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# Determination of the glucocorticoids prednisone, prednisolone, dexamethasone, and cortisol in human serum using liquid chromatography coupled to tandem mass spectrometry

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**Abstract**

Glucocorticoids are an important component of immunosuppressive therapy for solid organ transplantation. A method to quantitate prednisone, prednisolone, dexamethasone and cortisol in human serum has been developed. Analysis is performed utilizing reversed-phase liquid chromatography coupled to tandem mass spectrometry. The method was validated to a lower limit of quantitation of 5.4 ng/ml for prednisone and cortisol, and 10.7 ng/ml for dexamethasone and prednisolone, with error below 7% at the lower limits. The between-day relative standard deviations ranged 2.9–7.1%. Comparison of cortisol analysis to an established method using clinical samples yielded differences below 15% for 26 of 28 determinations.


*Keywords:* Prednisone; Prednisolone; Dexamethasone; Cortisol

## 1. Introduction

Glucocorticoids are important medications prescribed for immune-mediated conditions, such as solid organ transplantation, autoimmune diseases, and malignancies. Both synthetic and natural glucocorticoids possess anti-inflammatory and immunosuppressive properties [1]. For example, patients receiving chronic prednisone as a component of combination immunosuppressive therapy will have suppression of the hypothalamic pituatary adrenal (HPA) axis, resulting in suppression of endogenous cortisol concentrations, as compared to individuals not receiving prednisone [2]. Since the active component of prednisone is prednisolone, and endogenous cortisol adds to the immunosuppressive capacity, all three compounds should be measured for assessment of the suppression of the HPA axis in patients. Accordingly, it was our objective to develop a reliable method for quantitation of prednisone, prednisolone, and cortisol for analysis of serum from renal transplant patients receiving combination immunosuppressive therapy. Dexamethasone was also incorporated into the analytical method in anticipation of future clinical investigations. These four analytes are similar in chemical structure, mass, and spectral properties (Fig. 1) [1].

Various analytical methods have been used to determine glucocorticoid concentrations in biological samples. Documented methods for quantitation of these species utilize gas chromatography [3–6], high performance liquid chromatography (HPLC) [7–9] and capillary separations [10–12] coupled to various detectors. HPLC techniques for the targeted glucocorticoids require separation times of 10 min or more [7–9], use laboratory-intensive procedures [7], can utilize carcinogenic solvents [8], and, when using ultra-violet (UV) detection, suffer from inadequate limits of quantitation (LOQ) for anticipated clinical concentrations [8,9]. Although fluorescence detection can obtain detection limits of 100 pg/ml, precolumn derivatization is required [7]. Derivatization is also needed for gas chromatographic methods to render the analytes volatile [3–6].

Liquid chromatography coupled to mass spectrometry detection has been shown to produce low ng/ml quantitation of glucocorticoid mixtures in urine [13–15], solution [16], and for cortisol in serum [17] and plasma [18]. Past stud-

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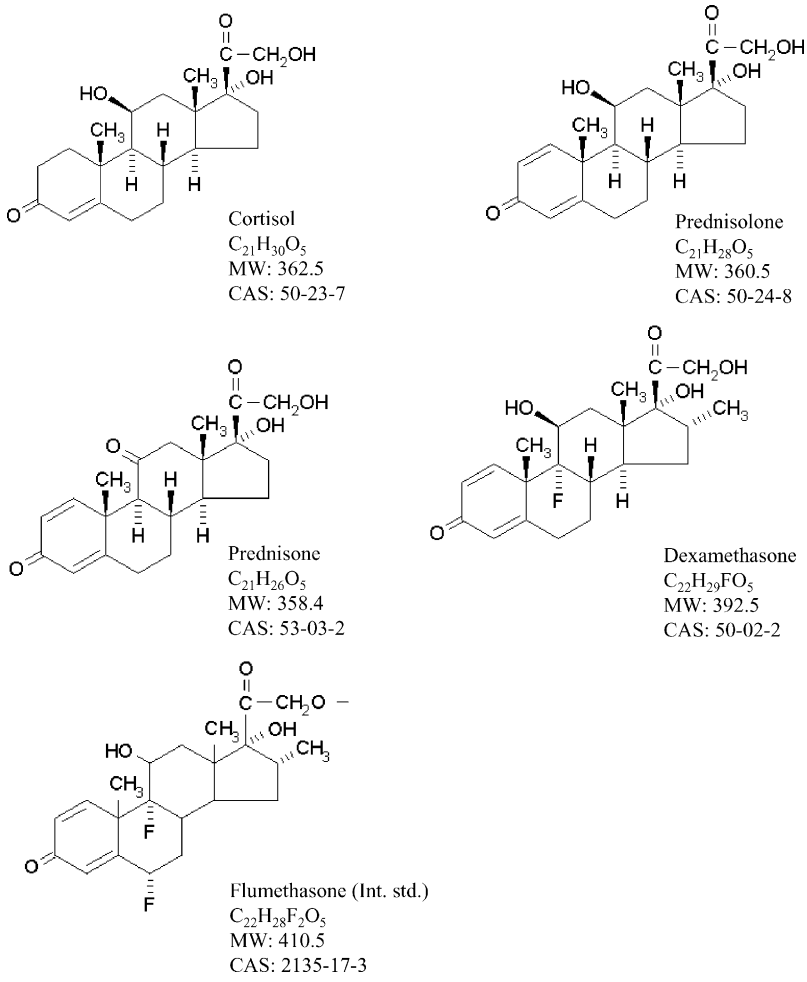


Fig. 1. Structures of the analytes and internal standard [23].

ies with glucocorticoids in renal transplant patients indicate that there is the potential for glucocorticoid concentrations in the low ng/ml range [19,20]. Here, we describe a simple, rugged method validated for the simultaneous quantitation of prednisone, prednisolone, cortisol and dexamethasone from 500 μl of human serum using liquid chromatography coupled to tandem mass spectrometry with electrospray ionization (ESI-LC/MS/MS). This method was developed for use in support of clinical pharmacology studies conducted in human renal transplant recipients.

## 2. Experimental

### 2.1. Chemicals

Glucocorticoids and flumethasone internal standard were purchased from Sigma–Aldrich (St. Louis, MO, USA). Water, acetic acid, ammonium acetate 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. Serum treated for the removal of cortisol and used for the preparation of standards and quality controls was purchased from Valley Biomedical (Winchester, VA, USA).

### 2.2. Instrumentation

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-ionspray source. The system was controlled through Analyst Software, version 1.1 from Applied Biosystems.

### 2.3. Separation conditions

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. The mobile phase solutions consisted of methanol and 5mM acetate buffer, pH 3.25. The mo-

bile phase was adjusted from 70 to 10% acetate buffer in 4.10 min. The flow rate was 400 μl/min. 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 tube directed to waste and the other to the mass spectrometer's source.

### 2.4. Preparation of samples

For standard solutions, 50 μl of 1 mg/ml stock solutions of prednisone and cortisol and 100 μl of 1 mg/ml stock solutions of dexamethasone and prednisolone were added to 1700 μl of a 50/50 methanol/acetonitrile solution. Serial dilutions of this mixture in 50/50 methanol/acetonitrile were used to make calibration standards. A second set of 1 mg/ml stock solutions were combined and serially diluted in serum to make control solutions. For control solutions, 100 μl of 1 mg/ml stock solutions of prednisone and cortisol and 200 μl of dexamethasone and prednisolone were added to 600 μl of 50/50 methanol/acetonitrile. Sixty microliters of this solution was diluted to 50 ml using human serum treated for the removal of cortisol. Serial dilutions of this solution were used to make control solutions. Controls were divided into 1200 μl aliquots prior to storage. Both calibration standards and controls were made in advance and stored at −70 °C for up to 3 months. A 1 μg/ml solution of flumethasone in methanol was stored at −20 °C for use as the internal standard.

For daily preparation of calibration standards, 50 μl of each standard analyte mixture and 100 μl of internal standard (1 μg/ml) were added to 500 μl of blank serum. To 500 μl of control solutions and patient samples, 50 μl of methanol and 100 μl of internal standard were added. After thoroughly mixing, all samples were centrifuged for 10 min, and then subjected to solid-phase extraction using Waters Corporation Oasis HLB 1 ml (30 mg) extraction cartridges. Briefly, each extraction cartridge was conditioned using 1 ml of methanol, followed by 1 ml of water using a Vac Elut SPS24 solid phase extraction manifold (Varian, Palo Alto, CA, USA). A 625 μl aliquot of each sample was then loaded on to the cartridges, followed by a wash using 1 ml of 5% methanol in water. Analytes were eluted using 1 ml of methanol. Samples were evaporated to dryness at 55 °C and reconstituted in 100 μl of 70% 5 mM acetate buffer, pH 3.25/30% methanol.

### 2.5. Optimization of MS/MS detection parameters

During method development, the optimized parameters for MS/MS detection of the analytes were determined. Each of the drugs was dissolved in 50% 5 mM acetate, pH 3.25/50% methanol to a concentration of 1 μg/ml. To observe how the instrument settings affect primary and fragment ions, analytes were directly infused into the instrument using a syringe pump (kdScientific Inc., Model 100, New Hope, PA, USA). The Applied Biosystems Analyst software "Quantitative Optimization" wizard was used to discern the optimal parameters.

### 2.6. Calibration procedures, accuracy and precision

Working curves were constructed for assay calibration on a daily basis at concentrations between 5 and 400 ng/ml, depending on the analyte. The analyst controlling software program was used to calculate the linear regression of the peak area ratios of internal standard and calibration sample versus nominal concentrations. All of the calibration curves were weighted by a factor of 1/(analyte concentration)$^2$. In order to accept the calibration, two criteria were mandated. First, any standards that did not fall within 15% of their nominal values upon back-calculation were excluded and the regression was performed again. Second, at least two-thirds of the standards' concentrations calculated using the regression were required to be within 15% of their nominal values. For example, when using eight standards to define a working curve, six were required to fall within 15% of the target concentrations. Otherwise, the entire analysis was repeated. Calibration standards were injected once, and randomized throughout the batch of injections.

For each analyte, the within and between-day precision was determined by testing six replicate samples of each control concentration on five different days. Four control concentrations were used. Over the five days, there were a total of 30 samples analyzed at each concentration. For each analyte, the lowest control sample concentration was prepared to be less than three times the LOQ. For the assay to be considered valid, at least two thirds of the samples at each control concentration had to be within 15% of the target value. Control samples were also randomized throughout each sample batch.

### 2.7. Lowest limit of quantitation and limit of detection

The LOQ was defined as the concentration for which analytes could be determined reproducibly within 15% of the targeted value and the signal-to-noise ratio was at least five. Six aliquots were determined for each analyte at the LOQ. To be considered acceptable as the LOQ, all of the samples were required to produce a result within 15% of the target value. The accepted LOQ concentration was then adopted as the lowest standard on the working curve for routine analysis.

The limit of detection was determined experimentally as the analyte concentration that produced a signal that was three times the noise level of a blank preparation. The limit of detection was determined through the addition of sequentially reduced analyte concentrations to serum and analyzing the samples to the point of obtaining a cortisol signal-to-noise level of three. For dexamethasone, prednisone, and prednisolone, detection limits were calculated from this chromatogram.

### 2.8. Accuracy and precision in independent lots of matrix

To assess the accuracy of the method in independent sources of serum, prednisone, prednisolone and dexamethasone were added to each of five independent serum lots (Valley Biomedical), resulting in an added concentration 4–5 times greater than the LOQ. The serum used was not treated for removal of endogenous cortisol. Three preparations of each serum lot were analyzed and assessed for precision and accuracy versus the added concentration. For comparison, a blank of each lot of matrix was also analyzed.

It was costly to purchase multiple lots of serum treated for removal of cortisol to conduct the same experiment to evaluate cortisol determination in multiple serum lots. Furthermore, untreated serum showed cortisol levels within the range of the working curve. Alternatively, the developed method was utilized to re-analyze clinical samples containing cortisol from a previous study. For the previous clinical study, samples were analyzed using a validated normal phase HPLC-UV method, with a lower limit of quantitation at 10 ng/ml [8]. Samples were stored at −70 °C for the 4-year interim.

### 2.9. Stability

The stability of samples after three repeated freeze-thaw cycles was determined to assess the integrity of the analytes upon reanalysis. 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. Six replicate samples of each of four controls were used for the comparison.

In some cases, prepared samples are re-injected into the LC/MS/MS system for repeat injection of the entire batch. This can occur if an instrumental failure should happen during analysis of a batch of samples. For determination of the stability of samples after sitting in the autosampler tray overnight, a set of standards and controls were prepared. Samples were analyzed once, allowed to sit in the autosampler for 24 h, and then analyzed a second time. Results of the first analysis were compared to the second analysis for determination of stability.

## 3. Results and discussion

### 3.1. Optimization of MS/MS detection parameters

For optimizing detection and fragmentation instrument settings, each species was directly infused into the mass spectrometer. The results are shown in Table 1. Comparison of the positive and negative ionization modes indicated that negative ionization produced better signal-to-noise ratio and reduced fragmentation. This is in agreement with published literature [18,21]. Collision-induced dissociation with nitrogen gas produced fragments that were thirty mass units lower than the precursor ions. This coincides with a $[M–H–CH_2O]^-$ fragment. The use of tandem-mass spectrometry for analyte quantitation enhanced selectivity of the method. In the developed chromatographic method, prednisolone and cortisol co-elute, with a two atomic mass unit difference in both the precursor and fragment ions. To determine if there was any interference between these two analytes, each compound was injected individually at concentrations five to ten times the upper limit of the working curve. For prednisolone, a peak for cortisol was found producing a signal thirty times less intense than the peak for prednisolone (see Fig. 2a). This height for cortisol is below the height of the lowest standard in cortisol's daily working curve. For cortisol, no prednisolone interference was found (see Fig. 2b). Furthermore, since three of the species are within six atomic mass units (cortisol, prednisone, and prednisolone), the instrument mass resolution was monitored frequently during validation and patient sample analysis, without notable drift between routine calibrations (every 1–2 weeks).

### 3.2. LC/MS/MS chromatographic characteristics

The chosen conditions resulted in elution of the four drug species and internal standard within 6 min (see Fig. 3a and b). It can be seen, in Fig. 3a, that two peaks are obtained for the precursor/fragment transition of prednisolone; the smaller peak co-elutes with prednisone. This signal was present for standards containing prednisone, and in fact this signal increased and decreased respective to prednisone concentration. Furthermore, this peak is not detected when prednisolone alone is injected (Fig. 2a), but was present in a 5 μg/ml prednisone solution (Fig. 2c). Thus,

Table 1
Optimized detection parameters for each species' precursor and fragment ion

| Analyte | Mass/charge: precursor/fragment | Declustering potential (V) | Focusing potential (V) | Excitation potential (V) | Collision energy (V) | Collision cell exit potential (V) |
|---|---|---|---|---|---|---|
| Dexamethasone | 391.0/361.0 | −31 | −190 | 10 | −16 | −3 |
| Prednisone | 357.1/327.2 | −21 | −120 | 10 | −12 | −3 |
| Cortisol | 361.1/331.1 | −26 | −160 | 10 | −14 | −3 |
| Prednisolone | 359.1/329.1 | −31 | −200 | 10 | −14 | −3 |
| Flumethasone (internal standard) | 373.1/343.0 | −21 | −130 | 10 | −12 | −3 |

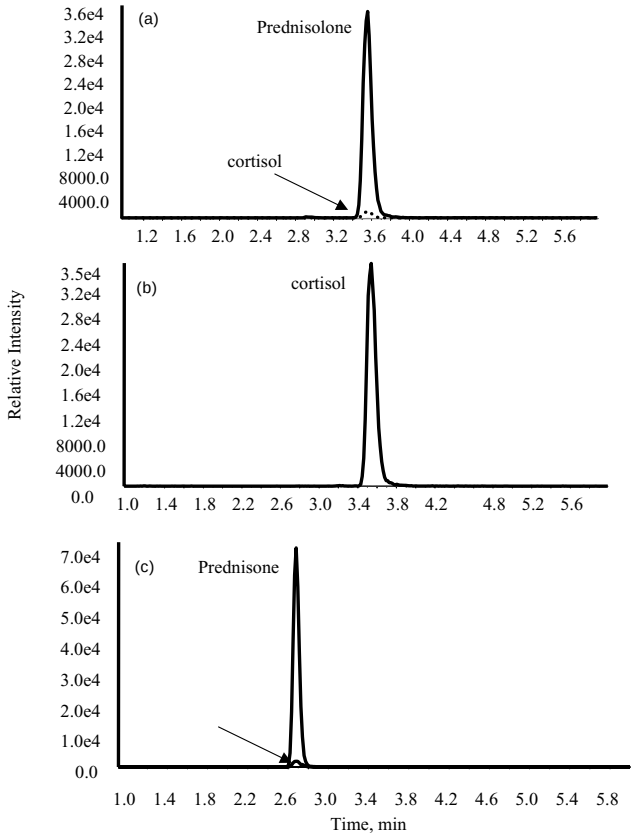


Fig. 2. Injection of 5 μg/ml of (a) prednisolone (b) cortisol, and (c) prednisone for determination of ion interference. Interfering precursor/fragment transitions are indicated with an arrow. In (a) the interfering peak corresponds in molecular weight and elution time with cortisol. In (c), since the peak indicated did not correspond to the elution time of the drug detected at that molecular weight transition (prednisolone), the peak was left unlabeled.

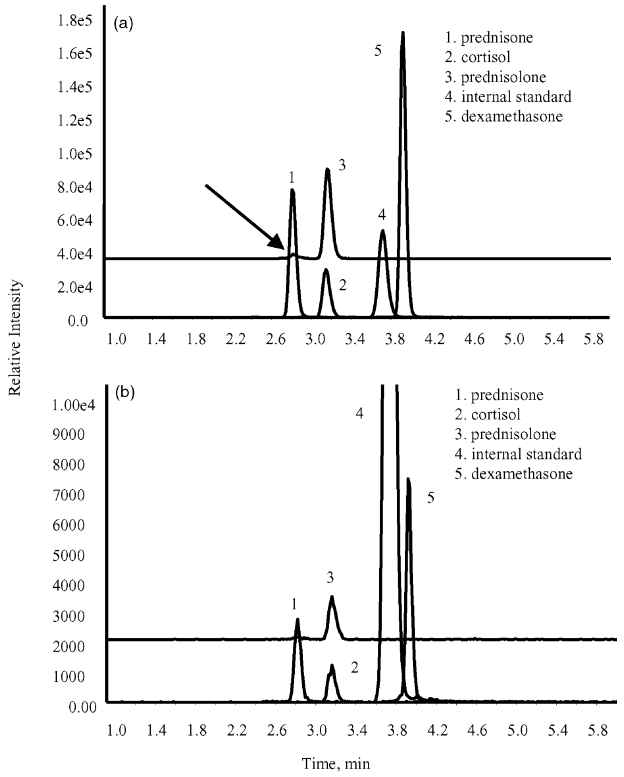


Fig. 3. (a) Example chromatogram of the highest standard, and, (b) a standard sample at the LOQ, or lowest standard. It can be seen that for (b), the signal co-eluting with prednisone at the precursor/fragment mass transition of prednisolone (indicated with an arrow) is below the limit of detection in (b). The *y*-axis does not apply to prednisolone, which has been offset for clarity.

we conclude that this peak is likely a contaminant of the purchased prednisone solid used to prepare our standards and quality control solutions. Although this signal is small enough not to interfere with quantitation, its resolution from prednisolone demonstrates the advantage of coupling a separation component to the mass spectrometer. It should be noted that this signal was not observed in patient samples.

Solid phase extraction (SPE) was utilized for sample preparation because the resulting extract produced no interference with quantitation during development. Published literature also describes established the use of liquid-liquid extraction of glucocorticoids from serum and plasma [8,17]. However, the solvents used (methylene chloride, ethyl acetate, hexane) are often harsh and expensive in terms of disposal. To a lesser-extent, on-line microextraction has been used for urine analysis of these drugs [13]. The automation of extraction techniques is attractive in terms of preparation time. However, it is known that the renal transplant patient samples will have a high level of lipid due to their state of health [22]. This poses a higher risk versus urine samples of obstructing the injector and electrospray source housing tubing connections.

The working range of the standard curve was chosen to accommodate expected clinical concentrations. For dexamethasone and prednisolone, the working range was 10.7–398 ng/ml; for prednisone and cortisol the working range was 5.4–194 ng/ml. Within these ranges, control samples 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 to and stored in serum (like patient samples). Control concentrations were made at four representative levels spanning the working curve. The control samples were used during method validation to determine the accuracy and precision of the assay, as shown in Table 2. It can be seen that for all of the analytes, the percent error was less than 15% at each control level for each analyte. In addition, the relative standard deviation (R.S.D.) was between 2.41 and 7.11% for the between- and within-day measurements.

### 3.3. Limits of quantitation and detection

The limits of quantitation and detection for this method were determined. Results are shown in Table 3. Relative

Table 2
Summary of assay accuracy and variability

| Analyte and calibration range (ng/ml) | Control concentrations | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Dexamethasone, prednisolone: 25.1 ng/ml cortisol, prednisone: 12.5 ng/ml | | | Dexamethasone, prednisolone: 50.1 ng/ml cortisol, prednisone: 24.9 ng/ml | | | Dexamethasone, prednisolone: 100 ng/ml cortisol, prednisone: 49.8 ng/ml | | | Dexamethasone, prednisolone: 200 ng/ml cortisol, prednisone: 100 ng/ml | | |
| | Mean | % Error | R.S.D. (%) | Mean | % Error | R.S.D. (%) | Mean | % Error | R.S.D. (%) | Mean | % Error | R.S.D. (%) |
| Within-assay variability, $n = 6$ | | | | | | | | | | | | |
| Dexamethasone: 10.7–389 | 25.2 | 0.8 | 4.40 | 50.3 | 0.40 | 4.65 | 95.2 | −4.80 | 2.17 | 177 | −11.5 | 1.89 |
| Prednisolone: 10.7–389 | 22.6 | −9.96 | 2.24 | 45.6 | −8.98 | 2.43 | 90.9 | −9.10 | 2.82 | 175 | −12.5 | 3.16 |
| Prednisone: 5.40–194 | 13.0 | 4.00 | 2.47 | 26.4 | 6.02 | 1.48 | 51.4 | 3.21 | 3.04 | 98.7 | −1.30 | 3.42 |
| Cortisol: 5.40–194 | 13.7 | 9.60 | 2.83 | 27.9 | 12.0 | 1.59 | 54.8 | 10.0 | 3.10 | 105 | 5.0 | 4.00 |
| Between-assay variability: (6 per day for 5 days: $n = 5$ using means) | | | | | | | | | | | | |
| Dexamethasone: 10.7–389 | 24.7 | −1.59 | 4.81% | 49.4 | −1.20 | 1.46 | 96.1 | −3.90 | 1.47 | 183 | −8.40 | 2.77 |
| Prednisolone: 10.7–389 | 22.4 | −10.8 | 3.99 | 45.8 | −8.58 | 3.63 | 92.3 | −7.7 | 2.90 | 179 | −10.5 | 4.30 |
| Prednisone: 5.40–194 | 12.8 | 2.40 | 5.18 | 26.1 | 4.82 | 3.70 | 51.8 | 4.02 | 2.97 | 100 | 0 | 3.69 |
| Cortisol: 5.40–194 | 13.7 | 9.60 | 4.80 | 27.9 | 12.0 | 4.02 | 55.6 | 11.6 | 3.82 | 108 | 8.0 | 4.44 |
| Calibration summary: $n = 5$ | Dexamethasone | Prednisolone | Cortisol | Prednisone | | | | | | | | |
| Mean (%R.S.D.) ($R$ = correlation constant; $m$ = slope; $b$ = $y$-axis intercept) | $R = 0.999$ (0.08) | $R = 0.998$ (0.16) | $R = 0.998$ (0.068) | $R = 0.999$ (0.063) | | | | | | | | |
| | $m = 6.66$ (2.36) | $m = 4.82$ (2.32) | $m = 4.29$ (4.74) | $m = 14.0$ (4.41) | | | | | | | | |
| | $b = 0.0060$–$0.019$ | $b = 0.0070$–$0.013$ | $b = 0.0057$–$0.012$ | $b = 0.0061$–$0.022$ | | | | | | | | |

Table 3
Limits of detection and quantitation

| Analyte | Limit of detection (pg/ml) | Lowest limit of quantitation (ng/ml), $n = 6$ | Accuracy at limit of quantitation: average % error (R.S.D.) |
|---|---|---|---|
| Dexamethasone | 225 | 10.7 | −6.65 (4.89) |
| Prednisolone | 225 | 10.7 | 2.51 (4.47) |
| Prednisone | 200 | 5.40 | −6.92 (2.61) |
| Cortisol | 575 | 5.40 | 5.61 (5.18) |

standard deviations at the LOQs ranged from 2.6 to 5.2%. The limit of detection was determined experimentally for cortisol, and calculated for the other analytes; results are shown in Fig. 4 and Table 3. Despite the vendor's treatment to remove endogenous cortisol, it was present in the serum blank, producing a distinct signal (Fig. 4b). For prednisone, prednisolone, and dexamethasone, no discrete peak could be identified in the blank serum. For cortisol, the quantity analyzed in Fig. 4a produced a signal approximately three times that found in the serum blank. Thus, this chromatogram represents the detection limit for cortisol. For the other analytes, detection limits were estimated through calculation. The resulting detection limits for cortisol, dexamethasone, prednisolone and prednisone are 570, 350, 225, and 350 pg/ml, respectively.

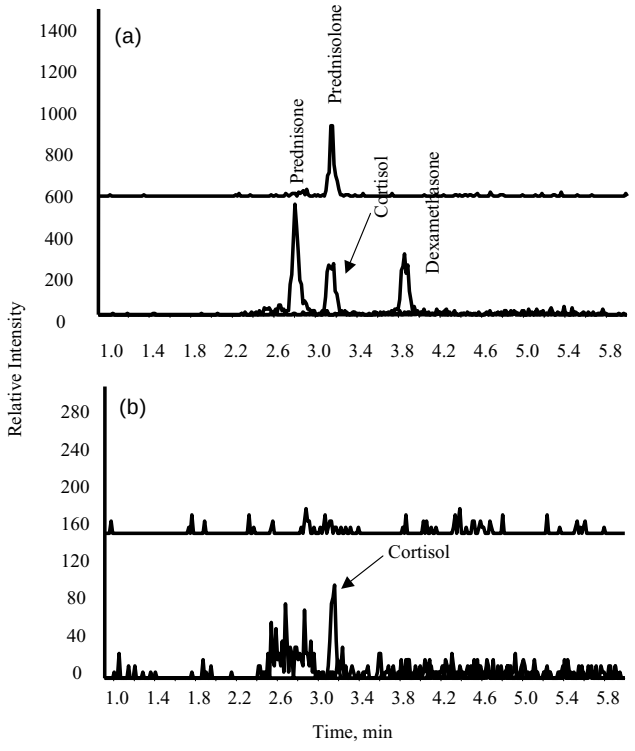


Fig. 4. (a) Chromatogram of analytes in an extracted serum standard sample of known concentration, treated to remove endogenous cortisol by the vender. The concentrations of dexamethasone, prednisolone, prednisone and cortisol are 1.14, 1.14 ng/ml, 570, and 570 pg/ml, respectively. (b) Blank serum, showing a peak for cortisol. The *y*-axis does not apply to prednisolone, which has been offset for clarity.

### 3.4. Accuracy and precision in independent lots of matrix

The accuracy of the method in independent serum matrices was determined. For dexamethasone, prednisone, and prednisolone, it was found that the method produced values within 15% of the target value in five independent serum matrices (Table 4). Since it was costly to obtain independent matrices of serum in which cortisol had been completely removed, we chose to analyze patient samples analyzed for cortisol from a previous study. Our method's results were compared to those previously obtained using a validated HPLC-UV method for analysis [8]. Results are shown in Fig. 5. It can be seen that results did not vary greater than 15% for all but two of the values, indicated with arrows. For these two data points, results were within 20% of each other. Positive correlation was seen, with a correlation constant equal to 0.99. It should be noted that the HPLC-UV method's lower limit of quantitation was 10 ng/ml, as determined through validation at that time. This experiment also serves to attest to the integrity of cortisol in these samples for the 4-year period that they were stored at −70 °C.

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

| | Dexamethasone | Prednisolone | Prednisone |
|---|---|---|---|
| Serum 1 | | | |
| TV[b] (ng/ml) | 41.8 | 41.8 | 20.9 |
| DV[b] (ng/ml) | 40.9 | 43.6 | 21.1 |
| R.S.D. (%) | 0.51 | 4.23 | 1.37 |
| Serum 2 | | | |
| TV (ng/ml) | 41.8 | 41.8 | 20.9 |
| DV (ng/ml) | 39.2 | 42.3 | 18.6 |
| R.S.D. (%) | 3.09 | 4.64 | 3.50 |
| Serum 3 | | | |
| TV (ng/ml) | 50.5 | 50.5 | 25.1 |
| DV (ng/ml) | 47.7 | 52.8 | 25.7 |
| R.S.D. (%) | 1.94 | 2.37 | 6.88 |
| Serum 4 | | | |
| TV (ng/ml) | 50.5 | 50.5 | 25.1 |
| DV (ng/ml) | 49.3 | 49.5 | 26.6 |
| R.S.D. (%) | 2.35 | 2.29 | 0.43 |
| Serum 5 | | | |
| TV (ng/ml) | 50.5 | 50.5 | 25.1 |
| DV (ng/ml) | 51.3 | 57.2 | 26.4 |
| R.S.D. (%) | 1.70 | 3.67 | 1.70 |

[a] Three replicates were prepared for each matrix.
[b] TV indicates target value, DV indicates average determined value, R.S.D. indicates the relative standard deviation of the results for three determinations.

Table 5
Stability of the analytes

| Analyte | Storage for 24 h at room temperature post-extraction | | | | Quantitation after three freeze-thaw cycles | | | |
|---|---|---|---|---|---|---|---|---|
| | 25.1 | 50.1 | 100 | 200 | 25.1 | 50.1 | 100 | 200 |
| Control concentration (ng/ml) (%R.S.D.) | | | | | | | | |
| Dexamethasone | 25.4 (4.45) | 50.4 (1.15) | 96.0 (1.76) | 177 (2.17) | 24.7 (7.11) | 49.5 (3.98) | 96.1 (2.94) | 183 (3.62) |
| Prednisolone | 22.4 (3.00) | 45.8 (1.10) | 90.9 (1.50) | 177 (0.73) | 22.4 (3.99) | 45.8 (3.63) | 92.3 (2.90) | 179 (4.30) |
| | 12.5 | 24.9 | 49.8 | 100 | 12.5 | 24.9 | 49.8 | 100 |
| Control concentration (ng/ml) (%R.S.D.) | | | | | | | | |
| Prednisone | 12.6 (3.85) | 25.5 (2.07) | 49.8 (1.58) | 96.4 (3.73) | 13.0 (2.26) | 26.1 (3.66) | 50.6 (2.58) | 98.9 (4.25) |
| Cortisol | 13.6 (2.59) | 27.6 (0.98) | 53.8 (1.57) | 102 (3.40) | 14.0 (3.94) | 27.9 (2.71) | 54.5 (2.65) | 105 (3.80) |

$n = 6$ for each.

### 3.5. Stability

Control samples analyzed after three repeated freeze-thaw cycles were evaluated versus the target concentrations for these samples. It can be seen, from the results in Table 5, that three cycles of freezing and thawing had minimal effect on the quantitation of samples at the four concentrations for these analytes. All of the analytes could be determined within 15% error at all control concentrations. This is also true for the samples allowed to sit in the auto-sampler tray for 24 h prior to reanalysis. Using a *t*-test for determination of statistical significance at a 95% confidence level, we found no statistical difference for both the repeated freeze-thaw and residence time in the auto-sampler when comparing the data control samples analyzed in a routine manner (one thaw, with same-day analysis).

### 3.6. Analysis of samples of unknown concentration from human subjects

Three example chromatograms from human subjects enrolled in an ongoing clinical study are included in Fig. 6a–c.

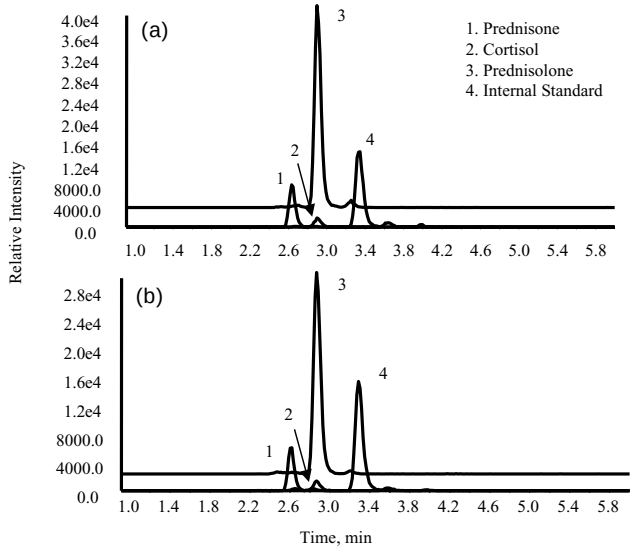


Fig. 6. Analysis of samples of unknown concentration from two different human subjects at various times after ingestion of prednisone. The resulting concentrations of prednisone, prednisolone and cortisol (in ng/ml) were (a) 26.7, 402, and 18.6, and, (b) 19.2, 299, and 13.9. Prednisolone has been offset for clarity.

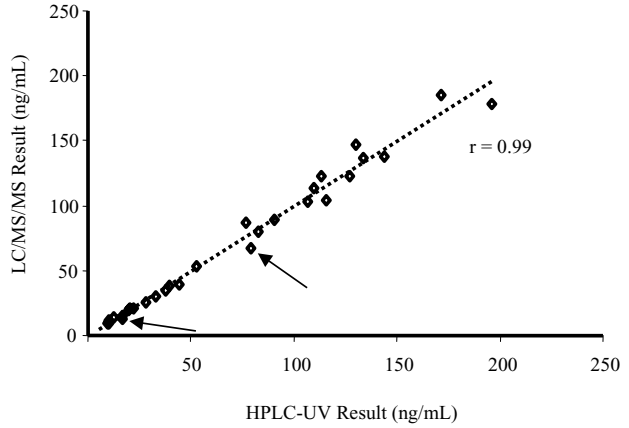


Fig. 5. Comparison of results obtained with HPLC-UV and LC/MS/MS for cortisol in patient serum. Arrows are used to indicate two data points which differed by more than 15%.

Samples were taken at various time points following the individuals' oral consumption of prednisone, which readily converts to prednisolone. The study monitored the quantitative levels of these two drugs, as well as cortisol in renal transplant patients.

## 4. Conclusions

A LC/MS/MS method was successfully developed for the quantitation of prednisone and cortisol between the concentrations of 5.4 and 196 ng/ml, and dexamethasone and prednisolone between the concentrations of 10.7 and 398 ng/ml. The method was validated for all four analytes in serum. Validation demonstrated the method's ability to analyze all four species from one 500 μl aliquot of sample, within a

6 min run time, with LOQs between 5 and 11 ng/ml, providing detection limits between 225 and 600 pg/ml. In addition, utilization of the method was able to produce concurrent quantitative results for cortisol in clinical samples versus analysis by HPLC-UV. The LC/MS/MS method is currently being used to support clinical pharmacology studies of these glucocorticoids in post-renal transplant patients of varying health status.

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## Appendix A

Calibration curve data

| Concentration of standard solution (ug/ml) | Dexa | % Error | Predl | % Error | Concentration of standard solution (μg/ml) | HC | % Error | Pred | % Error |
|---|---|---|---|---|---|---|---|---|---|
| (a) Day 1 | | | | | | | | | |
| 0.389 | 0.377 | −3.08 | 0.369 | −5.14 | 0.194 | 0.176 | −9.28 | 0.183 | −5.67 |
| 0.234 | 0.237 | 1.28 | 0.244 | 4.27 | 0.116 | 0.115 | −0.86 | 0.119 | 3.48 |
| 0.140 | 0.138 | −1.43 | 0.136 | −2.86 | 0.0698 | 0.0659 | −5.59 | 0.0661 | 0.30 |
| 0.0841 | 0.0856 | 1.78 | 0.0888 | 5.59 | 0.0419 | 0.0440 | 5.01 | 0.0444 | 0.91 |
| 0.0505 | 0.0525 | 3.96 | 0.0513 | 1.58 | 0.0251 | 0.0269 | 7.17 | 0.0262 | −2.60 |
| 0.0303 | 0.0303 | 0.00 | 0.0303 | 0.00 | 0.0151 | 0.0155 | 2.65 | 0.0152 | −1.94 |
| 0.0182 | 0.0175 | −3.85 | 0.0169 | −7.14 | 0.0090 | 0.00968 | 7.56 | 0.00878 | −9.30 |
| 0.0109 | 0.0110 | 0.92 | 0.0112 | 2.75 | 0.0054 | 0.00503 | −6.85 | 0.00539 | 7.16 |
| Slope | 6.39 | | Slope | 5.00 | | 4.64 | | 15.1 | |
| Intercept | 0.00827 | | Intercept | 0.00974 | | 0.00566 | | 0.00608 | |
| $r^2$ | 0.9994 | | $r^2$ | 0.9983 | | 0.9964 | | 0.9985 | |
| (b) Day 2 | | | | | | | | | |
| 0.389 | 0.352 | −9.51 | 0.350 | −10.0 | 0.194 | 0.177 | −8.76 | 0.177 | −8.76 |
| 0.234 | 0.238 | 1.71 | 0.241 | 2.99 | 0.116 | 0.122 | 5.17 | 0.121 | 4.31 |
| 0.140 | 0.139 | −0.72 | 0.138 | −1.42 | 0.0698 | 0.0678 | −2.87 | 0.0692 | −0.86 |
| 0.0841 | 0.0827 | −1.66 | 0.0865 | 2.85 | 0.0419 | 0.0457 | 9.07 | 0.0437 | 4.30 |
| 0.0505 | 0.0540 | 6.93 | 0.0523 | 3.56 | 0.0251 | 0.0247 | −1.59 | 0.0259 | 3.19 |
| 0.0303 | 0.0314 | 3.63 | 0.0311 | 2.64 | 0.0151 | 0.0153 | 1.32 | 0.0149 | −1.32 |
| 0.0182 | 0.0190 | 4.40 | 0.0187 | 2.75 | 0.0090 | 0.00872 | −3.11 | 0.00898 | −0.22 |
| 0.0109 | 0.0103 | −5.50 | 0.0105 | −3.67 | 0.0054 | 0.00544 | 0.74 | 0.00537 | −0.56 |
| Slope | 6.53 | | Slope | 4.87 | | 4.16 | | 14 | |
| Intercept | 0.0168 | | Intercept | 0.00719 | | 0.00752 | | 0.0178 | |
| $r^2$ | 0.9976 | | $r^2$ | 0.9981 | | 0.9975 | | 0.9985 | |
| (c) Day 3 | | | | | | | | | |
| 0.389 | 0.351 | −9.77 | 0.369 | −5.14 | 0.194 | 0.181 | −6.70 | 0.185 | −4.64 |
| 0.234 | 0.225 | −3.85 | 0.231 | −1.28 | 0.116 | 0.117 | 0.86 | 0.116 | 0.00 |
| 0.140 | 0.137 | −2.14 | 0.138 | −1.43 | 0.0698 | 0.0679 | −2.72 | 0.0692 | −0.86 |
| 0.0841 | 0.0844 | 0.36 | 0.0863 | 2.62 | 0.0419 | 0.0422 | 0.72 | 0.0447 | 6.68 |
| 0.0505 | 0.0544 | 7.72 | 0.0525 | 3.96 | 0.0251 | 0.0258 | 2.79 | 0.0257 | 2.39 |
| 0.0303 | 0.0344 | 13.5 | 0.0313 | 3.30 | 0.0151 | 0.0163 | 7.95 | 0.0146 | −3.31 |
| 0.0182 | *Sample loss*: *technical* Error | | | | 0.0090 | *Sample loss*: *technical* Error | | | |
| 0.0109 | 0.0103 | −5.50 | 0.0107 | −1.83 | 0.0054 | 0.00522 | −3.33 | 0.00540 | 0.00 |
| Slope | 6.75 | | Slope | 4.86 | | 4.21 | | 13.6 | |
| Intercept | 0.018 | | Intercept | 0.00692 | | 0.0075 | | 0.0159 | |
| $r^2$ | 0.9953 | | $r^2$ | 0.9992 | | 0.9983 | | 0.9990 | |

Cells that are italic were not utilized in the calculation of the calibration curve equation.

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