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Fifteen years of operation of a high-performance liquid chromatographic assay for prednisolone, cortisol and prednisone in plasma

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Abstract

A high-performance liquid chromatographic (HPLC) assay first described in 1979 has been modified and revalidated for the simultaneous determination of prednisone, cortisol and prednisolone in human plasma using betamethasone as an internal standard. Revisions include: mobile phase composition; use of a precolumn, automated injector, integrator, and computer software; improved sensitivity and quantitation; thorough investigation of stability, variation, and specimen type; and inclusion of suggested quality control criteria. Plasma-based drug standards are extracted with methylene chloride and washed with sodium hydroxide followed by a water wash. After evaporation of solvent and reconstitution with mobile phase, the extracts are then injected onto a silica gel column (Zorbax SIL) for chromatography with UV absorbance at 254 nm. Calculated limits of quantitation are 10 ng/ml and limits of detection are less than 5 ng/ml. Intra- and inter-day coefficients of variation for quality control samples for all three corticosteroids are less than 11.2%. Recovery and stability data are also provided. Several drugs that may be coadministered do not interfere with the analysis.

1. Introduction

Rose and Jusko [1] published a normal-phase high-performance liquid chromatographic method (HPLC) for the quantitation of several corticosteroids in 1979. Since then, the assay has been used for many pharmacokinetic and pharmacodynamic studies [2–11]. As new technologies have emerged, some modifications were made to the methodology. More rigorous guidelines for quality control required that the methodology be validated further. Certain studies necessitated modifications to accommodate their quantitation needs [13–18]. This report summarizes the information that has culminated after 15 years of use of this method.

2. Experimental

2.1. Instrumentation

The HPLC system consists of a Model 510 solvent delivery system, Model 441 UV fixed-

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wavelength (254 nm) absorbance detector, and a Wisp 712 automatic sample injector (Waters Associates, Milford, MA, USA). Chromatographic separations are obtained with a Zorbax SIL (Dupont, Wilmington, DE, USA) column (250 × 4.6 mm I.D., 5–6 μm particle size) attached to a 2 cm × 2 mm I.D. guard column (Upchurch Scientific, Oak Harbor, WA, USA) packed with HC Pellosil (30–38 μm particles) (Whatman, Clifton, NJ, USA). Peak heights are recorded on a Hewlett-Packard 3392a Integrator (Avondale, PA, USA).

2.2. Reagents

HPLC grade methylene chloride (Lot No. BD942) and heptane (Lot No. BC 812) used for sample extraction and mobile phase preparation were purchased from Burdick and Jackson (Baxter Health, Muskegon, NJ, USA). Glacial acetic acid (Lot No. D22832) purchased from J.T. Baker (Phillipsburg, NJ, USA) and absolute alcohol (U.S.P.) purchased from Quantum Chemical (Tuscola, IL, USA) were also used in the mobile phase preparation.

Cortisol (11α,17ß,21-trihydroxypregn-4-ene-3,20-dione), betamethasone (9α-fluoro-16ß-methylprednisolone), prednisone (17α-21-dihydroxy-1,4-pregnadiene-3,11,20-trione), and prednisolone (11ß,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione) were purchased from Sigma (St. Louis, MO, USA). Anhydrous sodium sulfate (Lot No. 912064) and sodium hydroxide (Lot No. 780718) were purchased from Fisher Scientific (Fairlawn, NJ, USA).

2.3. Preparation of calibration and quality control standards

Plasma-based standards and quality control standards are prepared from pooled human plasma which has been "stripped" of endogenous glucocorticoids using pharmaceutical-grade decolorizing carbon (Norit A) (Amend Drug and Chemical Co., Irvington, NJ, USA) as follows: 4 g of charcoal per 100 ml of citrate-phosphate-dextrose-anticoagulated plasma are stirred at room temperature for 2 h. The mixture is centrifuged for 6 or more hours at 35 300 *g* in a Beckman J2-HS Centrifuge at 4°C. Finally, the plasma supernatant is filtered through a series of Acrodisc filters (Gelman Sciences, Ann Arbor, MI, USA): 5 μm, 1.2 μm, and 0.45 μm.

To the stripped plasma, various microliter volumes of corticosteroid solutions in ethanol are added such that the total ethanol content is always less than 0.1%. Seven standards are ordinarily prepared beginning at 10 ng/ml and may range as high as 1000 ng/ml. These concentrations may be adjusted to encompass the range of corticosteroid concentration expected for a study. (For example, females on oral contraceptives exhibit higher cortisol concentrations than do normal volunteers [5]). Three controls are prepared in a similar fashion; their concentrations are fixed at a median concentration of the standards and at concentrations above the lowest and below the highest standards. According to good laboratory practice, control and standard stock solutions as well as controls and standards themselves are prepared from separate weighings of analytical grade reagent by different technicians. Standards and controls are aliquotted at > 1.0 ml into 1.5-ml polypropylene tubes and stored sealed at ≤ −20°C.

2.4. Sample handling

Patient samples are collected into glass tubes containing heparin and centrifuged at 1500 *g* for 10 min. The plasma is transferred into polypropylene tubes and kept frozen at −20°C until analysis. It is allowable to collect serum (no anticoagulant) or EDTA-anticoagulated patient samples as well.

2.5. Extraction procedure

To 25 × 125 mm screw-capped centrifuge tubes, 1.0 ml of plasma-based standard or quality control sample or patient sample which has been equilibrated to room temperature, is added. Then 50 μl of internal standard (betamethasone, 200 ng) and 15 ml methylene chloride are added. The tubes are tightly capped and shaken

horizontally for 20 min. The tubes are then centrifuged at 1500 *g* for 15 min, and the creamy interface and aqueous layer aspirated. The organic phase is then washed with 0.1 ml of 0.1 *M* NaOH followed by a 1.0-ml double distilled water wash. After aspiration of the aqueous phase, 1.0 g of anhydrous sodium sulfate is added to dry the organic phase. The organic phase is transferred to a 20-ml glass scintillation vial and evaporated to dryness under a nitrogen stream at $\leq 37°C$.

2.6. Chromatography

The residue obtained by evaporation is reconstituted with 200 μl of mobile phase and a 175-μl aliquot is injected onto the column. The mobile phase consists of methylene chloride–heptane–glacial acetic acid–ethanol (600:350:10:35, v/v). (The ethanol content may be adjusted to obtain suitable chromatographic conditions.) During preparation, the mobile phase is filtered through a 0.22-μm GV Millipore filter to de-gas and remove particulates. The mobile phase is maintained at a flow-rate of 2.0 ml/min.

2.7. Calculations

Standard curve regression analysis was computed using a variance-stabilizing transformation method (VST) without forcing through zero [19]. Means and standard deviations were calculated using Lotus 1-2-3 software functions on an IBM-type personal computer equipment [20].

2.8. Defining assay characteristics

Linearity

Direct injections of steroids onto the chromatographic system were performed using amounts from 1 to 1000 ng. The linearity was assessed by determining if the peak heights increased in a proportional fashion.

Limit of detection

The limit of detection was assessed at a signal-to-noise ratio of 4:1.

Limit of quantitation

The lower (LOQ) and upper (HOQ) limits of quantitation were determined as the standard (lowest or highest) which consistently predicted steroid concentrations within 10% of its nominal value.

Precision and accuracy

Intra-day (within-run) precision was evaluated by analyzing three known concentrations of steroids in nine replicates. Inter-day (between-day) precision was assessed by measuring the same controls used for the intra-day precision study (in duplicate) over eighteen analytical runs. The accuracy of each study was determined by the percent difference of the mean concentration from the known concentration:

$$\%\text{Error} = \frac{[(\text{mean concentration} - \text{nominal concentration})}{\text{nominal concentration}]} \cdot 100\%$$

Precision is expressed as the coefficient of variation:

$$\%\text{C.V.} = \frac{(\text{standard deviation} \cdot 100\%)}{\text{mean}}$$

Assay specificity

Direct injections of various drugs that might be considered as concomitant therapy were chromatographed to determine potential interferences. Whenever possible, extracts of samples from patients or volunteers on these therapies but not taking corticosteroids were analyzed as well. Pre-dose samples from patients/volunteers also provided specificity with respect to endogenous components.

Steroid recovery

The assay recovery for each steroid was assessed at concentrations of 250 and 600 ng/ml. The peak heights obtained from three extracted plasma samples and from three direct injections of the same amount of steroid (*e.g.* 250 and 600 ng) in mobile phase were compared. The mean assay recovery of each steroid was computed using the following equation:

Percent recovery =

$$\left\{\frac{(\text{peak height}_{\text{extracted drug}})}{(\text{peak height}_{\text{direct injection}})}\right\} \cdot 100$$

Corticosteroid stability studies

To determine the stability of drugs, samples from a study [10] which had been kept for 38 months at −20°C and analyzed using the same HPLC method, were compared. Samples had been freeze-thawed two times prior to the stability study. Process stability was assessed utilizing controls and standards. First, four identical suitability injections of 50 ng of all steroids were made for evaluation of proper operation of the HPLC system. Next, four sets of each quality control sample were chromatographed prior to an initial standard curve, followed by single quality controls, and followed by a second standard curve. Lastly, four of each quality control sample were chromatographed. The analysis time between the first quality control injection and the last was 833 min (for a 48-vial tray).

Specimen type

Serum, stripped plasma, and plasma anticoagulated with heparin or ethylenediaminetetraacetic acid (EDTA) were spiked with corticosteroids and assayed for corticosteroid content. Endogenous cortisol in serum and unstripped plasma was measured prior to addition of corticosteroids and subtracted from results prior to statistical analysis. Results of each group (n = 5 per group) were tested for each corticosteroid using one-way analysis of variance (ANOVA) and followed by Bonferroni t-tests between groups.

3. Results

Fig. 1 shows chromatograms of plasma blank, standards, and patient samples. Retention times ranged from *ca.* 9 min for prednisone to 21 min for prednisolone.

Table 1 summarizes the various characteristics of the assay methodology.

3.1. Linearity

The quantification of corticosteroid standards was found to be linear from 10 to 1000 ng.

3.2. Limit of detection and quantification

The detection limit was found to be 5 ng/ml for each of the steroids. The lowest standard, 10 ng/ml, was found to consistently predict its own value within 10% and was therefore determined to be the lower limit of quantitation. The highest standard, 1000 ng/ml, was similarly determined to be the high limit of quantitation.

3.3. Precision and accuracy

The intra-day precision (%C.V.) was 3.8% or better. The accuracy was found to be within 7% or better of the nominal value. The inter-day precision (%C.V.) was found to be 8% or better. The accuracy was also found to be within 7% of the nominal value.

3.4. Specificity

Table 2 lists various compounds that were injected onto the chromatographic system which showed no interferences. Those compounds which were injected as extracted patient samples are designated as well.

3.5. Recovery

Mean extraction and processing recoveries for the corticosteroids ranged from 72 to 78%; all standard deviations were less than 4%.

3.6. Stability

The mean ($\pm$ S.D.) ratio of calculated steroid concentrations (new/old result) indicated that the compounds are stable over 3 years at ≤ -20°C: 1.25 ($\pm$ 0.34) for prednisone ($n = 20$), 1.02 ($\pm$ 0.15) for cortisol ($n = 14$), and 1.10 ($\pm$ 0.09) for prednisolone ($n = 20$).

Process stability results are shown in Fig. 2. Least-squares regressions of quality control val-

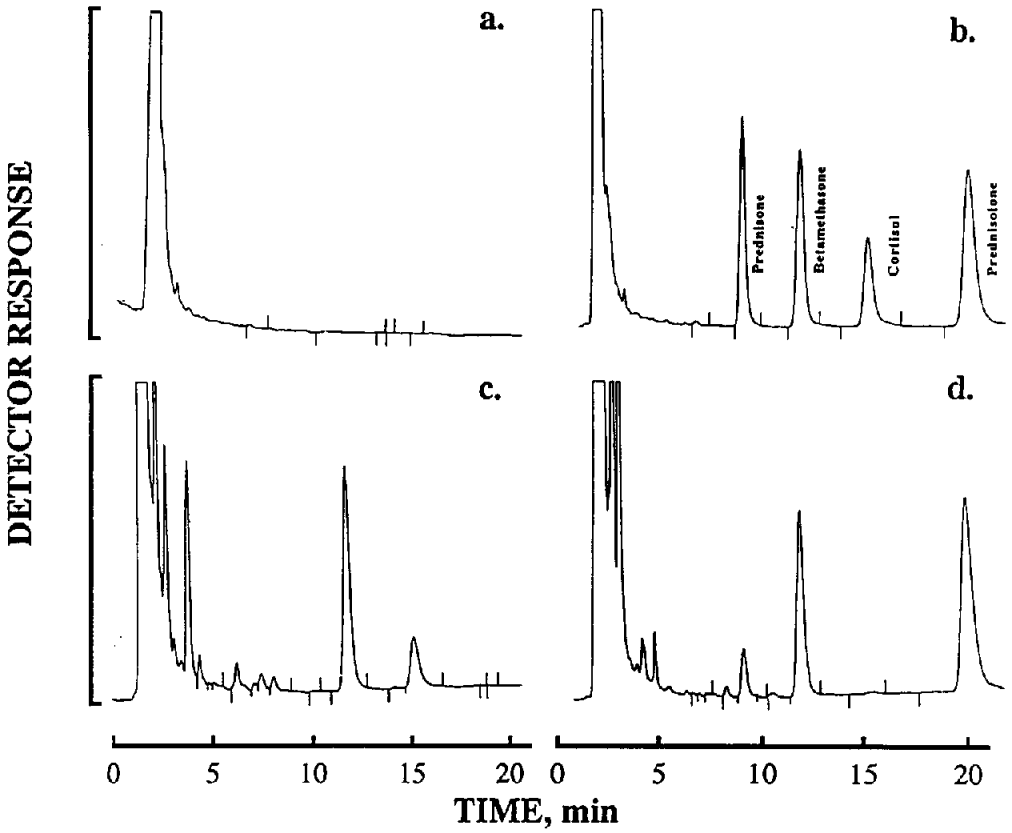

Fig. 1. Chromatograms of: (a) blank standard, (b) standard containing 150 ng/ml prednisone, 147 ng/ml cortisol, and 250 ng/ml prednisolone (internal standard betamethasone, 200 ng), (c) predose patient sample containing cortisol, and (d) postdose patient sample with cortisol suppressed. Full scale represents 4 mV.

ues *vs.* time yield a significant difference ($p < 0.05$) for three of the nine groups tested. The low control for prednisone indicates an 8% lower result after 833 min (19.9 at $t = 0$, 18.3 ng/ml at $t = 833$ min). The high controls for cortisol and prednisolone indicate a rise in sample concentrations of 3 and 5% during the same time period.

3.7. Specimen type

ANOVAs for cortisol and prednisolone assayed in various types of serum or plasma showed no statistical differences ($p < 0.05$) between specimen types; the ANOVA for prednisone showed a significant difference between serum, EDTA-plasma, heparinized plasma and stripped plasma. The Bonferroni tests revealed that the only significant difference was found between EDTA-anticoagulated plasma and stripped plasma when prednisone was measured. Notably, the mean of these two groups differed by only 4 ng.

4. Discussion

Since the 1970s, many HPLC assays for corticosteroids have been developed and published. The two most common methods are ours [1] which was cited 140 times (according to the Science Citation Index) and that of Frey *et al.* [21], cited 113 times. A review of the literature reveals that although such assay methods are

Table 1
Characteristics of operation of the corticosteroid assay

Parameter	Prednisone	Cortisol	Prednisolone
Detection limit (ng/ml)	5	5	5
LOQ (ng/ml)	10	10	10
HOQ (ng/ml)	1000	1000	1000
Intra-assay statistics (at 20–630 ng/ml)			
Precision (C.V.%)	≤ 3.8%	≤ 3.1%	≤ 3.6%
Accuracy (% error)	≤ ± 7.2%	≤ ± 11.3%	≤ ± 5.1%
Inter-assay statistics (at 20–630 ng/ml)			
Precision (C.V.%)	≤ 7.2%	≤ 6.4%	≤ 8.0%
Accuracy (% error)	≤ ± 3.4%	≤ ± 7.3%	≤ ± 6.5%
Recovery[a] (%)			
250 ng/ml	72.0%	71.7%	72.1%
600 ng/ml	73.8%	77.2%	78.0%

[a]Betamethasone at 4 μg/ml = 75.8%.

numerous, most are quite similar. Prior to chromatography, drugs in biological samples must be partially isolated by liquid–liquid [1,21] or solid-phase extraction [22,23]. A variety of reversed- [24,25] and normal-phase [12,26] chromatographic procedures are possible, but most methods utilize ultraviolet absorbances at 240 to 254 nm. Limits of detection for these methods typically are 5 ng/ml or higher. Use of HPLC fractionation with subsequent radioimmunoassay [27] or derivatization with a fluorescence marker [28] allow for lower limits of quantitation, but are technically more demanding. Despite the advances in laboratory technologies during the past decade, the present methodology has continued to adequately serve our analytical needs for pharmacokinetic and pharmacodynamic studies.

Table 2
Drugs which do not interfere with the corticosteroid assay

Cyclosporine*
Rapamycin*
Tacrolimus (FK506)*
Ketoconazole*
Tenidap*
Ethinylestradiol*
Levonorgestrel*
Tetrahydrocortisone
5-β-Pregnone-3α-11β-17α-21-tetrol-20-one
5-α-Pregnone-3α-11β-17α-21-tetrol-20-one
11-β-Hydroxyetiocholanolone
11-Ketoetiocholanolone

* Including plasma samples from treated patients.

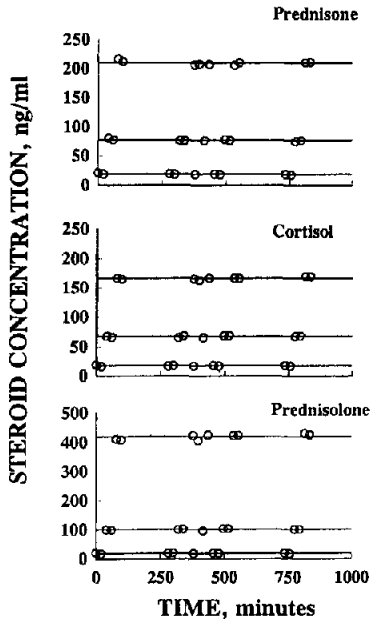

Fig. 2. Analytical results for quality control samples of three corticosteroids maintained in the automated injector tray for the indicated times.

This HPLC methodology is shown to present a precise, reproducible, specific and accurate method to measure prednisone, prednisolone and cortisol in human and animal plasma or serum. Although samples were stable for at least three years, we recommend assaying samples as soon as feasible. Standards and controls should be prepared in batches that will suffice for completion of a study. Process stability changes are modest and, since the control samples for each specific corticosteroid did not demonstrate consistent trends, the significance found is probably not meaningful. The same argument applies to the statistical difference found between EDTA-anticoagulated plasma and stripped plasma for prednisone. Since the difference between the means was only 4 ng, which is lower than the limit of quantitation for this method (10 ng/ml), this difference is most likely random and not meaningful.

Occasionally, patient groups will demonstrate an unidentified chromatographic peak just prior to or after components of interest. By lowering the ethanol content of the mobile phase, most of these potential interferences can be eliminated. Also, sometimes a large peak will appear within ten minutes after the last component of interest (prednisolone) and interfere with the next sample chromatogram. Careful prescreening of new patient groups will allow the technician to adjust the chromatographic parameters as needed.

This assay has also been utilized in our laboratory for pharmacokinetic studies in rats [7,8] and rabbits [13]. Both tissue and plasma samples from animals were analyzed. The tissues must first be homogenized in buffer and 1 g or less of the homogenate is analyzed. Of course, cortisol is not present in either of these species, but corticosterone may be easily measured. Methylprednisolone [16,17] and cortexolone [15] can also be quantitated by making minor adjustments to the methodology.

We suggest that the assay be implemented with the following considerations [29]:

Quality control: With each set of patient samples, a standard curve and two curves of each quality control samples at three different concentrations (low, mid and high range), should be run. Chromatographic parameters should be tested daily prior to sample analysis. A series of four identical injections of the compounds of interest should be made to test the proper operation of the HPLC system. The relative standard deviation for the measurement of the peak height of each compound should not exceed 5%. Retention times must be ≥ 0.5 min apart. The chromatographic conditions are judged to be operational if these criteria are met.

Data acceptability: A standard curve is considered acceptable if the curve contains at least 5 (out of 7 used) standard concentration values with all standards exhibiting $\leq \pm 15\%$ of their nominal concentration. The percent error of the predicted value should not exceed $\pm 15\%$ of the nominal value in more than two of the six quality control samples with at least one quality control at each concentration falling within range.

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