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Research Article

PHARMACOKINETIC DETERMINATION OF METHYLPREDNISOLONE IN INDIAN HEALTHY VOLUNTEERS BY LC-MS/MS

Das Dibya[1], Halder Dhiman[2], Bose Anirbandeep[3], Maji Himangshu Sekhar[1*], Pal Tapan Kumar[2, 3]

1. Department of Pharmaceutical Technology, JIS University, Kolkata, West Bengal, India.
2. Bioequivalence Study Centre, Dept. of Pharm. Tech., Jadavpur University, Kolkata, West Bengal, India.
3. TAAB Biostudy Services, Jadavpur, Kolkata, West Bengal, India.

ABSTRACT

Methylprednisolone is a synthetic glucocorticoid. It relieves inflammation and uses to treat thyroid, colitis, allergies, asthma and many more. This study aims to develop a bio-analytical method for measuring methylprednisolone in human plasma, accurate, sensitive, rapid, precise and simple. In this study, Propranolol used as an Internal Standard (IS). Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) instrument was used to identify and quantify the drug. Gradient conditions were used to establish the method; with a flow rate of 0.5 ml/min, we used 0.1 % formic acid in Milli-Q water and 0.1 % formic acid in methanol as mobile phases. A C18 Phenomenax Kinetex (50x3 mm, 5µ) column was used to isolate the analyte and the IS. The total chromatographic run time 7.0 min. The extraction of analyte and IS from plasma is done by a simple protein precipitation technique. The method was very selective and sensitive. Lower limit of quantification was 12.50 ng/ml. Linearity range between 12.50 ng/ml - 800ng/ml. The precision and accuracy results from 5 validation batches at five different concentration levels were well within acceptable ranges. The analyte was stable under different stability conditions. The developed method was found reproducible and very simple. The Retention Time of the analyte and IS was 3.53 min and 3.24 min, respectively. The methodology was validated in accordance with EMEA and USFDA regulations. The particular study has been carried out expertly on the pharmacokinetic study and BA/BE study in healthy human male volunteers. No Serious Adverse Events were noted in the clinical phase.

KEYWORDS: Methylprednisolone, LC-MS/MS, Method Development and Validation, Healthy human volunteers, Application in BA/BE Studies.

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CORRESPONDENCE: **Himangshu Sekhar Maji*** ✉ hsmaji@jisuniversity.ac.in **Address** - Department of Pharmaceutical Technology, JIS University, 81,Nilgunj Road, Agarpara, Kolkata,West Bengal, India.

INTRODUCTION

Methylprednisolone is a (6S,8S,9S,10R,11S,13S,14S,17R)-11,17-dihydroxy-17-(2-hydroxyacetyl)-6,10,13-trimethyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthren-3-one[(1)]. Because of their well-known anti-inflammatory and immunosuppressive effects, synthetic glucocorticoids are commonly prescribed for a large number of diseases, including allergic, inflammatory, and autoimmune disorders [(2)].

Methylprednisolone (Figure 1) is a methylated prednisolone derivative that belongs to the glucocorticoid or corticosteroid drug class. The medication has anti-inflammatory properties[(3)] and treatment of arthritis, eye disorders, extreme allergic reactions, blood and immune system disorders. It reduces symptoms including swelling, discomfort, and allergic reactions by lowering the immune system's response to various diseases. In humans, 76-78 % of methylprednisolone was bound to plasma proteins. According to the literature, several LC-MS methods for determining methylprednisolone in biological samples such as human plasma[(4,5)], tissue samples[(6,7,8)], human urine[(9,10)], pig fat[(11)], bovine milk samples[(12,13)], and rat plasma[(14)] have been published. Just two techniques can be compared to the current work out of all the methods previously mentioned. Recently published an LC-MS/MS procedure for determining methylprednisolone in human plasma using a multi-stage solid-phase extraction (SPE) sample preparation technique. The protein precipitation technique (PPT) was used to pre-treat the samples first, after that it was processed by the online SPE technique[(4)]. Another approach for determining methylprednisolone, as well as other glucocorticoids and immunosuppressive drugs. This approach entails complexities such as gradient elution, a standard mobile step consisting of two or more buffers with pH change, a larger sample volume greater than 500 µl and a long chromatographic run time (>7 min), all of which are inconvenient if used for routine drug analysis in pharmacokinetic and BA/BE studies. The remaining analytical methods described so far were unsuitable for routine drug testing. We need a suitable bio-analytical technique for its detection and quantification to research the safety, tolerability, and pharmacokinetics of new formulations. In bio-analytical applications, sensitive and fast analytical techniques such as LC-MS are now the most commonly used. The current research paper details a rapid, simple and selective LC–ESI-MS/MS assay method for the quantification of methylprednisolone in human plasma using propranolol as an internal standard (IS)[(5)]. The proposed LC–ESI-MS/MS

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approach is designed to measure methylprednisolone concentrations in pharmacokinetic and bioequivalence studies. The methodology was validated in accordance with USFDA [15] and EMEA[16] regulations.

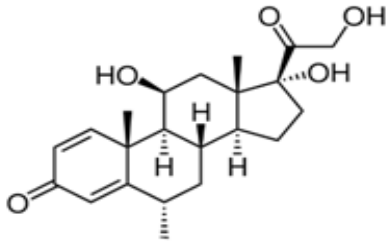

Figure 1: Structure of Methylprednisolone

MATERIAL AND METHODS

Chemicals and Reagents

The sample of methylprednisolone (99.2%) was procured from Steril Gene Life Sciences (P) Ltd., whereas propranolol was obtained from Subham Biopharma. HPLC grade formic acid and methanol and acetonitrile were procured from JT Baker. LC-MS grade water was used for the analysis using Milli-Q water purification at our laboratory. Blank human plasma samples were obtained from M/S TAAB Bio-study Services.

LC-MS/MS instrument and conditions

A HPLC system consisting of a binary LC–20AD prominence pump, an autosampler (SIL-20A) was used for the study. We used a C18 Phenomenex Kinetex (50x3 mm, 5µ) column. The processed samples were injected into the column in aliquots of 10 µl. An isocratic mobile phase consisting of 0.1 % formic acid in Milli-Q water and 0.1 % formic acid in methanol was used to isolate the analyte from the endogenous components. The analyte was quantified using a mass spectrometric detection system, the AB Sciex API-2000, in positive ion mode. The nebulizer gas (GS1), auxiliary gas (GS2), curtain gas, and collision gas were set to 45 psi, 45 psi, 38 psi, and five psi, respectively, as source-based parameters. The source voltage was set to 5500 V, and the interface temperature was set to 400 ºC. The various compound dependent parameters like declustering potential (DP), collision energy (CE), collision cell exit potential (CXP) and entrance potential (EP) were set at 46 V, 44 V, 11 V, 4.6 V for methylprednisolone and 28 V, 25 V, 2 V, 8.50 V for the IS propranolol. For methylprednisolone, multiple reaction monitoring (MRM) modes were used to track the transition pairs of m/z 375.4 precursor ion (Figure 2) to m/z 161.03 product ion (Figure 3), and m/z 260.3 precursor ion to m/z 116.1 product ion for the IS. The transaction's dwell time was set to 100 ms. The data was collected using Analyst software version 1.6.3.

Preparation of spiked plasma samples

In DMSO, a stock solution of methylprednisolone and propranolol at a concentration of 1 mg/ml were prepared, and further dilutions (working solutions) of methylprednisolone and propranolol were prepared. The stock solutions were made and then used to make calibration standards and quality control samples. The working concentration of propranolol was 10 µg/ml and prepared in the same diluent. The total samples were prepared in blank human plasma as a bulk. Calibration standards were prepared at seven (7) concentration levels of 12.50ng/ml, 25ng/ml, 50ng/ml, 100ng/ml, 200ng/ml, 400ng/ml and 800 ng/ml. Similarly, quality control (QC) samples at concentrations of 12.50 (LLOQ), 37.50 (LQC), 300 (MQC), 600 (HQC) ng/ml. The plasma samples were stored at −20 ± 2 °C.

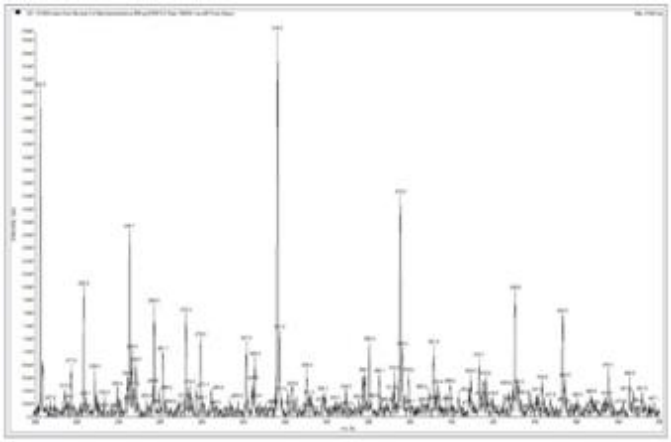

Figure 2: Q1 Scan of Methylprednisolone

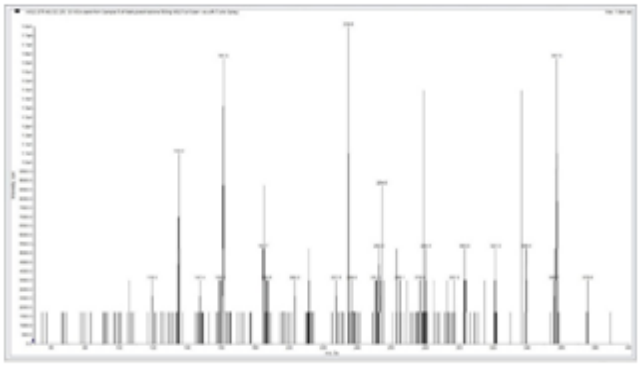

Figure 3: MS2 Scan of Methylprednisolone

Sample extraction protocol

At first, withdraw the spiked blank human plasma samples from the deep freezer and allow them to thaw at room temperature. Then we add 150 µl of aliquot and 50 µl internal standard (Propranolol 10 µg/ml) into 2 ml eppendorf and vortex for 60 s. Then added 300 µl of acetonitrile and 10 minutes vortex. After that, Cold Centrifuge at 12000 rpm for 5 min at 4˚C. At last Separate 300 µl of the supernatant organic layer and transfer into autosmpler vials, and inject the sample into LC-MS/MS. The sample extraction procedure was executed by protein precipitation technique (PPT).

Bio-analytical method development and validation

The procedure was subjected to validation experiments that followed the USFDA guidelines for all of the recommended parameters [17,18]. There are specificity, selectivity, linearity, carryover, precision, accuracy, recovery, and matrix effect and stability studies.

Specificity, selectivity and linearity

The assay's specificity and selectivity were demonstrated by the representative chromatograms of the mobile phase run and the extract of blank plasma recorded for samples. An

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unweighted least square regression analysis was used to evaluate the calibration curve's linearity. The linearity graph depicted a representative calibration curve of methylprednisolone from human plasma.

Carryover

After HQC, blank samples were injected to eliminate the possibility of interference from an injected run from the previous run. It qualifies under guideline if the carryover from a high concentration injection is <20% of the region of LLOQ.

Precision and accuracy

The LLOQ, LQC, MQC, and HQC were used to assess between-run precision and accuracy. On day 1st d, five (5) replicates of each quality control (QC) concentration were tested, and five replicates of each quality control concentration were tested on days 2nd d and 3rd d. The concentrations of quality control samples were calculated using three separate calibration curves tested with quality control samples.

A total of five (5) replicates of each quality control concentration were used to assess within-run precision and accuracy. On day 1, the LLOQ, LQC, MQC, and HQC were tested. The concentrations of quality control (QC) samples were calculated using LIN1 calibration curves. The percent variance (%CV) was used to measure precision, while the percent nominal was used to measure accuracy.

Recovery

To ensure the method's reproducibility, quality control samples spiked with the analyte spiked in blank plasma are used to measure the recovery.

Matrix Effect (ME)

The amount of erratic value created during analysis due to endogenous substances in a biological matrix is known as the matrix effect. The change may result in a significant deviation from the actual value, which is prohibited by the guideline. As a result, the ME was determined using quality control criteria spiked plasma at various concentrations.

Stability

In the present study, the freeze-thaw (FT), auto sampler (AS) stability, short term (ST), Bench top (BT) and long term (LT) had been performed as per the regulatory guidelines.

Pharmacokinetic study design

In healthy Indian male volunteers (n = 24), pharmacokinetic analysis of methylprednisolone was performed. The protocol was approved by the HURIP Independent Ethics Committee after all of the volunteers gave written informed consent. Orally, a single dose of methylprednisolone tablet (16 mg) was given with ± 240 ml water to all the volunteers, and blood samples were collected at 0.25 h, 0.5 h, 1 h, 1.5 h, 1.75 h, 2 h, 2.25 h, 2.5 h, 3 h, 4 h, 5 h, 6 h, 8 h, 10 h, 24 h and 48 h post-dose. At each time point, 5 ml of blood was obtained in K_2EDTA vacutainer tubes. In addition, a pre-dose sample was taken to rule out any potential plasma interferences. Plasma was removed from the samples and stored at -20 °C±2 °C min. Along with QC tests, plasma samples were spiked with the IS and processed at different concentrations. The software tool SAS Version 9.1.3 was used to calculate the pharmacokinetic parameters of methylprednisolone.[19].

RESULTS AND DISCUSSION

Specificity, selectivity and linearity

In human blank plasma, no interfering peaks were observed at analyte retention times. It was discovered that the developed analytical method was precise and selective in its application. The R2 value of 0.9926 (Figure 4) was recorded for the seven (7) points calibration curve of the analyte. The developed method has a linearity present over a range of 12.50 ng/ml to 800 ng/ml for the analyte.

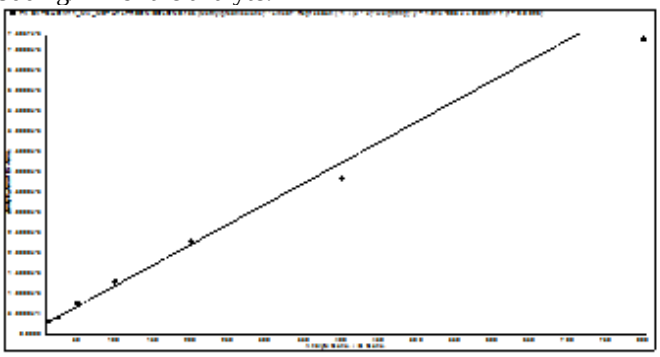

Figure 4: Calibration Curve of Methylprednisolone

Carryover

For desired precision and accuracy of an analytical method, carryover evaluation is essential. No significant carryover was found in the blank sample after injection HQC sample. The carryover was not affected the proposed method.

Precision and accuracy

Inter day and Intra-day precision and accuracy results are shown in Table 1a and Table 1b.

Table 1a: Inter day precision and accuracy results

QC Samples (ng/ml)		Interday (Between Run)			
		Mean Concentration (ng/ml)	SD	%CV	Accuracy (%)
LLOQ	12.50	12.76	1.43	11.19	102.06
LQC	37.50	38.76	3.19	8.25	103.37
MQC	300	296.38	27.37	9.24	98.79
HQC	600	561.03	52.51	9.36	93.51

Table 1b: Intra-day precision and accuracy results

QC Samples (ng/ml)		Intra-day (Within Run)			
		Mean Concentration (ng/ml)	SD	%CV	Accuracy (%)
LLOQ	12.50	11.84	0.81	6.87	94.72
LQC	37.50	40.51	2.14	5.28	108.02
MQC	300	263.27	8.92	3.39	87.76
HQC	600	540.07	19.42	3.60	90.01

Recovery Analyte

The projected protein precipitation technique method, showing acceptable recoveries for the analyte and the IS. The recovery results securely for analyte at LQC, MQC and HQC concentrations were 94.9%, 95.5% and 94.6%, respectively.

Matrix Effect (ME)

There was no significant ME, ion suppression, or enhancement when the Area under curve ratios of extracted quality controls and Internal Standard were compared to the Area under curve ratios of unextracted quality controls, and Internal Standard (IS) obtained from the injecting aqueous solution prepared at the same concentrations.

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Stability

The stability studies study results shown in table 2.

Table 2: Stability studies

Storage Conditions	Interday (Between Run)			
	QC Samples (ng/ml)		Mean (ng/ml)	Accuracy (%)
Freshly Thawed	LQC	37.50	39.14	
	MQC	300	339.37	
	HQC	600	633.89	
Freeze and Thaw Stability at -20°C	LQC	37.50	35.74	91.32
	MQC	300	332.07	97.85
	HQC	600	542.93	85.65
Autosampler Stability 24 h	LQC	37.50	34.87	89.09
	MQC	300	301.10	88.72
	HQC	600	601.07	94.82
Short Term Stability 24 h	LQC	37.50	36.71	93.81
	MQC	300	288.72	85.07
	HQC	600	547.42	86.36
Bench Top Stability for 8 h	LQC	37.50	38.72	98.95
	MQC	300	295.56	87.09
	HQC	600	544.38	85.88
Long Term Stability at -20 °C for 10 d	LQC	37.50	37.65	96.20
	MQC	300	311.55	91.80
	HQC	600	583.80	92.10

As per the guideline, the acceptance criteria LLOQ is 80%-120%, LQC, MQC and HQC is 85%-115%. As per the Inter day and Intra-day precision and accuracy results and stability studies result, all the accuracy values are in the acceptance range as per the guidelines.

Pharmacokinetic study (n=24)

Pharmacokinetic parameters in healthy human volunteers with the Reference and Test Formulation shown in table 3.

Table 3: Pharmacokinetic parameters results

Pharmacokinetic parameters	Reference Formulation		Test Formulation	
C_{max} (ng/ml)	Mean	124.50	Mean	119.04
	± SD.	5.95	± SD.	7.70
t_{max} (h)	Mean	1.23	Mean	1.67
	± SD.	0.29	± SD.	0.12
AUC $_{0-t}$ (ng. h/ml)	Mean	1281.28	Mean	1176.99
	± SD.	275.03	± SD.	196.80
AUC $_{0-\infty}$ (ng. h/ml)	Mean	1548.23	Mean	1691.06
	± SD.	241.47	± SD.	346.49
k_{el} (h^{-1})	Mean	0.040	Mean	0.039
	± SD.	0.004	± SD.	0.004
$t_{1/2}$ (h)	Mean	17.40	Mean	17.84
	± SD.	1.81	± SD.	1.90
Relative Bioavailability (%)	100 %		91.86%	

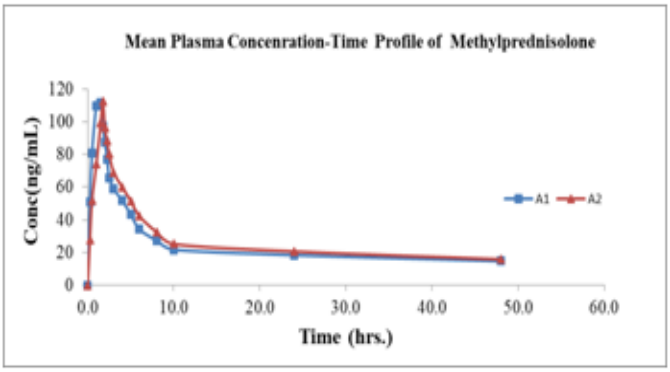

Figure 5: Mean Plasma concentration-time Profile of Methylprednisolone.

CONCLUSION

For quantification of methylprednisolone in human plasma, the LC–MS/MS bio-analysis described here is easy, efficient, specific, and sensitive and the method was validated according to USFDA and EMEA guidelines. The analyte was consistently and reliably recovered from human plasma using a simple protein precipitation technique extraction procedure. For this reason, minimizes the chances of errors, saves considerable time. We used 100 µl plasma for validation plasma analysis and 150 µl plasma for human plasma analysis. The method provided good linearity. The analytes stability in plasma samples and aqueous samples has been rigorously tested under a variety of storage conditions. The sample retention time was less than 3.6 min, making it an appealing approach for high-throughput methylprednisolone bio-analysis. The approach was found to be accurate and repeatable enough to support the pharmacokinetic studies in healthy human volunteers. The C_{max} of the test drug was 119.04 ± 7.70 (ng/ml) and T_{max} was 1.67 ± 0.12 (h). The developed and validated method was useful for bioavailability and bioequivalence (BA/BE) investigations. And, depending on the findings of all validation parameters, routine therapeutic drug monitoring with the appropriate precision and accuracy. During the clinical process, no SAEs were observed.

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