

Simultaneous Determination of Plasma Prednisolone, Prednisone, and Cortisol Levels by High-Performance Liquid Chromatography

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Summary: Recipients of organ transplants remain particularly dependent on prednisolone as part of their maintenance immunosuppression. Despite this, the pharmacokinetics of prednisolone have never been fully characterized in these patients, and consequently dosing remains empirical. Accurate monitoring of prednisolone, its primary metabolite prednisone, and endogenous cortisol suppression in such patients may provide a means of improving the clinical outcome by adjusting for variability in prednisolone pharmacokinetics and pharmacodynamics. Measurement of endogenous cortisol may provide an independent marker of prednisolone pharmacodynamics. A simple isocratic reverse-phase high-performance liquid chromatography procedure, using betamethasone as an internal standard, was developed to quantify plasma prednisolone, prednisone, and cortisol simultaneously. The steroids were extracted from 0.5 mL plasma with 3 mL (1:1 v/v) ethyl acetate/tert-methyl butyl ether and 0.1 mL phosphoric acid, washed in 0.1 mol/L NaOH before a final drying step and reconstitution in mobile phase for injection. Separation was achieved using a Supelcosil LC-18-DB, 150 × 4.6-mm, 5- μ m particle size, reverse-phase column attached to a Newguard 15 × 3-mm, RP8 guard column maintained at 25°C, with ultraviolet detector set at 254 nm. The mobile phase consisted of 16% isopropanol in water containing 0.1% trifluoroacetic acid, set at a flow rate of 1.2 mL/min. The assay was linear up to 1,002 μ g/L for prednisolone, 982 μ g/L for prednisone, and 545 μ g/L for cortisol. Mean intra-assay and interassay imprecision levels were 6.0% and 7.2%, respectively, for prednisolone, 5.8% and 7.2% for prednisone, and 5.6% and 7.9% for cortisol. Intra-assay inaccuracy was <7% of nominal values for prednisolone, prednisone, and cortisol. The lower limit of quantification was 7 μ g/L for prednisolone and prednisone and 10 μ g/L for cortisol. Corticosteroid recoveries were 73%, 74%, and 90% for prednisolone, prednisone, and cortisol, respectively. The authors describe a robust, inexpensive, and simple method suitable for therapeutic drug monitoring or pharmacokinetic studies of prednisolone; it may also be used to measure the suppression of endogenous cortisol production. **Key Words:** Prednisolone—Cortisol—Corticosteroids—Immunosuppression—HPLC.

Corticosteroids are synthetic glucocorticoids whose anti-inflammatory and/or immunosuppressive properties are widely used for the symptomatic treatment of many

disorders, including asthma and arthritis. Prednisolone also remains a vital component of the maintenance immunosuppressive regimen of transplant recipients for the prevention of allograft rejection (1). Corticosteroid dosage is currently adjusted on empirical grounds alone, and protocols are primarily designed to reduce doses to a low maintenance level as soon as possible, without taking into consideration differences in the pharmacokinetic

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characteristics of each transplant recipient. Consequently, there is considerable variation in the response to fixed doses of corticosteroids between patients (2,3). Measurement of the suppression of endogenous cortisol production may be used as a surrogate marker of this pharmacodynamic response to prednisolone (3). Monitoring of prednisolone, prednisone, and cortisol may, therefore, provide an intermediate therapeutic end point for steroid dosage adjustment, accounting for variability in both prednisolone pharmacokinetic and pharmacodynamic characteristics. In patients receiving prednisolone, immunoassay techniques are unsuitable for monitoring because of the cross-reactivity between prednisolone and endogenous cortisol (4).

We describe a reverse-phase high-performance liquid chromatography (HPLC) method using a C18 column for the simultaneous measurement of prednisolone, prednisone, and endogenous cortisol. The method can also be used to determine other corticosteroids, such as cortisone and 6- α -methylprednisolone, but we did not validate the assay for measurement of these analytes.

MATERIALS AND METHODS

Instrumentation

The HPLC system consisted of a Hewlett Packard (Stockport, UK) 1100 series: column, autosampler fitted with a 100- μ L sample loop, quaternary pump, and a variable ultraviolet (UV) wavelength detector, set at 254 nm. Separation was achieved using a Supelcosil LC-18-DB, 150 \times 4.6 mm 5- μ m particle size (SUPELCO, Poole, UK) attached to a Newguard 15 \times 3 mm, RP8 (Hichrom Ltd., Reading, UK), maintained at 25°C with an 1100 series oven. The mobile phase was 16% isopropanol in water containing 0.1% trifluoroacetic acid set at a flow rate of 1.2 mL/min.

Chemicals and Reagents

Corticosteroids and other reagents were purchased from Sigma-Aldrich (Poole, UK), human serum cortisol standards containing <1% sodium azide from Wallac Oy (Turku, Finland), and HPLC-grade solvents and deionized water from Rathburn Chemicals (Walkerburn, UK). Normal plasma for quality control and calibration samples containing sodium citrate anticoagulant was supplied by the National Blood Services, UK. Plasma samples containing EDTA anticoagulant were obtained from lung transplant recipients who had been recruited to a prospective clinical trial at Papworth Hospital,

Cambridge, UK. The trial was approved by the Huntingdon Local Research Ethics Committee (5).

Calibration and Quality Control Standards

Stock solutions containing 313 mg/L prednisolone and 307 mg/L prednisone were prepared by dissolving appropriate amounts of these analytes in methanol. A stock solution containing 1 mg/mL betamethasone internal standard was prepared in methanol and diluted in deionized water to a working solution of 2.5 mg/L. A combined prednisolone/prednisone substock containing 12.5 mg/L prednisolone and 12.3 mg/L prednisone was prepared by diluting an aliquot of each stock solution with deionized water. Aliquots of the combined substock were then diluted with deionized water to give eight working calibrators of 25–5,010 μ g/L prednisolone and 24.6–4,910 μ g/L prednisone. To 500- μ L drug-free plasma samples, 100- μ L volumes of working calibrators were added to produce final plasma concentrations of 5.0, 10.0, 125.2, 250.5, 501.0, and 1,002.0 μ g/L prednisolone and 4.9, 9.8, 24.6, 49.1, 98.2, 245.5, 491.0, and 982.0 μ g/L prednisone.

Four in-house quality control samples containing final concentrations of 37.6, 62.5, 125.2, and 750 μ g/L prednisolone and 36.8, 61.38, 122.8, and 738 μ g/L prednisone were prepared by diluting aliquots of the combined substock described above in drug-free plasma. Human serum cortisol calibrators contained 11.0, 26.4, 71.2, 218, and 545 μ g/L cortisol.

Patient samples, quality control samples, and cortisol calibrators were kept in aliquots of 500 μ L in 1-mL airtight polypropylene tubes and stored at -70° C until use. Calibration samples were spiked with 100 μ L working calibrator, and to ensure that quality controls, cortisol standards, and patient samples were of equal volume, 100 μ L deionized water was added before the extraction of these samples.

Sample Preparation

Aliquots of 500 μ L normal plasma for calibration, quality control sample, cortisol calibrator, or patient samples, equilibrated at room temperature, were added to 12-mL polypropylene centrifuge tubes. To the quality controls, cortisol calibrators, and patient samples, 100 μ L deionized water was added, and 100 μ L working calibration standard was added to tubes with normal plasma. Then, 100 μ L betamethasone (2.5 mg/L) internal standard, 100 μ L 5% phosphoric acid, and 3 mL (1:1 v/v) ethyl acetate/tertiary methyl butyl ether were added

to all samples. The tubes were tightly capped and vigorously mixed on a vertical reciprocating shaker for 30 minutes before being centrifuged for 5 minutes at 1,500*g*. The upper organic layer was then aspirated and transferred to new 12-mL centrifuge tubes and washed with 250 μL 0.1 mol/L NaOH on the reciprocating shaker for 5 minutes, followed by centrifugation at 1,500*g* for 5 minutes. The organic layer was aspirated once again and transferred to 5-mL glass culture tubes and evaporated to dryness in an Automatic Environmental SpeedVac (Savant, NY) for 2 hours at room temperature.

Chromatography

The residue was reconstituted with 200 μL 16% isopropanol in water, vortexed twice for 10 seconds with a 5-minute interval, followed by centrifugation at 8,000*g* for 5 minutes. A 100- μL aliquot of the reconstituted solution was then injected onto the column. The separation of cortisol from prednisolone was optimized by varying the isopropanol concentration between 15% and 20% (v/v) and adjusting the flow rate to ensure complete elution within 45 minutes. The baseline output was rezeroed after approximately 2 minutes when elution of the solvent front was completed and again after 8 minutes, just before the elution of prednisone. The mobile phase was recycled continuously for a total of 40 samples with no effect on chromatographic performance. If rapid results were required, the assay run time could be reduced significantly by introducing a mobile phase gradient. Thus, after the elution of prednisolone between 18 and 20 minutes, the proportion of isopropanol in the mobile phase could be increased, giving faster elution of the more hydrophobic betamethasone internal standard. (However, because the composition of the mobile phase is altered, it is then not appropriate to recycle the mobile phase, and as a result assay costs are increased.)

The sensitivity of the assay was optimized by initially testing for the best UV detection wavelength, signal attenuation, and column temperature. Corticosteroid peak areas were integrated with HP 1100 Chemstation software. Calibration curves of peak area ratio (steroid/internal standard) against concentration in $\mu\text{g/L}$ were plotted for prednisolone, prednisone, and cortisol.

Assay Characteristics and Validation

The HPLC assay validation was carried out according to guidelines recommended by the Food and Drug Administration (6). The specificity and selectivity of the assay were investigated by comparing the retention times

of steroids detected in an assay of drug-free plasma with those from an assay of extracted calibrator and those from a directly injected pure aqueous corticosteroid mix, run as five replicates. Retention times for various compounds that could interfere with the elution of prednisolone, prednisone, or cortisol, including cortisone and 6- α -methylprednisolone, were also evaluated by injecting pure analyte directly onto the column. Peak selectivity was measured with reference to the nearest eluting peak to the analyte of interest.

Assay linearity was assessed by direct injections of calibrators in the range 5–1,002 $\mu\text{g/L}$ for prednisolone, 5–982 $\mu\text{g/L}$ for prednisone, and 10–545 $\mu\text{g/L}$ for cortisol. The lower limit of detection was defined as the concentration at which the signal-to-noise ratio was no less than 3. Similarly, the lower limit of quantification was defined as the concentration at which the signal-to-noise ratio was no less than 10 (6). Both parameters were calculated with the calibrator containing the lowest concentration of analyte.

Intra-assay imprecision and inaccuracy were assessed by analyzing quality controls as six replicates during a single day. Mean, standard deviation, and coefficient of variation values were calculated for each quality control. The inaccuracy of the estimates for each quality control was determined as the difference between the mean measured concentration and the nominal concentration as a percentage of the nominal concentration. Interassay imprecision was evaluated in 20 assays run on separate days with two quality controls containing analyte concentrations within the therapeutic range. This was again expressed as coefficient of variation. The recoveries of prednisolone, prednisone, and cortisol were assessed by comparing recorded peak areas from different concentrations of extracted calibrator with corresponding peak areas derived from equal concentrations of directly injected aqueous analytes. Recovery was expressed as the percentage of the recorded peak area for directly injected material.

RESULTS

Typical chromatograms of drug-free plasma spiked with internal standard and a quality control sample containing 125 $\mu\text{g/L}$ prednisolone and 123 $\mu\text{g/L}$ prednisone are shown in Figure 1. Comparison of the assays showed the absence of any endogenous interfering peak at the retention times for prednisone and prednisolone. The assays also showed satisfactory selectivity and baseline

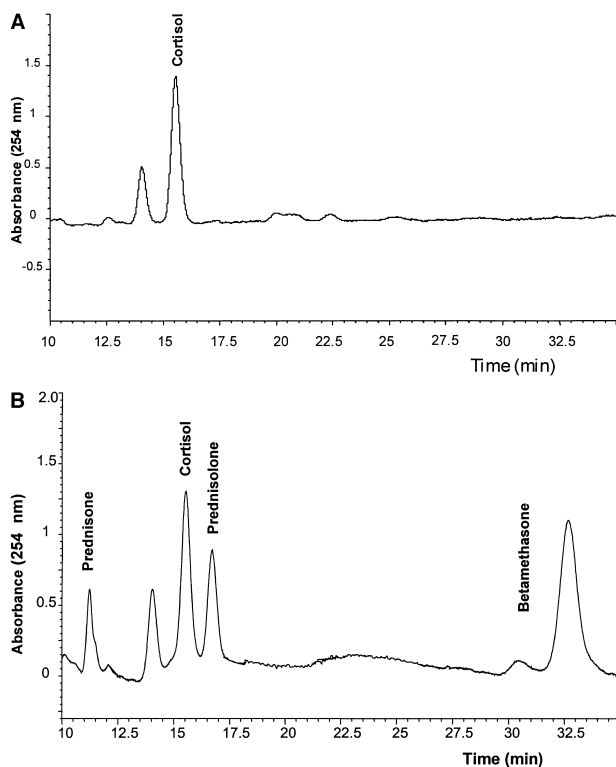


FIG. 1. Representative chromatograms of the assay. (A) Extracted drug-free plasma. (B) Extracted quality control sample containing 123 µg/L prednisone and 125 µg/L prednisolone

resolution between prednisolone and cortisol. The average retention times and selectivities for a variety of steroids are shown in Table 1. Validation results of linearity, lower limit of detection, lower limit of quantification, and assay repeatability, accuracy, and recovery are shown in Tables 2 and 3. The method has since been applied to measure prednisolone, prednisone, and cortisol concentrations in >2,000 samples collected during pharmacokinetic studies of 50 lung and 50 heart transplant recipients. Figure 2 shows the range and median trough corticosteroid concentrations for 40 heart

transplant recipients who received 15 mg oral prednisolone. Notable variation was observed in trough prednisone, prednisolone, and cortisol concentrations between patients.

DISCUSSION

We have described a simple, precise, and accurate method for the simultaneous measurement of prednisolone and its pharmacologically inactive metabolite prednisone in plasma. The assay can also be used to quantify endogenous cortisol production and thus may provide a pharmacodynamic measure of the inhibitory effects of exogenous steroids on the pituitary–adrenal axis. Early pharmacokinetic studies of prednisolone used radioimmunoassay techniques. Although such methods achieved higher sensitivity, precision was poor and the detection of cortisol was not possible because of cross-reactivity between cortisol and prednisolone (7). Also, several steroids could not be measured in a single plasma sample, so overall costs were high (8). During the past 20 years, several HPLC methods for corticosteroid quantification have been developed. Initially these were predominantly normal-phase techniques (7,9) with polar silica stationary phases and organic-based mobile phases. These early systems suffered from poor selectivity because steroids formed hydrogen bonds with the silica (4). The use of relatively viscous organic solvents also produced unwanted background noise, decreasing the limit of quantification (4). However, refinement of this technology has led to significant improvement (10). Numerous reverse-phase methods (4,11,12) have also been described in the past 20 years, mostly of a remarkably similar design.

The method developed by Frey et al in 1979 (7), improved further during 20 years of use and implemented in many pharmacokinetic studies since, displays mean intra-assay and interassay repeatability of <6%, a lower limit of quantification of 10 µg/L, and sample recovery of about 75%. A review of these popular methods

TABLE 1. Retention times for various corticosteroids detected using the assay measured over five consecutive injections and respective selectivity data

Drug	Retention (min)	Selectivity*	Reference peak
Prednisone	11.8 ± 0.17	2.4	Cortisone
Cortisone	13.2 ± 0.15	NA	NA
Cortisol	16.4 ± 0.10	1.5	Prednisolone
Prednisolone	17.7 ± 0.09	1.5	Cortisol
Betamethasone	34.1 ± 0.11	6.7/2.7	Prednisolone/methylprednisolone
Methylprednisolone	40.5 ± 0.11	NA	NA

NA, not applicable.

* The selectivity for each analyte was calculated with reference to the closest eluting peak.

TABLE 2. Precision and accuracy data

Nominal concentration (µg/L)	Intraassay			Interassay		
	Observed concentration* (µg/L)	CV (%)† precision	CV (%)‡ accuracy	Observed concentration§ (µg/L)	CV (%) precision	CV (%) accuracy
Prednisolone						
37.6	35.1 ± 3.0	7.8	6.7	34.8 ± 2.9	8.3	7.6
62.5	58.6 ± 3.5	6.0	6.3			
125.2	131.4 ± 7.4	5.6	5.0	133.1 ± 8.1	6.1	6.3
750	769.0 ± 33.7	4.4	2.7			
Prednisone						
36.8	34.6 ± 2.4	7.0	6.0	34.1 ± 2.8	8.2	7.3
60.4	57.6 ± 3.5	6.1	6.2			
122.6	117.2 ± 6.6	5.6	4.6	115.2 ± 7.3	6.3	6.0
738	719.0 ± 30.7	4.2	2.7			
Cortisol						
11.0	10.5 ± 0.5	5.1	4.4	10.2 ± 0.9	6.3	6.0
71.2	66.5 ± 4.1	6.2	6.5	66.2 ± 4.9	7.4	7.0

* Each value represents mean ± SD of six replicates.

† The coefficient of variation of the observed concentration.

‡ The coefficient of variation of the difference between the observed concentration and the nominal concentration.

§ Each value represents mean ± SD of 20 replicates.

indicates that our assay compares favorably, in terms of specificity, sensitivity, precision, accuracy, and analytic recovery, for the simultaneous determination of prednisolone, prednisone, and cortisol. Recent methods of increasing complexity describe attempts to improve sensitivity by sequential fractionation by HPLC and radioimmunoassay (10) or by the use of precolumn fluorometric derivatization (11), which can increase the limit of detection to 0.1 µg/L. Other advances include the development of solid-phase extraction techniques, which allow analytic recoveries of almost 100% (11).

In common with other assays (12,13), we encountered problems with inadequate separation of cortisol from prednisolone during the development of the assay. Slightly increasing the polarity of the mobile phase improved baseline resolution, giving satisfactory separation of cortisol from prednisolone. We also encountered an interfering peak close to the elution of betamethasone. This was found only with extracted samples and was later identified as a plasticizer contaminant from the extraction tubes. Changing to a polypropylene extraction

tube (catalog #57.527, Sarstedt; Leicester, UK) eliminated this problem.

The use of betamethasone as an internal standard in this method increased the sample turnaround time considerably. However, it provides elution at a relatively clean part of the assay run (as opposed to fludrocortisone, which showed interference with cortisol); it allows the method to be adapted for the measurement of methylprednisolone, which elutes after betamethasone; and it has a relatively low therapeutic application in transplant recipients (compared with dexamethasone, for example) (10).

Recovery of cortisol from cortisol serum solution was found to be 90%, greater than other analytes recovered from normal plasma. Cortisol recovery was measured based on recovery from a lyophilized human serum matrix containing known concentrations of cortisol reconstituted in deionized water. These solutions appeared to

TABLE 3. Assay parameters

Compound	Range (µg/L)	LLD (µg/L)	LLQ (µg/L)	Recovery (%)
Prednisone	5-982	2.1	7	74.1
Prednisolone	5-1,002	2.1	7	73.2
Cortisol	9-545	3.3	10	90.0
Betamethasone (IS)				79.9

LLD, lower limit of detection; LLQ, lower limit of quantification; IS, internal standard.

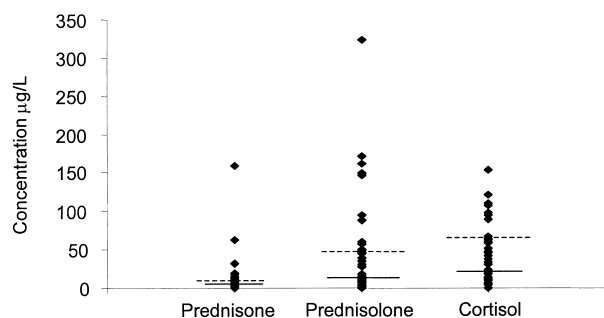


FIG. 2. Trough concentrations of corticosteroids in 40 heart transplant recipients who received a morning dose of 15 mg oral prednisolone. Solid and dashed lines represent median and upper quartile concentrations, respectively.

give a better extraction efficiency than normal plasma. Such cortisol standard solutions have been used consistently throughout the assay validation and analysis of unknowns; therefore, the error introduced as a result of using a solution different from normal plasma would be minimal. Ideally, normal human plasma stripped of endogenous cortisol and spiked with different concentrations of cortisol should be used for the measurement of cortisol recovery.

In our laboratory, 40 samples can be tested in a single run, but this was restricted by the capacity of our centrifugal evaporator. Including the 2-hour evaporating procedure, the time required to extract and reconstitute 40 samples was about 6 hours. Assuming a run time of 42 minutes per sample, the whole analysis of the 40 samples took 28 hours. A cost analysis showed that the consumables for a 40-sample run amounted to about £1. The economy of the method is one of its main features. During the course of an assay run, only 350 mL isocratic water-isopropanol mobile phase is used, and it is recycled continuously. The use of a recycled water-based mobile phase greatly reduces solvent costs and obviates the strict safety and storage regulations associated with methods that use organic-based mobile phases (4,7,10). Introducing an in-line filter proximal to the guard column further reduces costs by lengthening the life of the expensive guard and analytic columns. This becomes useful if the reconstituted material for chromatographic injection contains lipid or particulate matter, which cannot be separated by prior centrifugation. In our laboratory, only one analytic column was required for >1,000 samples.

The assay has been applied in pharmacokinetic studies of 50 lung and 50 heart transplant recipients. Figure 2 shows the range and median morning trough concentrations of prednisolone, prednisone, and cortisol in 40 heart transplant recipients 24 hours after receiving a 15-mg oral dose of prednisolone. Currently, we are trying to determine whether the variability in the response to prednisolone can be characterized by monitoring the concentrations of corticosteroids in transplant recipients. If a strong relationship can be shown, we may then use this assay in routine therapeutic monitoring of prednisolone as a guide to steroid dosage individualization. The assay would also have potential applications in other populations requiring corticosteroid therapy, such as those with

arthritis and asthma, as well as uses in the diagnosis of various endocrine disorders.

In conclusion, we describe a simple, accurate, inexpensive, and safe HPLC method for the simultaneous determination of plasma prednisolone, prednisone, and cortisol, suitable for pharmacokinetic studies, therapeutic drug monitoring, and diagnostic purposes.

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