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Original Research Paper

Reliable Estimation of Prednisolone, A Glucocortico- Steroid in Human Plasma by LC-MS/MS

Science

KEYWORDS : prednisolone; prednisolone-d6; solid phase extraction, LC-MS/MS; human plasma

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ABSTRACT

An accurate and precise method was developed and validated using LC-MS/MS to quantify prednisolone in human plasma. The merits of this method include solid phase extraction with high extraction recovery using deuterated internal standard (IS) and short chromatographic run time. The analyte and prednisolone-d6 (IS) were extracted from 500 µL plasma volume using solid-phase extraction on OASIS WCX (1cc, 30mg) cartridges. Chromatographic analysis was performed on a Gemini C18 (150 × 4.6 mm, 5 µm) column using acetonitrile-0.5 % acetic acid in water (50:50, v/v) as the mobile phase. Tandem mass spectrometry in positive ionization mode was used to quantify prednisolone and prednisolone-d6 by multiple reaction monitoring. Entire data processing was done using Watson LIMSTM software which provided excellent data integrity, high throughput with improved operational efficiency. The calibration graph was linear in the range of 2.0–1000 ng/mL. The accuracy and precision values for intra- and inter-batch values ranged from 94.7 to 103.8 % and 1.1 to 6.3 %, respectively. The mean overall recovery across all quality control levels was ≥ 80 % for both analyte and IS, while matrix factors ranged from 1.00 to 1.06 which were evaluated for the assessment of matrix effect.

INTRODUCTION

Prednisolone (PRED), (11β)-11,17,21-Trihydroxy pregna-1,4-diene-3,20-dione (**Fig. 1**), is a synthetic glucocorticoid, a derivative of cortisol, used to treat a variety of inflammatory and autoimmune conditions and some cancers. It is the active metabolite of the drug prednisone [1] and is used especially in patients with liver failure, as these individuals are unable to metabolize prednisone into active PRED; it is primarily metabolized via the liver enzyme, 11-β-hydroxydehydrogenase [2]. PRED is a man-made form of a natural substance (corticosteroid hormone) made by the adrenal gland. It is used to treat conditions such as arthritis, blood problems, immune system disorders, skin and eye conditions, breathing problems, cancer, and severe allergies. It decreases the immune system's response to various diseases to reduce symptoms such as pain, swelling and allergic-type reactions. As a glucocorticosteroid, unauthorized or adhoc use of PRED during competition via oral, intravenous, intramuscular or rectal routes is banned under World Anti-doping Agency (WADA) anti-doping rules [3]. The drug may be used in competition with a TUE (Therapeutic Use Exemption), in compliance with WADA regulations. Local or topical use of PRED during competition as well as any use out of competition is not regulated.

Earlier methods developed for the detection for PRED are based on gas chromatography (GC) [4, 5] coupled with mass spectrometry (MS) [6, 7], which allows for distinction of prednisolone from endogenous corticosteroids; since the derivatization step prior to GC–MS analysis is cumbersome and has limited application. Other methods including high-performance liquid chromatography (HPLC) and radioimmunoassay have been widely used for the determination of prednisolone in biological fluids [8–11]. However, these methods do not meet the modern drug discovery and development needs in terms of short run time, high sensitivity, and highly efficient sample preparation procedures.

The objective of the work described here was to develop and validate a sensitive, simple, and robust high-throughput LC–MS/MS method that could be used easily for the determination PRED in human plasma. The SPE sample preparation process is one of the preferred techniques used in assay development because it provides higher purity extracts without matrix interference than either LLE or PPT. After successful development and validation of this automated method, it can be utilized in sample analysis for a clinical pharmacokinetic study. The assay demonstrated accuracy, reproducibility, and rigor in a high-throughput analysis of samples in established dynamic linear range.

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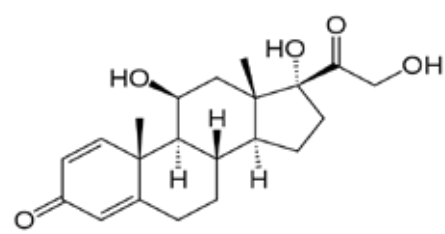

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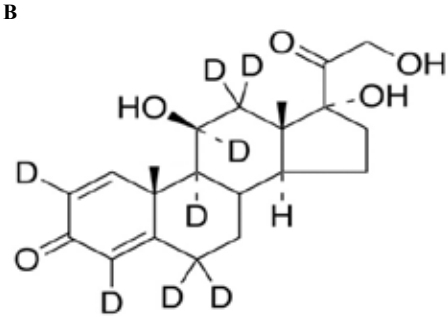

Fig. 1. Chemical structure of (A) Prednisolone and (B) Prednisolone-d6

Chemicals and materials

Reference standard of PRED (99.4 %) and PRED-d6 (IS, 98.0 %) were procured from Clearsynth Labs Ltd. (Mumbai, India). Acetic acid and *ortho*-phosphoric acid were purchased from Merck Pvt. Ltd. (Mumbai, India). HPLC grade acetonitrile and methanol were procured from Spectrochem Pvt. Ltd. (Mumbai, India). OASIS WCX (1cc, 30mg) extraction cartridges were obtained from Waters (MA, USA). Milli-Q water was prepared from Millipore water purification system (Bangalore, India). Blank human plasma with K_3EDTA as anticoagulant was obtained from Supratech Micro Pathology (Ahmedabad, India) and was stored at –20 °C until use.

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CHROMATOGRAPHIC AND MASS SPECTRO-METRIC CONDITIONS

A Shimadzu LC-VP HPLC (Kyoto, Japan) system interfaced with MDS SCIEX API-4000 (Toronto, Canada) triple quadrupole mass spectrometer and equipped with electro spray ionization having positive ionization mode was used in the present work. Chromatographic analysis of PRED was carried out on Phenomenex Gemini C18 (150 × 4.6 mm, 5 µm) column using acetonitrile-acetic acid in water (0.5 % *v/v*) (50:50, *v/v*) as the mobile phase delivered at a flow rate of 1.0 mL/min. The column temperature was 40 °C and the auto sampler temperature was maintained at 5 °C. The pressure of the system was 1000 psi. The total eluant from the column was split in 75:25 ratio; flow directed to the ion spray interface was equivalent to 250 µL/min.

Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor protonated precursor → product ion transitions at *m/z* 361.3 → 325.3 for PRED and *m/z* 367.1 → 331.3 for PRED-d6. The source dependent parameters maintained for PRED and IS were Gas 1 (Nebuliser gas): 50.0 psig; Gas 2 (heater gas flow): 50.0 psig; ion spray voltage: 5500 V, turbo heater temperature: 450 °C; entrance potential: 10 V; collisional activation dissociation: 10 psig and curtain gas (nitrogen): 20 psig. The optimum values for compound dependent parameters like declustering potential, collision energy and cell exit potential were set at 80 V, 46 eV and 15 V for PRED and IS respectively. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 200 ms. Analyst software version 1.4.2 was used to control all parameters of LC and MS. Watson LIMS™ software version 7.4 was used for regression and final data processing.

Calibrators and quality control samples

The standard stock solution of PRED (1.0 mg/mL) was prepared by dissolving accurately weighed reference standard in methanol. Calibration standards (CSs) and quality control (QC) samples were prepared by spiking blank plasma with stock solution. CSs were made at concentrations of 2.00, 4.00, 10.0, 25.0, 50.0, 100, 200, 500, 1000 ng/mL, while QC samples were prepared at five concentration levels, 800 ng/mL (HQC, high quality control), 400/90.0 ng/mL (MQC1/2, medium quality control), 6.00 ng/mL (LQC, low quality control) and 2.00 ng/mL (LLOQ QC, lower limit of quantification quality control). Stock solution (1.0 mg/mL) of the internal standard was prepared by dissolving accurately weighed PRED-d6 in appropriate volume of methanol. Its working solution (0.5 µg/mL) was prepared by appropriate dilution of the stock solution in methanol. Standard stock and working solutions used for spiking were stored at 5 °C, while CSs and QC samples in plasma were kept at -70 °C until use.

Plasma sample preparation

Prior to analysis, CSs and QC samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 500 µL of spiked plasma sample, 50 µL of IS (0.5 µg/mL), 300 µL of *ortho*-phosphoric acid in water (4 % *v/v*) was added and vortexed for 10s, followed by centrifugation at 2147 × g for 3 min at 10 °C. Load 0.750 mL of prepared samples on extraction cartridges OASIS WCX (1cc, 30mg) which was previously conditioned with 1.0 mL of methanol followed by 1.0 mL of acetic acid in water (0.5 % *v/v*). Elute the samples by applying positive or negative pressure of gravitational force with respect to solid phase extraction assembly. Wash the cartridges with 1.0 mL of acetic acid in water (0.5 % *v/v*) followed by 1.0 mL of methanol in water (5 % *v/v*). Elute the content from the cartridges with 1 mL of acetic acid in water (0.5 % *v/v*): methanol (50:50 *v/v*). The elution solution was collected and evaporated to dryness in a thermostatically controlled water-bath maintained at 40 °C under a gentle stream of nitrogen. The dried samples were reconstituted with 100 µL of reconstitution solution of acetic acid in water (0.5% *v/v*): acetonitrile (20:80 *v/v*), briefly vortexed and 10 µL was used for injection in the chromatographic system.

VALIDATION PROCEDURE

The bioanalytical method was validated as per the USFDA guidelines [12]. System suitability test was performed by injecting six successive injections using aqueous standard mixture of PRED and IS at the start of each batch. Carryover effect of autosampler was verified by sequentially injecting extracted blank plasma → ULOQ sample → extracted blank plasma → LLOQ sample.

The selectivity of the method for endogenous plasma matrix components was evaluated in ten different batches of blank plasma (7- normal K_3EDTA plasma and 1 each of lipemic, haemolysed and heparinised plasma). These sets were processed along with freshly prepared CSs and qualifying QC samples in duplicate using normal plasma lots. As per the acceptance criterion, % accuracy of lipemic and haemolytic samples should be within 85 to 115%.

The linearity of the method was determined by analysis of five calibration curves containing nine non-zero concentrations. The area ratio response for analyte/IS obtained from MRM was used for regression analysis. The calibration curves were analyzed individually by using least square weighted ($1/x^2$) linear regression. The lowest standard on the calibration curve was accepted as the LLOQ, if the analyte response was at least ten times more than that of drug free (blank) extracted plasma.

Intra-batch accuracy and precision for PRED was determined by analyzing six replicates of QC samples along with calibration curve standards on the same day. The inter-batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive days.

The extraction recovery of PRED and IS was estimated by comparing the mean area response of samples spiked before extraction to that of extracts with post-spiked samples (spiked after extraction) at three QC levels. Matrix effect, expressed as matrix factors (MFs) was assessed by comparing the mean area response of post-extraction fortified samples with mean area of solutions prepared in mobile phase solutions (neat standards).

The standard stock solutions of PRED and IS were evaluated for short term and long term stability at 25 °C and 5 °C respectively. The analyte stability in spiked plasma samples was evaluated by measuring the area ratio response (PRED/IS) of stability samples against freshly prepared standards having identical concentration. Bench top (at room temperature), processed sample stability at room temperature and at refrigerated temperature (5 °C), dry extract (-20 °C), freeze-thaw (-20 °C and -70 °C) and long term (-20 °C and -70 °C) stability of PRED in plasma was studied at LQC and HQC levels.

Method ruggedness study was done with two precision and accuracy batches. The first batch was analyzed by a different analyst while the second batch was studied on two different columns of the same make having different batch no. Dilution integrity experiment was evaluated by preparing the spiked standard at 1500 ng/mL concentration for 1/10 and 1/2 dilutions in the screened plasma. The pre-

cision and accuracy for dilution integrity standards at 1/10 (150 ng/mL) and 1/2 (750 ng/mL) dilution were determined by analyzing the samples against freshly prepared calibration standards.

RESULTS AND DISCUSSION

LC-MS/MS method development

MS and tandem MS were obtained by the infusion of PRED solution via a tee connection between the LC column and mass spectrometer inlet. The ESI conditions were optimized so as to have predominant protonated precursor $[M-H]^+$ ions at *m/z* 361.3 for PRED and *m/z* 367.1 for IS in the Q1 MS full scan spectra. In the product ion mass spectrum the most consistent and intense fragments were observed at *m/z* 325.3 and 331.3 for PRED and IS respectively by applying 46 eV collision energy.

In the present work, SPE was carried out on OASIS WCX (30 mg, 1 cc), which required minimal steps for sample cleanup and ensured quantitatative and precise recovery at all QC levels for the analyte and IS (**Table 1**). Initially, sample was treated with *ortho*-phosphoric acid to breakdown plasma protein binding of PRED by precipitating the proteins as PRED is 75 % protein bound.

TABLE NO. 1
EXTRACTION RECOVERY AND MATRIX FACTOR FOR ANALYTE & IS

QC levels	Prednisolone	Prednisolone-d6
	Extraction recovery (%)	
HQC	85.7	88.4
MQC-1	83.1	84.2
MQC-2	81.6	81.3
LQC	83.7	85.6
	Matrix factor	
HQC	1.01	1.03
MQC-1	1.06	1.09
MQC-2	1.04	1.03
LQC	1.00	1.12

Chromatographic conditions were suitably optimized under isocratic conditions to get adequate response, acceptable peak shape and a short analysis time on Gemini C18 (150 × 4.6 mm, 5 µm) column. Various combinations of organic diluents (methanol/ acetonitrile) together with acidic buffers (ammonium formate/formic acid, ammonium acetate/acetic acid) with different ionic strengths (2-8 mM) in the pH range of 3.5-5.5 were tested. The best mobile phase system which afforded adequate retention and peak shape was acetonitrile-0.5 % acetic acid in water (50:50, *v/v*). The analyte and IS were eluted within 3.5 min with retention times of 2.23 and 2.22 min respectively. PRED-d6, a deuterated IS adequately compensated for any variability during sample extraction and MS ionization. Representative MRM ion chromatograms in **Fig. 2a-d** verify the selectivity of the method to differentiate and quantify the analyte from endogenous components in the plasma matrix.

Validation results

The precision values for system suitability ranged from 0.05 to 0.24 % for the retention time and 0.41 to 2.14 % for the area ratio response of PRED/IS. The evaluation of autosampler carry-over was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. There was practically negligible carry-over (≤ 0.003 %) during carryover experiment in extracted blank plasma (without IS and analyte) after

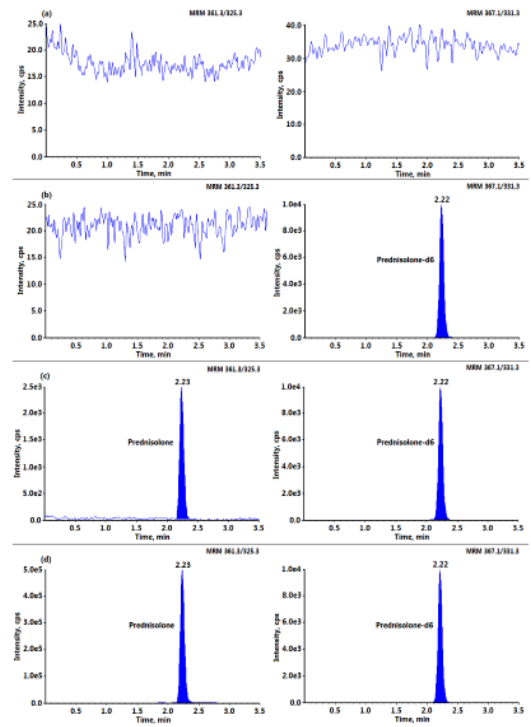

Fig. 2. Representative MRM ion-chromatograms of (a) double blank plasma (without analyte and IS), (b) blank plasma with working solution of prednisolone-d6, IS (c) prednisolone at LLOQ & IS and (d) prednisolone at ULOQ & Isubsequent injection of highest CS at the retention time of PRED and IS.

All five calibration curves showed good linearity ($r^2 \geq 0.9995$) through the studied concentration range of 2.00-1000 ng/mL. The accuracy and precision (% CV) observed for the calibration curve standards ranged from 99.2 to 101.1 % and 1.08 to 2.70 % respectively.

The intra-batch and inter-batch precision and accuracy results for PRED across five QC levels are shown in **Table 2**. The intra-batch precision (% CV) ranged from 1.14-6.07 % and the accuracy was within 94.8-102.8 %. Similarly for inter-batch experiments, the precision varied from 1.84-5.57 % and the accuracy was within 94.6-101.0 %.

TABLE NO. 2
INTRA- & INTER-BATCH PRECISION & ACCURACY

Nominal concentration (ng/mL)	Intra-batch		
	Mean conc. (ng/mL)	% CV	% Accuracy
HQC (800)	783	1.14	97.8
MQC-1 (400)	404	1.19	101.0
MQC-2 (90.0)	92.6	2.49	102.8
LQC (6.00)	5.69	2.97	94.8
LLOQ (2.00)	1.96	6.07	98.0
	Inter-batch		
HQC (800)	790	1.84	98.7
MQC-1 (400)	404	1.95	101.0
MQC-2 (90.0)	90.4	2.96	100.4
LQC (6.00)	5.68	2.69	94.6
LLOQ (2.00)	2.01	5.57	100.5

The mean extraction recovery and matrix factors for PRED are shown in **Table 1**. The recovery obtained was consistent, ranging from 81.3 to 88.4 % across four QC levels for both PRED and PRED-d6. As co-eluting matrix components can directly impact the overall reliability of a validated method, therefore it is suggested to compute matrix factors

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to assess the matrix effect. For the analyte and IS, matrix factors ranged from 1.00 to 1.12.

The stock solutions kept for short-term and long-term stability as well as spiked plasma samples showed no evidence of degradation under all studied conditions. Samples for short-term stability remained stable up to 10 h, while the stock solutions of PRED and IS were stable for minimum period of 114 days at refrigerated temperature of 5 °C. No significant degradation was observed for the analyte during sample storage and any of the processing steps during extraction. The detailed results for stability studies are presented in **Table 3**.

TABLE NO. 3
STABILITY OF PREDNISOLONE IN PLASMA UNDER VARIOUS CONDITIONS

Stability conditions	% change at two levels
Bench top stability at room temperature (25 °C), 9 h	HQC: -5.81 LQC: 7.17
Freeze & thaw stability after 5th cycle at -20 °C	HQC: -4.54 LQC: 2.83
Freeze & thaw stability after 5th cycle at -70 °C	HQC: -3.90 LQC: 5.14
Autosampler reinjection reproducibility at 5±3 °C, 90 h	HQC: -4.26 LQC: 6.17
Wet extract stability at 25 °C, 4 h	HQC: -1.58 LQC: 2.59
Dry extract stability in deep freezer at -20°C, 75 h	HQC: -3.47 LQC: 0.98
Long term stability at -20 °C, 68 days	HQC: -2.89 LQC: 1.54
Long term stability at -70 °C, 68 days	HQC: -3.33 LQC: 6.57

The precision (% CV) and accuracy for method ruggedness with different columns, analysts and equipments ranged from 1.42 to 2.70 % and 97.8 to 103.0 % respectively at five QC levels. The precision (% CV) for dilution reliability of 1/2 and 1/10th were between 1.59 and 1.96 %, while the accuracy results were within 101.1-101.7 % respectively, which is within the acceptance limit of 15 % for precision (% CV) and 85 to 115 % for accuracy.

Conclusion

The proposed validated LC-MS/MS assay provides a reliable and rugged approach for the quantitation of PRED in human plasma The SPE procedure afforded highly selective separation of the analyte and IS from endogenous components enabling quantification of 2.00-1000 ng/mL employing 500 μL plasma volume. The method is extensively validated for matrix effect and stability under different storage conditions. The analytical method was proven to be consistent and reproducible for determination of PRED from human plasma with minimum interference and short chromatographic run time (3.5 min). The bioanalytical methodology for PRED described in the present work can be highly useful for therapeutic drug monitoring of PRED for analysis of routine samples of single dose or multiple dose pharmacokinetics and also for the clinical trial samples.

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