone during the post-transplant period [3,10,11], an assay that provides concurrent measurement of prednisone, its metabolite prednisolone, and endogenous cortisol along with MPA and MPAG would facilitate TDM for both immunosuppressives [3,12]. The glucocorticoids, methylprednisolone and dexamethasone were also added as analytes to this assay since these drugs are often prescribed for immunologic disorders or oncologic diseases and may be used concurrent to MPA. The objective of this research was to develop a multiple analyte assay for MPA, MPAG and all glucocorticoids to address these needs and provide a clinical tool for TDM.

2. Experimental

2.1. Chemicals and reagents

Analytical grade prednisone, prednisolone, 6-α-methylprednisolone, hydrocortisone (cortisol), dexamethasone, mycophenolic acid, 5,5-diphenylhydantoin (internal standard utilized for MPA) and flumethasone (internal standard utilized for all other components) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Mycophenolic acid glucuronide was donated by Roche (Palo Alto, CA, USA). HPLC grade water, acetic acid, ammonium acetate, hydrochloric acid and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Acetonitrile was obtained from VWR (South Plainfield, NJ, USA). All solvents used in sample preparation and chromatographic separations were of HPLC grade. Human plasma was treated for the removal of endogenous hydrocortisone in order to be utilized for the preparation of standards and quality controls and was purchased from Bioresources Technology Inc. (Ft. Lauderdale, FL, USA). Plasma was double charcoal stripped, delipidated by the supplier. Treated human plasma was tested in-house to confirm the absence of hydrocortisone detection.

2.2. Apparatus

The LC/MS/MS system consisted of an Agilent Technologies 1100 series autosampler (Palo Alto, CA, USA) pump, degasser, and an Applied Biosystems PE/Sciex, API 3000 mass spectrometer (Foster City, CA, USA) equipped with a Turbo-ion spray source. The source and collision gas was nitrogen. The voltage was held at −4500 V and the source temperature at 350 °C. The mass spectrometer was operated utilizing multiple-reaction-monitoring (MRM) in negative ionization mode using a TurboIon spray interface. The resolution used in this method for Q1 and Q3 was Unit/Unit. The system was controlled through Analyst Software, version 1.4 from Applied Biosystems. Analytes were separated on a Waters Corporation (Milford, MA, USA) Symmetry C18 column, 30 mm × 2.1 mm i.d., 3.5 μm particle size, preceded by a Waters Symmetry Shield guard column, 10 mm × 2.1 mm i.d. The injection volume was 10 μL. Two mobile phases (A and B) were used in method. Mobile phase A consisted of 95% 5 mM ammonium acetate buffer pH 3.5 and 5% methanol mobile phase B consisted of 95% methanol and 5% 5 mM ammonium acetate buffer pH 3.5. The 4 min gradient separation started with 70% mobile phase A and 30% mobile phase B; final percentages were 10% mobile phase A and 90% mobile phase B, respectively. The flow rate of the mobile phase was 400 μL/min. Refer to Table 1 for mass transitions of the analytes. Prior to entering the electrospray source housing, the flow was split 1:1 using a PEEK tubing splitter (Upchurch Scientific, Oak Harbor, WA, USA), with one split line directed to waste and the other to the mass spectrometer's turbo-ion spray source.

2.3. Preparation of calibration standards and quality controls

Stock solutions were stored at a concentration of 1 mg/mL in methanol at −70 °C for up to 6 months. The stock solution of methylprednisolone was protected from light in an amber vial or by wrapping the vial in aluminum foil. Standard analyte mixtures for calibration standards utilized aliquots of the 1 mg/mL of analyte stock solutions combined in a 10.0 mL volumetric flask consisting of: 30 μL of hydrocortisone and prednisone, 40 μL of prednisolone and dexamethasone, 60 μL of methylprednisolone,

Table 1
Optimized detection parameters for each species' precursor and product ion and calibration curve ranges with performance

Analyte	Mass/charge: precursor/product (amu)	Calibration curve range in ng/mL (lowest to highest limit of quantitation)	Number of acceptable calibrants: *n* within ±15[a]/*n* total over 5 days
MPA	319.1/190.9	656–13600	38/40
MPAG	495.1/191.0	6750–50,000	35/35
HC	361.1/331.1	3.60–300	54/55
Dexa.	391.0/361.0	4.80–400	55/55
MPredl.	373.1/343.1	7.20–600	51/55
Pred.	357.1/327.2	3.60–300	53/55
Predl.	359.1/329.1	4.80–400	55/55
5,5-DPH (IS)	251.1/102.1	–	–
Flumethasone (IS)	409.1/379.1	–	–

[a] Per FDA bioanalytical guidelines May 2001: all calibrants must be within ±15% target except at the LLOQ where ±20% is allowed.

Table 2a
Identification of analyte concentrations at each control level

Control I	Control II	Control III	Control IV
MPA: 1.03 μg/mL	MPA: 2.94 μg/mL	MPA: 8.40 μg/mL	
MPAG: 14.0 μg/mL	MPAG: 25.0 μg/mL	MPAG: 40.0 μg/mL	MPredl.: 480 ng/mL
MPredl.: 20.6 ng/mL	MPredl.: 58.8 ng/mL	MPredl.: 168 ng/mL	Dexa., Predl.: 320 ng/mL
Dexa., Predl.: 13.7 ng/mL	Dexa., Predl.: 39.2 ng/mL	Dexa., Predl.: 112 ng/mL	HC, Pred.: 240 ng/mL
HC, Pred.: 10.3 ng/mL	HC, Pred.: 29.4 ng/mL	HC, Pred.: 84.0 ng/mL	

3000 μL of MPA and 5000 μL of MPAG. This solution was then diluted to a total volume of 10 mL using methanol. Syringes were used to add stock solutions to prepare standard analyte mixtures and accuracy was ±1% or less. Serial dilutions in methanol were used to make standard analyte mixtures and these were added to blank plasma to produce the calibration curve. For MPA and MPAG, 8 and 7 calibrators were utilized, respectively. A second set of 1 mg/mL stock solutions were combined and serially diluted in plasma for quality control solutions. The following volumes were combined in a 100 mL volumetric flask: 24 μL of prednisone and hydrocortisone, 32 μL of prednisolone and dexamethasone, 48 μL of methylprednisolone, 2400 μL of MPA and 4000 μL of MPAG. Syringes were used to add stock solutions to prepare quality control solutions and accuracy was ±1% or less. This mixture was then diluted to 100 mL with plasma. Serial dilutions of the control mixture were used to prepare four levels of quality control concentrations. Controls were divided into 1200 μL aliquots. Refer to Table 2a for the four levels of quality control concentrations. Both calibration standards and controls were stored at −70 °C for up to 3 months.

An internal standard solution mixture was made at a concentration of 1 μg/mL for both flumethasone (Flu) and 5,5-diphenylhydantoin (DP) in methanol. This mixture was stored at −20 °C. These two internal standards were utilized to accommodate the differing concentration ranges expected within clinical samples. The corticosteroid component and MPAG utilized Flu for internal standardization. MPA utilized DP as the internal standard.

2.4. Sample extraction

For assay calibration standards, 50 μL of each standard analyte mixture was added to 500 μL of blank plasma. To 500 μL of each control or patient sample, 50 μL of methanol was added. Once the samples were vortexed, 750 μL of 0.1 M HCl and 100 μL of internal standard solution were added with repeated vortexing. All samples were centrifuged (Beckman, Wakefield, MA, USA) for 10 min at 1200 × *g* (ambient temperature) and then subjected to solid-phase extraction using Oasis HLB 1 mL (30 mg) cartridges (Waters Corporation, Milford, MA, USA). Prior to sample extraction, each cartridge was conditioned using 1 mL of methanol, followed by 1 mL of HPLC grade water using a Vac Elut SPS24 solid phase extraction manifold (Varian, Palo Alto, CA, USA). A 1 mL aliquot of the acidified sample was then loaded onto the cartridges, followed by a 1 mL HPLC grade water wash. Analytes were eluted using 1 mL of

Table 2b
Interday summary of assay accuracy (%error) and variability (%R.S.D.)

Analyte	Control I		Control II		Control III		Control IV	
	%Error	%R.S.D.	%Error	%R.S.D.	%Error	%R.S.D.	%Error	%R.S.D.
MPA	−1.94	6.36	+2.72	3.63	−5.00	3.81		
MPAG	−0.71	8.77	+4.40	6.97	−4.25	6.84		
HC	+0.97	6.64	+2.04	5.41	+1.31	4.68	−1.92	7.51
Dexa.	−7.30	5.66	−5.87	4.24	−6.88	3.90	−9.25	3.57
MPredl.	+5.34	7.23	+6.63	5.83	+3.21	5.20	−4.15	5.70
Pred.	−5.83	6.03	−4.42	5.19	−8.10	5.16	−15.2	7.74
Predl.	+7.30	4.67	+9.18	3.19	+9.20	3.90	+6.13	4.07

Table 2c
Intraday assay accuracy (%error) and variability (%R.S.D.) for day 1 ($n = 6$ each control level)

Analyte	Control I			Control II			Control III			Control IV		
	Mean	%Error	%RSD	Mean	%Error	%R.S.D.	Mean	%Error	%R.S.D.	Mean	%Error	%R.S.D.
MPA	0.994	−3.50	3.99	2.96	0.68	0.96	7.49	−10.8	0.76			
MPAG	0.814	−3.33	6.02	26.4	5.67	5.62	38.4	−4.04	6.47			
HC	10.4	1.36	4.91	28.7	−2.38	5.45	80.1	−4.64	5.46	219.0	−8.61	4.72
Dexa.	13.0	−5.35	3.89	35.2	−10.2	2.95	100.4	−10.4	1.11	281.8	−11.9	3.87
MPredl.	21.5	4.53	5.65	59.8	1.67	5.08	163.7	−2.58	4.21	441.7	−7.99	4.56
Pred.	9.50	−7.77	4.93	27.3	−7.26	5.84	74.9	−10.8	6.55	202.0	−21.7	8.27
Predl.	15.0	9.49	5.35	41.9	6.85	2.72	117.8	−3.42	2.60	327.7	2.40	4.53

methanol. The eluants were evaporated to dryness for 30 min at 55 °C using a Zymark Turbovap LV (Hopkinton, MA, USA) and reconstituted in 100 μL of 70% mobile phase A/30% mobile phase B.

2.5. Optimization of mass spectrometry

Optimization of detector parameters for tandem mass spectrometric detection of the analytes was determined through direct infusion of each analyte or internal standard at a flow rate of 7.5 μL/min using a syringe pump (KD Scientific Inc., Model 100, New Hope, PA, USA). Each of the drugs were dissolved in 50% 5 mM ammonium acetate buffer, pH 3.5/50% methanol with a concentration of 1 μg/mL for observation of the precursor ion scan (Q1 scan). The Analyst software "Quantitative Optimization" wizard was utilized to discern the effect of instrument potential settings on precursor (Q1) and product (Q3) ions and optimize each potential sequentially. This scan was compared to the same scan of a blank solution. Ions produced that were unique to the sample were designated. For the corticosteroids, internal standards and MPA, the highest abundance precursor ion was utilized for product ion tandem mass spectrometry optimization. For MPAG, several ion transitions were monitored since this analyte was expected to be at a greater concentration with potential of detector overload. This was resolved by repeated testing of the method to determine the appropriate ion for MPAG. Fig. 1b displays the Q1 to Q3 ion transitions for MPA and MPAG.

2.6. Validation studies

A linear regression of 1/(analyte concentration)^2 was constructed for each analyte using the ratio of calibration sample and internal standard peak area responses. The data collected for standardization focused on the following three acceptance requirements as required by the FDA [17]. First, at least six calibrators had to be within 15% of the nominal concentrations using back calculation from the constructed regression including the lower limit of quantitation (LLOQ) where within 20% of nominal was allowed [17]. Second, FDA states 75% non-zero calibrators meet calculation criteria; any calibrators outside this criteria were excluded and the regression was recalculated [17]. Calibrators were injected once, and randomized throughout the batch of injections, as were quality controls. Two blank plasma samples, one with and without internal standard, were also run with each curve in every run.

For each analyte, the intraday and interday accuracy and precision were determined by testing six replicate samples of each control on five different days. At least three control concentrations were used. For each analyte, the lowest control sample concentration was prepared to be less than three times the LLOQ. Acceptance criteria required that at least two-thirds of the samples at each control concentration were within 15% of the target value and at least one at each concentration level must be acceptable. Control samples were also randomized throughout each sample batch.

The LLOQ was defined as the lowest concentration that analytes could be determined reproducibly within 20% of the targeted value with a signal-to-noise ratio of at least five. Six aliquots were determined for each analyte at the LLOQ on five consecutive days. All of the samples were required to produce a result within 20% of the target value to be an acceptable LLOQ. The accepted LLOQ concentration was then adopted as the lowest standard on the working curve for later routine analysis.

The accuracy of the assay in independent sources of plasma was assessed in five plasma lots. Three preparations of analyte mixture was prepared with each plasma lot and then analyzed and assessed for precision and accuracy. For comparison, a blank of each lot of matrix was also analyzed. To assess matrix suppression, the same five lots were extracted without adding the analyte mixture until just prior to evaporation and their results compared to samples of the analyte mixture without matrix extract added.

Dilution of samples were assessed for accuracy. A human plasma control sample was prepared at concentrations above the highest calibration level of analytes and was diluted at factors of 2-, 5-, and 10-fold with blank human plasma. Each dilution was assayed in triplicate.

The stability of samples after three repeated freeze–thaw cycles was determined to assess the integrity of the analytes. Control samples were analyzed after thawing once, and compared to the same samples that were frozen and thawed three times. Following each thaw, samples were allowed to sit at room temperature for at least 4 h before refreezing for 12 h. Three replicate samples of each of two controls were subjected to this treatment and compared to untreated controls. To determine the stability of plasma samples at room temperature, three additional replicates of two controls were maintained at room temperature (at 26 °C) for 18 h prior to preparation and analysis. These measured values of these samples were then compared to the expected nominal concentration.

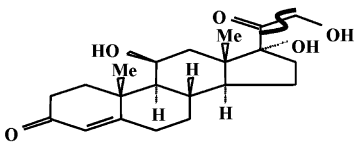
2.7. Calculations

All calculations were performed in Microsoft® Office Excel 2003 using the function formulas for mean ("average") and standard deviation ("stdev"). Error was also calculated as a percentage; the nominal value was subtracted from the measured value and the difference was divided by the nominal value.

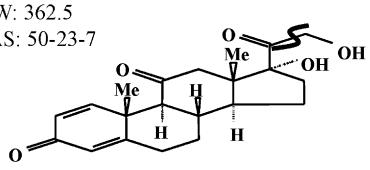
3. Results and discussion

For optimizing detection and fragmentation instrument settings, each species was directly infused into the mass spectrometer. As previously indicated, Table 1 provides the precursor and product ion pairs monitored for the analytes, and Fig. 1a shows the fragmentation of the analytes. Previous comparison of the positive and negative ionization modes for corticosteroids indicated that negative ionization produced better signal-to-noise ratio and a reduced number of fragments. This is in agreement with other published literature [16,30]. Collision-induced dissociation with nitrogen gas produced fragments that

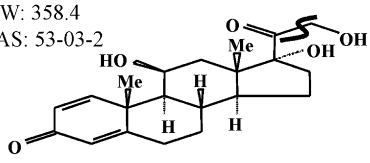
(a)



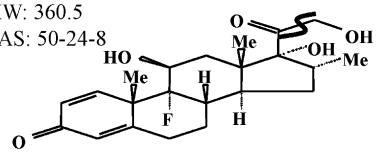
Cortisol
$C_{21}H_{30}O_5$
MW: 362.5
CAS: 50-23-7



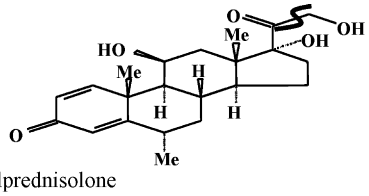
Prednisone
$C_{21}H_{26}O_5$
MW: 358.4
CAS: 53-03-2



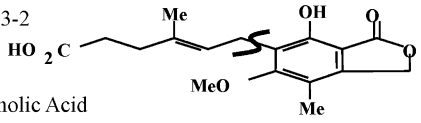
Prednisolone
$C_{21}H_{28}O_5$
MW: 360.5
CAS: 50-24-8



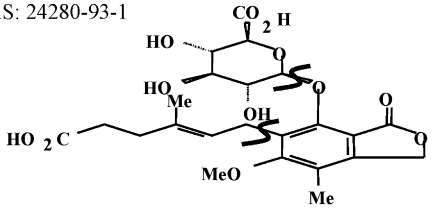
Dexamethasone
$C_{22}H_{29}FO_5$
MW: 392.5
CAS: 50-02-2



6-α-methylprednisolone
$C_{22}H_{30}O_5$
MW: 374.47
CAS: 83-43-2



Mycophenolic Acid
$C_{17}H_{20}O_6$
MW: 320.34
CAS: 24280-93-1



Mycophenolic Acid Glucuronide
$C_{23}H_{28}O_{12}$
MW: 496.46
CAS: 31528-44-6

(b)

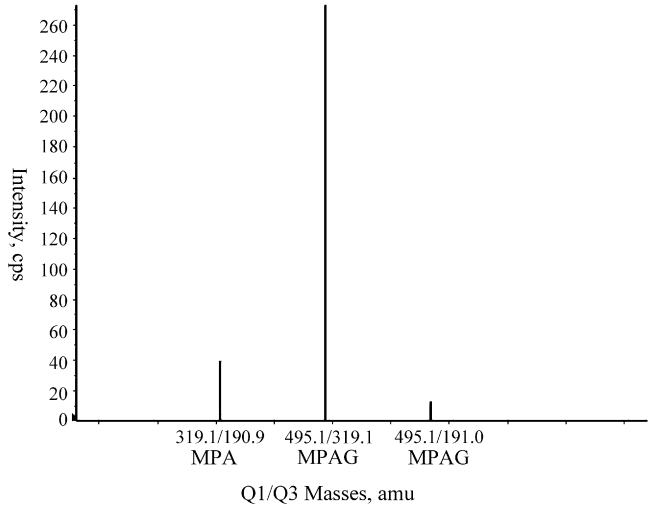


Fig. 1. (a) Structures of the analytes with proposed fragmentation correspondent to the masses of fragments detected utilized for quantitation reference. (b) Q1–Q3 ion transitions for MPA and MPAG.

were thirty mass units lower than the precursor ions. This coincides with a $[M-H-CH_2O]^-$ fragment. For MPA and MPAG, both indicate a precursor of $[M-H]^-$. The fragment for MPA at a mass of 190.9 amu indicates a loss of 129 amu, which would suggest a loss corresponding to the 3-methyl-hexanoic acid ($C_7H_{13}O_2$) group. Fig. 1b displays the Q1/Q3 ion transitions for MPA and MPAG. Our instrument resolution has 0.1 amu resolution so the difference between the MPA and the MPAG precursor fragments is within this uncertainty and the Q3 transition ion is fundamentally the same. As stated previously, the fragmentation pattern chosen for MPAG is not derived from the most intense precursor ion. The most intense product ion of 366 amu produced

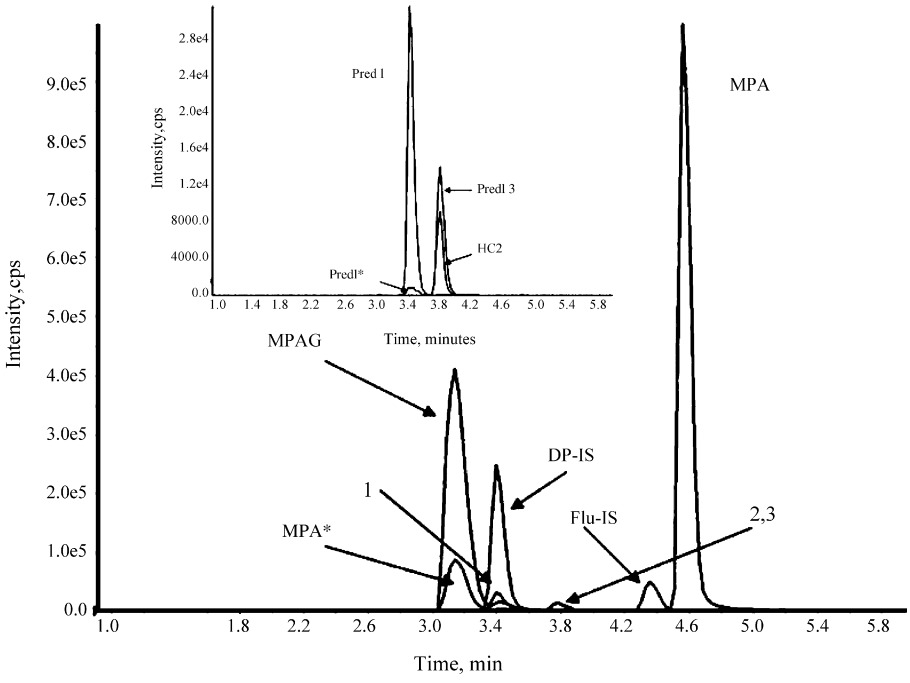


Fig. 2. Extracted chromatogram of medium quality control showing all analytes: MPA, MPAG, internal standards (DP-IS and Flu-IS), and steroids 1, 2, 3. Inset shows same chromatogram extracted only for steroids: Pred. or 1, HC or 2, and Predl. or 3. Post column production of MPA and Predl. are highlighted as *.

a signal in excess of 10^8 in infusion solutions less than 1 μM. As a result, a secondary fragment was chosen for analysis. Analysis of the most intense precursor and product ion for MPAG at relevant plasma concentrations produced a signal that was potentially deteriorating for the MS/MS instruments and difficult to calibrate due to intensity. An effect of this signal in terms of suppression of the other analytes was not observed. If plasma specimens are diluted to address this analytical issue, then the corticosteroids measurements would result in undetectable concentrations. In addition, our goal was to develop a multi-analyte assay requiring a single sample injection. Therefore, the precursor ion of the highest intensity and the product ion of the second highest intensity were chosen for quantitation. As a result, we were able to maintain selectivity and reproducibility, but reduce the signal. Choosing a less intense precursor ion was considered in developing the assay such as one corresponding to the loss of the glucuronide. Since the user can control fragmentation conditions, we reasoned that choosing a less intense product ion would likely be more reproducible in the designated matrix.

The use of tandem-mass spectrometry for analyte quantitation enhanced selectivity of the method for these closely related compounds. For example, prednisolone and hydrocortisone co-elute, and only have a two atomic mass unit difference in precursor and product ion mass without interference. However, due to the use of correspondent precursor and product ions, these analytes can be distinguished. This has been previously described in a similar method developed in our laboratory for serum analysis of corticosteroids [16].

Use of chromatography in this case is of equal importance. Upon in-source fragmentation, an identical precursor-product ion pair for MPA was obtained when MPAG was fragmented. However, with chromatography, we were able to separate MPA and MPAG sufficiently such that the identical ion pair did not interfere with the quantitation of MPA (see Fig. 2). Without this chromatographic separation, MPA and MPAG would co-elute and produce a falsely high response correspondent with MPA. This necessity has been reported and described previously by other MPA and MPAG assay publications [12–15].

Table 3
Limits of quantitation ($n = 6$ each day over 5 days)

Analyte	No. within 20% accuracy	Lower limit of quantitation	No. of analysis acceptable[a]
MPA	27/27[b]	656 ng/mL	5/5
MPAG	25/30	6.75 μg/mL	5/5
HC	25/30	3.60 ng/mL	4/5
Dexa.	27/30	4.80 ng/mL	5/5
MPredl.	17/24[b]	7.20 ng/mL	3/4
Pred.	30/30	3.60 ng/mL	5/5
Predl.	30/30	4.80 ng/mL	5/5

[a] A batch is acceptable when at least 66.6% of the values are within 20% of the target concentration for any one batch.
[b] Samples were lost due to handling or instrumental error, reducing the total number analyzed.

Table 4
Quantitation results for analytes in independent sources of matrix[a]

Plasma lot anticoagulant	Analyte	MPA (10,000)[b]	MPAG (16,700)[b]	Dexa. (133)[b]	HC (100)[b]	Predl. (133)[b]	Pred. (100)[b]	Mpredl. (200)[b]
1 Heparin	DV, ng/mL	9,793	18,400	128	90.3	128	92.5	199
	%Error	−2.07	10.2	−3.51	−8.67	−3.51	−7.50	−0.67
2 Heparin	DV, ng/mL	9,543	17,400	125	91.0	127	91.8	195
	%Error	−4.57	4.19	−5.76	−9.00	−4.51	−8.17	−2.33
3 Heparin	DV, ng/mL	8,857	18,300	123	90.3	131	86.5	185
	%Error	−11.4	9.58	−7.52	−9.67	1.75	−13.5	−7.67
4 EDTA	DV[a] ng/mL	10,533	19,600	132	106.7	136	99.3	211
	%Error	5.33	17.6	−0.75	6.67	2.26	−0.97	5.50
5 EDTA	DV, ng/mL	9,137	18,400	124	89.7	126	84.4	187
	%Error	−8.63	10.2	−6.52	−10.3	−5.26	−15.6	−6.50
Overall %error, mean of five matrices		−5.3%	+10%	−3.8%	−6.9%	−3.9%	−9.9%	−4.3%

DV: average determined value; R.S.D.: the relative standard deviation of the results for three replicates in each matrix.
[a] Three replicates were prepared for each matrix (mean R.S.D. = 2.75 ± 1.45%).
[b] TV (ng/mL): target value.

The working ranges of the calibration curves were chosen to accommodate expected clinical concentrations achieved with commonly prescribed doses of glucocorticoids and mycophenolic acid. The resulting range of working curve calibrators, ion transitions utilized for quantitation, calibration curve statistics and calibration performance in terms of percent error is summarized in Table 1. The required analysis concentrations of MPA and MPAG are markedly higher than the corticosteroids, with assay upper limits at 13.6 and 50.0 μg/mL, respectively.

The overall acceptance results of assay calibrants over five batches are summarized in Table 1. A 93–100% of calibrants for each analyte were acceptable over the 5 days tested. All calibration curves used a linear fit with a weighting of 1/(analyte concentration)^2. The resulting calibration concentrations for corticosteroids, MPA and MPAG were clinically relevant for the patient sample concentrations to be analyzed.

Control samples containing all the analytes in plasma were used to measure the validity of the analysis on a daily basis. While solutions used to make standard samples were stored in solvent, control concentrations were added and stored in plasma to reflect similar storage conditions to patient samples. Results of the analysis of the control samples in terms of accuracy and precision are shown in Tables 2b and 2c with a summary for four interday variation experiments and one intraday. For all of the analytes except prednisone, no more than 2 of the total 24 control samples deviated from the nominal concentration to greater than

Table 5
Stability of the analytes (n = 6 for each)

Analyte	Storage for 18 h at 26 °C pre-extraction, n = 3		Three freeze–thaw cycles, n = 3	
Dexa. Predl.	13.7 (ng/mL)[a]	112 (ng/mL)[a]	13.7 (ng/mL)[a]	112 (ng/mL)[a]
Mean (R.S.D.)	13.4 (3.01)	104 (4.46)	12.7 (2.08)	102 (1.14)
Mean (R.S.D.)	14.7 (7.06)	121 (8.33)	15.5 (13.1)	117 (4.17)
Pred. HC	10.3 (ng/mL)[a]	84 (ng/mL)[a]	10.3 (ng/mL)[a]	84 (ng/mL)[a]
Mean (R.S.D.)	9.21 (2.59)	72.8 (5.22)	9.88 (5.67)	75.4 (1.20)
Mean (R.S.D.)	9.90 (7.88)	86.1 (4.67)	11.6 (6.04)	81.3 (2.10)
Mpredl.	20.6 (ng/mL)[a]	168 (ng/mL)[a]	20.6 (ng/mL)[a]	168 (ng/mL)[a]
Mean (R.S.D.)	22.8 (2.67)	167 (2.40)	23.0 (2.65)	168 (0.60)
MPA	1.03 (μg/mL)[a]	8.40 (μg/mL)[a]	1.03 (μg/mL)[a]	8.40 (μg/mL)[a]
Mean (R.S.D.)	1.07 (2.36)	8.10 (2.75)	1.01 (2.48)	7.76(0.54)
MPAG	14.0 (μg/mL)[a]	25.0 (μg/mL)[a]	14.0 (μg/mL)[a]	25.0 (μg/mL)[a]
Mean (R.S.D.)	13.9 (3.25)	26.1 (2.30)	14.7 (2.18)	26.8(1.88)

[a] Control concentrations.

15% at any calibrator concentration. For all analytes, the relative standard deviation (R.S.D.) was between 0.76% and 9.57% for the between-day and within-day measurements. The prednisone high control was not valid for either the interday and intraday measurements evaluation, and only two of the four testing days produced acceptable results (within 15% of the target concentration) at the high control concentration. However, the other three levels of control were acceptable and allowed for a calibration range of 3.60–136 ng/mL, which is an adequate clinical range for this analyte as it is quickly metabolized to prednisolone.

The lower limit of quantitation for these analytes was determined using six replicates of the lowest calibration standard in five separate analyses. Results are summarized in Table 3. Acceptability for each day of the assay also requires 2/3 of the controls (four of six) on any day to be within 20% of the nominal value at the LLOQ. For the analytes: MPA, prednisone and prednisolone, all sample replicates produced results within 20% of the target nominal value. For dexamethasone, MPAG, methylprednisolone and hydrocortisone, the analysis of the replicates produced results within 20% of the target value for greater than 2/3 of the total number of samples.

The accuracy of the method in independent plasma matrices is summarized in Table 4. Matrices 1–3 contained the anticoagulant, sodium heparin additive while matrices 4 and 5 contained EDTA additive with no differences noted during testing. Overall the means of the matrices were well within 15% of the desired target and all analytes except MPAG met each individual matrix target within 15%. For MPAG, one matrix showed 17.2% deviation from the target value. Notably, all MPAG values were positively biased with a range of 4–17% and a median of 10%. Future modifications of the method to improve accuracy and lessen bias will be sought. For analytes such as hydrocortisone and MPA, where proficiency testing programs are available, the accuracy of assay will be tested.

The response of the analytes versus their neat response determined a small consistent amount of matrix suppression: −10.9% for dexamethasone, −9.7% for prednisone, −6.6% for prednisolone, −5.1% for hydrocortisone, −10.4% of methylprednisolone, −9.8% for MPA and −4.5% for MPAG. Yet since their accuracies were acceptable, as stated above, the internal standards used compensated well.

Accuracy results for dilutions of 2×, 5× and 10× were within 15% error of nominal concentration. Therefore samples at least 10-fold higher than the calibration limit could be accurately quantitated. Accuracies ranged from +7% to −14%.

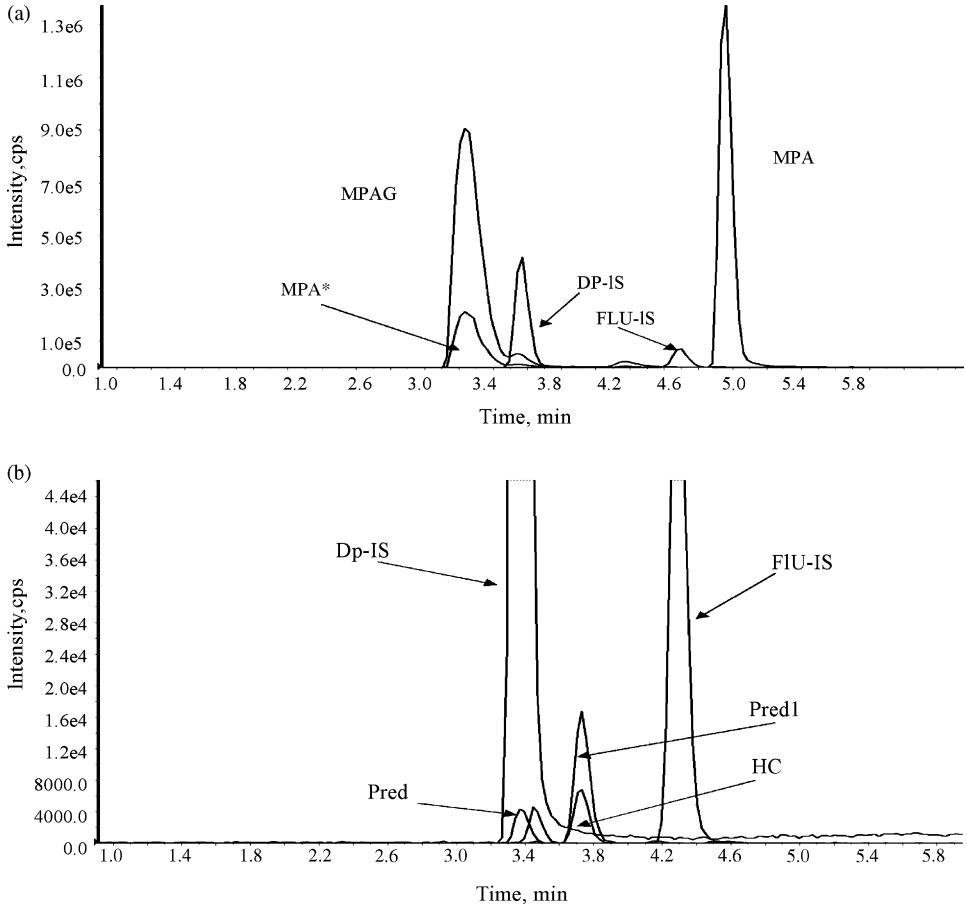


Fig. 3. (a) Patient sample drawn 2 h after mycophenolate mofetil (1250 mg) and prednisone (2.5 mg) doses. Ion pairs for MPA, MPAG and internal standards (DP-IS and Flu-IS) are extracted from the sample chromatogram. Plasma concentrations are 112,000 ng/mL (MPAG) and 10,500 ng/mL (MPA). (b) Patient sample drawn 2 h after mycophenolate mofetil (1250 mg) and prednisone (2.5 mg) doses. Ion pairs for Pred., Predl., HC, and internal standards (DP-IS and Flu-IS) are extracted from the sample chromatogram. Plasma concentrations are 5.84 ng/mL (Pred.), 72.2 ng/mL (Predl.) and 32.9 ng/mL (HC).

The stability of the analytes was tested using two different treatments. One treatment involved controls that were frozen and thawed three times prior to analysis. The second treatment controls were allowed to reside at room temperature (26 °C) in plasma for 18 h prior to analysis. Two control concentrations were utilized for each analyte. These treated controls were quantified using untreated calibration standards and compared to untreated controls. From the results in Table 5, it can be seen that three cycles of freezing and thawing had minimal effect on the quantitation of samples at the two concentrations for these analytes. All of the analytes could be determined within 15% error of nominal concentration at both control concentrations. This is also true for the samples allowed to sit at room temperature prior to preparation.

Example chromatograms from a human subject enrolled in an ongoing clinical study are included in Fig. 3a and b. Samples were taken at various time points following oral MPA and prednisone. The concentrations of the analytes are indicated in the figure legend.

4. Conclusions

It was anticipated that assay development for simultaneous analysis of MPA, its glucuronide (MPAG) with glucocorticoids in plasma would be a challenge based upon the large concentration range clinically achieved for each analyte [14]. It has been reported that MPA and MPAG plasma concentrations during the post-transplant period are in the microgram per milliliter range [15,16]. However, glucocorticoid concentrations such as prednisone, prednisolone, methylprednisolone, dexamethasone and hydrocortisone are present in patients in the range of nanogram per milliliter. Most analytical methodology is inadequate for accommodating quantitation of the higher concentrations of MPA and MPAG in combination with the lower concentrations of glucocorticoids in plasma. It has been reported that high performance liquid chromatography (HPLC) coupled with ultraviolet detection (UV) has proven appropriate for the expected concentrations of MPA and MPAG [15]. However, HPLC–UV methods are inadequate and inefficient for analysis of glucocorticoids at the appropriate concentrations in comparable time. Therefore, the primary goal of these experiments was to develop an assay for combination immunosuppressive regimens that required a reduced volume of plasma.

There are several analytical methods developed for the quantitation of corticosteroids during organ transplantation or immunologic diseases [14,16,18]. The various methods utilize HPLC [14,19,20], gas chromatography (GC) [21–24], and capillary separations [25–28] coupled with various detectors. Assay methods using these techniques for simultaneous analysis in biological media can require long separation times [14,19,20], use laboratory-intensive procedures and harsh solvents [14,19], and can require derivatization for GC or fluorescence based techniques [19,21–24]. In addition, the use of ultra-violet detection provides rugged analyses, but inadequate quantitation limits for the anticipated clinical concentrations [14,20]. However, the development of HPLC methods coupled with tandem mass spectrometry has provided the determination of lower analyte concentrations for mixtures of corticosteroids in biological media [29,12,30,31]. A recent assay report describes the simultaneous determination of corticosteroids in human serum using a small sample volume while achieving low detection concentrations for multiple analytes [16]. This method has been utilized for assessment of glucocorticoid pharmacokinetics and pharmacodynamics in renal transplant recipients [16]. Since combination immunosuppressive therapy includes a calcineurin inhibitor, mycophenolic acid with low doses of glucocorticoids, we further developed our multi-analyte assay to include MPA and MPAG resulting in a novel analytical method.

A limitation of the testing of this method was failure to include cortisone in specificity tests for prednisolone monitoring. Cortisone and prednisolone have molecular weights less than 1 amu apart and should fragment to the same product ion. If cortisone chromatographically co-elutes with the prednisolone analyte, it likely will be measured falsely as prednisolone. However, when this assay was used to monitor 55 patients of whom 25 were not receiving prednisone dosing, no false positives for prednisolone were observed, indicating that cortisone was not falsely detected as prednisolone. A peak for the precursor-product ion pair was detected prior to and fully resolved from prednisolone, which was most likely the endogenous cortisone.

Since HPLC–UV is not an adequate method of analysis for low concentrations of simultaneous corticosteroids in biological media, development for this analysis used LC/MS/MS. Our previous validation for the analysis of corticosteroids in serum indicated that negative electrospray ionization would provide detection with reduced interference within these complex samples [16]. To accommodate the expected higher concentrations in plasma, MPAG was monitored using an ion transition of less abundance which reduced the amount of current (signal) and avoided detector signal overload. However, this adjustment was not necessary for MPA under these analytical conditions. The development and validation of this multi-analyte assay using LC/MS/MS for glucocorticoids and MPA/MPAG provides a unique and efficient approach for conducting clinical research in areas such as pharmacokinetics, drug interactions and therapeutic drug monitoring of combination immunosuppressive regimens prescribed during the post-transplant period.

Acknowledgements

We acknowledge the Shared Instrumentation Grant, No. S10RRR14572 from the National Center for Research Resources, National Institutes of Health, which was used for acquisition of the development mass spectrometer. The Kapoor Foundation provided support for the purchase of a second mass spectrometer which was utilized for the validation work. Supervisor and technical support included Donna Ruszaj, Linda Hale, Kelly Tooley, and Kim Keil. A portion of funding was received from the University at Buffalo through the Interdisciplinary Research and Creative Activities Fund (IRCAF) to support this project.

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