wjpmr, 2019,5(3), 300-306

SJIF Impact Factor: 4.639

WORLD JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH
www.wjpmr.com

Research Article
ISSN 2455-3301
WJPMR

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF BUDESONIDE IN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

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Article Received on 21/01/2019 **Article Revised on 09/02/2019** **Article Accepted on 01/03/2019**

ABSTRACT

Budesonide is a nonhalogenated corticosteroid exhibits potent glucocorticoid and weak mineralocorticoid activity. In the present research work, a novel and rapid LC-MS/MS method was developed and validated for the determination of budesonide in human plasma. Levonorgestrel was used as an internal standard (IS) to quantify budesonide in plasma. Sample preparation was performed by simple liquid-liquid extraction method and chromatographed on a C8 column with isocratic mobile phase of acetonitrile and 5mM ammonium formate in 0.1% formic acid in the ratio of 60:40, v/v used for the separation of extracted analyte. The budesonide linearity was established in the range of 0.10 – 3.00 ng/mL. Variety of validation parameters namely precision and accuracy, linearity, dilution integrity, recovery and matrix effect were well within the acceptable limits. Also, the stability of analyte was extensively evaluated in plasma as well in extracted samples and results met the acceptance criteria as per the regulatory guidelines.

KEYWORDS: Budesonide, human plasma, liquid-liquid extraction, LC–MS/MS, pharmacokinetics.

I. INTRODUCTION

Budesonide is a glucocorticoid used in the management of asthma, the treatment of various skin disorders, and allergic rhinitis. Budesonide is one of the most extensively used inhaled glucocorticoids and has a high ratio of topical anti-inflammatory to systemic activity. Budesonide decreases airway hyperresponsiveness and reduces the number of inflammatory cells and mediators present in the airways of patients with asthma. The drug acts by way of a cytoplasmatic glucocorticoid receptor (GR), to which it binds after passing through the cell membrane. This inhibits the synthesis of pro-inflammatory factors and induces anti-inflammatory factors and the transcription of β2-receptor genes.[1-3] Budesonide is approved for the treatment of mild-to-moderate active Crohn's disease. Budesonide is subject to high first-pass metabolism (80-90%).[4,9]

A review of literature reveals many LC-MS/MS methods,[7,9] have been reported for determination of budesonide alone or simultaneously along with other drugs in dog plasma,[7,8] human plasma,[9-5] human sputum samples,[31] and urine or serum samples,[14,15] Of all the methods reported earlier few methods were comparable with the present method in human plasma. Szeitz *et al.,* 2014,[9] reported a method for the determination of cortisol and budesonide simultaneously using UPLC-MS/MS. Dexamethasone used as an internal standard. The LLOQ was 1.0 ng/mL and employs LLE for sample preparation. The run time was 4 min which is high and not suitable routine for drug analysis. Qu *et al.,* 2007,[10] reported a highly sensitive method for the determination of budesonide, dexamethasone, triamcinolone acetonide, and dexamethasone acetate simultaneously in the range of 5-5000 pg/mL. This method employs SPE for the sample preparation and chromatographed on micro LC column. The run time was >5 min, which is considered high period. Also, since this method is used for the determination of Budesonide and other corticosteroids, it can create a conflict in the determination of analyte of interest Similarly, Wang *et al.,* 2003,[11] was reported a similar method for the determination of budesonide and its metabolites. 9-fluoro-hydrocortisone was used as an internal standard. This method was also employed SPE technique for the sample preparation. A semi-automated method for quantification of budesonide in human plasma was developed and validated by Nilsson *et al.,* 2014,[12] This method used 96-well SPE plates making it expensive for research, Likewise, most

of the reported methods are having limitations in terms of plasma volume, extraction procedure and chromatographic run time.

In view of above, the authors developed and validated a simple and rapid LC-MS/MS method for the determination of budesonide in human plasma. Levonorgestrel was used an inter standard. A simple liquid-liquid extraction (LLE) method was employed for the sample preparation in 50μL of human plasma. The method was fully validated and applied to a pharmacokinetic study in healthy male volunteers.

II. EXPERIMENTAL

2.1 Standards And Chemicals Standards and chemicals

Budesonide reference sample (98% pure) was obtained from Aarati Industries Limited Mumbai India. Levonorgestrel (99% pure) was used as an internal standard and was obtained from Clearsynth Labs Limited, (Mumbai, India). LC-MS grade methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, USA). Analytical grade formic acid was purchased from Merck Ltd (Mumbai, India). Water used for the LC–MS/MS analysis was prepared by Milli Q water purification system procured from Millipore (Bangalore, India).

2.2. LC–MS/MS Instrument and Conditions

An HPLC system (Shimadzu LC-20 AD (Make: Shimadzu Corporation, Japan) consisting of a Zorbax Eclipse XBD C_8 column (100 mm × 4.6 mm; 3.5 μM), a binary LC–20AD prominence pump, an auto sampler (SIL–HTc) and a solvent degasser (DGU–$20A_3$) was used for the analytical study. An aliquot of 10 μL of the processed samples were injected into the column, which was kept at 40ºC. An isocratic mobile phase consisting of a mixture of acetonitrile and 5mM ammonium formate in 0.1% formic acid in the ratio of 60:40, v/v was used to separate the analyte from the endogenous components and delivered at a flow rate of 1 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantification was achieved with MS–MS detection in positive ion mode for the analyte and the IS using an API-4000, AB Sciex, Applied Biosystems, (Foster City, CA, USA) equipped with a Turboionspray ™ interface. The optimized chromatographic conditions were listed in Table 1.

2.3 Preparation of Plasma Standards and Quality Controls

Standard stock solution of budesonide and levonorgestrel IS (1 mg/mL) were prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in methanol and water (60:40, v/v; diluent). The IS working solution (100 ng/mL) was prepared by diluting its stock solution with diluent. Calibration samples were prepared by spiking 980 μL of control K_2 EDTA human plasma with the 20 μL working standard solution of the analyte as a bulk, to obtain budesonide concentration levels of 0.10, 0.21, 0.30, 0.61, 1.21, 1.81, 2.41 and 3.01 ng/mL as a single batch at each concentration. Similarly, quality control (QC) samples were also prepared as a bulk based on an independent weighing of standard drug, at concentrations of 0.11 (LLOQ), 0.26 (LQC), 0.52 (MQC1), 1.58 (MQC2) and 2.63 ng/mL (HQC) as a single batch at each concentration. All the QC and CC Samples were stored in the freezer at –70 ± 10ºC and used at the time of analyses.

2.4 Processing of the plasma samples

After complete thawing, the samples were vortexed to mix for 10s prior to spiking. A 50 μL aliquot of human plasma sample was mixed with 10 μL of the 100 ng/mL of levonorgestrel internal standard working solution. After vortexing for 15s, a 3 mL of *tert*-butyl methyl ether and n-hexane (70:30, v/v) was added using Dispensator Organic (Brand GmbH, Wertheim, Germany). The sample was shaken for 10 min using a reciprocating shaker (Scigenics Biotech, Chennai, India) and then centrifuged for 5 min at 4000 rpm on Megafuse 3SR (Heraeus, Germany). The clear organic layer (2 mL) was transferred to a 5 mL glass test tube and evaporated at 40°C under a gentle stream of nitrogen. The dried extract was reconstituted with 250 mL of the mobile phase and a 10 μL aliquot of it was injected into the LC-MS/MS system.

III. Bioanalytical Method Validation

The validation of the above method was carried out as per the US FDA guidelines. The parameters determined were selectivity, specificity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability.[16]

IV. Pharmacokinetic Study Design

A single dose fed state pharmacokinetic study of budesonide 3 mg capsule was conducted in 7 healthy male subjects (n=7). The blood sample was withdrawn at pre-dose (0.00) and 0.5, 1.5, 2, 2.5, 3, 3.33, 3.67, 4, 4.33, 4.67, 5, 5.33, 5.67, 6, 6.5, 7, 7.5, 8, 8.5, 10, 12, 16, 20, 24 and 36 h of post-dose in to 5 mL K2 EDTA vacutainer collection tubes. The collected samples were then placed in a refrigerated centrifuge and centrifuged at 3200 rpm for 10 minutes to separate the plasma. The resulting plasma collected samples were stored in a deep freezer maintained at –70+10ºC. The subject samples were spiked with the IS and processed as per the sample processing procedure. The linear pharmacokinetic parameters were estimated for budesonide by using Phoenix WinNonlin 5.2 software package by employing non-compartmental model for the study.

V. RESULTS AND DISCUSSION

5.1 Mass Spectrometry

Mass parameters were tuned in both positive and negative ionization modes using electrospray ionization source. The budesonide intensity response obtained in positive mode was much higher than those in negative

ion mode since the analyte and IS having the ability to accept protons. During the optimization of Q1MS scan ion spray voltage (ISV) and declustering potential (DP) were suitably altered to get maximum precursor ion response and detection of the parent ion. Later, collision gas (CAD) and collision energy (CE) were successfully modified to obtain the most intense and reproducible product ion during the product ion scan. Finally, multiple reaction monitoring (MRM) mode was chosen with parent and product ion and all the above optimized parameters were once again confirmed. The quadrupoles were set at unit resolutions. A dwell time of 200 ms was used to avoid cross talk in quantification. Protonated form of analyte and IS, $[M+H]^+$ ion was the parent ion in the Q_1 spectrum and was used as the precursor ion to obtain Q_3 product ion spectra. The most sensitive mass transition was observed from transition pairs of m/z 431.3 precursor ion to the m/z 323.1 for budesonide and m/z 313.3 precursor ion to the m/z 245.2 product ion for the IS.

5.2 Method Development

The method development includes mobile phase selection, flow rate, column type and dimensions, injection volume. For LC-MS analysis commonly used organic solvents are acetonitrile and methanol and widely used buffers are ammonium acetate, ammonium formate, formic acid and acetic acid. Hence a combination of buffers was tried during method development. The strength of the buffer solutions was evaluated in the range of 1 to 15 mM. Additionally, weak acid like formic acid and acetic acid were also checked in the range of 0.02 to 0.3 % for their suitability. It was observed that 5mM ammonium formate in 0.1% formic acid and acetonitrile (40:60, v/v) as the mobile phase was most appropriate to give best sensitivity, efficiency and peak shape. Zorbax Eclipse XBD C_8, 100 x 4.6 mm, 3.5 µm column gave good peak shapes and response even at lowest concentration level for the analyte. The mobile phase was operated at a flow rate of 1 mL/min. The retention time of analyte and the IS were low enough (2.0 and 2.7 ± 0.3min, respectively) allowing a short run time of 4.0 min.

5.3 Extraction Process

To develop an effective and sensitive method with minimum or no matrix effect, solid phase extraction was tested. Strata X polymeric sorbent, Bond Elut Plexa, Oasis HLB, and Orpheus C18 cartridges were checked for their suitability. The recovery of analyte obtained with all the cartridges were good and sufficient to quantify, but not reproducible. Hence LLE was tested with diethyl ether, TBME, hexane, dichloromethane, 2 propanol and ethyl acetate and their combination. The recovery results obtained for budesonide with TBME, ethyl acetate and ethyl acetate in combination with diethyl ether (50:50, v/v) was in the range of 40-50%. Matrix effect for all the analyte was more with 2-propanol and dichloromethane. Finally, good recovery results were achieved with *tert*-butyl methyl ether and n-hexane (70:30, v/v). Addition of acidic and basic buffers to plasma sample does not increase analyte recovery. Due to unavailability of isotope labelled compound to serve as IS, many other structural similar compounds were tested and levonorgestrel was found to be best for the present purpose and used.

5.4 Selectivity and chromatography

Best chromatographic results were obtained with optimized mobile phase acetonitrile and 5mM ammonium formate in 0.1% formic acid (60:40, v/v) with the flowrate of 1.00 mL/min. Method selectivity was assessed by analyzing the blank plasma samples obtained from 8 individual human plasma samples in which 6 were normal and one lipemic and one hemolyzed. No interference was observed from endogenous components in all the plasma lots tested at the retention time (RT) of budesonide and the IS Figure 1A. Similarly, Figure 1B shows the absence of IS interference to MRM channel of the analyte at the retention time. Figure 1C depicts a representative ion–chromatogram for the LLOQ sample (0.10ng/mL).

5.5 Matrix effect

Matrix effect was checked at LQC and HQC levels in eight human plasma lots including hemolytic and lipemic. No significant matrix effect was observed in all the eight lots of human plasma for budesonide at low (LQC) and high (HQC) concentrations. The %RSD of budesonide for IS normalized matrix factor at LQC and HQC level was found to be 6.67% and 4.45%, respectively and IS normalized factor was 1.01 for LQC and 0.98 for HQC.

5.6 Linearity, sensitivity, precision and accuracy

The eight–point calibration curve was found to be linear over the concentration range of 0.10 –3.01 ng/mL for budesonide. Five acceptable precision and accuracy batches were analyzed in this range. Correlation coefficient (r^2) results were >0.99 for all five linearity batches. Sensitivity was tested at a concentration of 0.11 ng/mL which is the lowest level of concentration (LLOQ) At this concentration, the precision and accuracy of budesonide was 8.56% and 110.90%.

The results for intra–day and inter–day precision and accuracy in plasma quality control samples are summarized in Table 2.

5.7 Extraction efficiency

The extraction efficiency of budesonide was determined by comparing the detector response of analyte at three distinct levels of extracted low, medium2 and high-quality control samples with detector response obtained from un-extracted aqueous quality control samples. The mean extraction efficiency of budesonide was 76.80% with a precision ranging from 2.75% to 6.71%. The mean extraction efficiency of internal standard was 71.58% with a precision ranging from 5.50% to 8.05%.

The recoveries of analyte and IS was good and reproducible.

5.8 Stability Studies

In the different stability experiments carried out viz. bench top stability (16 h), In-injector stability (66 h) repeated freeze–thaw cycles (4 cycles), post-processing stability (64 h at 2–8 °C) Re-injection reproducibility (45 h), and long term stability at –70 °C for 55 days the mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels (Table 3). Thus, the results were found to be within the acceptable limits during the entire validation.

5.9 Dilution integrity

Dilution integrity (DI) samples were analyzed along with precision and accuracy batch. 12 sets of plasma samples were prepared by spiking 1.66 times of ULOQ (5.02ng/mL). These samples were processed by diluting them two-and-four folds with screened blank plasma. Dilution factor 2 and 4 was used to quantify these QCs under the undiluted calibration curve standards (Batch-4). Budesonide %RSD for a dilution factor of 2 & 4 was 5.84% and 5.10%, respectively. Likewise, Budesonide accuracy, for a dilution factor of 2 & 4 was 103.62% and 105.37%, respectively.

5.10 Pharmacokinetic study results

To verify the sensitivity and selectivity of this method in a real–time situation, the present method was used to test for budesonide in human plasma samples collected from healthy male volunteers (n = 7). The mean plasma concentrations vs time profile of budesonide is shown in Figure 2. The maximum concentration (C_{max}) in plasma (1.64±0.28 ng/mL) for budesonide was attained at 4.81±0.42 h (t_{max}) The area under the plasma concentration–time curve from time zero to last measurable time point ($AUC_{0–t}$) and area under the plasma concentration time curve from time zero to infinity time point ($AUC_{0–inf}$) for budesonide were 16.52±3.44 and 17.06±3.70 ng h/mL, respectively. The terminal half–life ($t_{½}$) was found to be 5.82±1.30 h.

Table 1: Optimized LC-MS/MS conditions.

Parameter	Budesonide	Levonorgestrel
Detection	Positive	Positive
Monitoring mode	Multiple reaction monitoring (MRM)	
Ionization mode	Positive ion mode	
m/z	431.3 (parent) and 323.1 (product)	313.3 (parent) and 245.2 (product)
Ion Spray Voltage (ISV)	5000 V	5000 V
Temperature (TEM^{0}C)	500 ^{0}C	500 ^{0}C
Curtain Gas (CUR)	35 psi	35 psi
Collision Gas (CAD)	8 psi	8 psi
GS1	40 psi	40 psi
GS2	35 psi	35 psi
Declustering Potential (DP)	90 V	81 V
Collision Energy (CE)	41 V	27 V
Collision Cell Exit Potential (CXP)	11 V	11 V
Entrance Potential (EP)	10 V	10 V
Dwell time	200 ms	200 ms

Table 2: Precision and accuracy data for budesonide.

Quality control	Run	Concentration found Mean±SD (ng/mL)	Precision (%)	Accuracy (%)
Intra–day (*n*=12)				
LLOQ		0.114 ± 0.00	6.75	106.62
LQC		0.261 ± 0.02	7.94	100.54
MQC1		0.565 ± 0.02	3.67	108.70
MQC2		1.721 ± 0.09	5.49	108.98
HQC		2.733 ± 0.19	7.27	103.81
Inter–day (*n*=30)				
LLOQ		0.113 ± 0.00	7.81	105.86
LQC		0.266 ± 0.01	6.73	102.33
MQC1		0.543 ± 0.03	6.26	104.47
MQC2		1.665 ± 0.09	5.77	105.41
HQC		2.843 ± 0.19	6.85	107.98
Nominal concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 0.107, 0.260, 0.520, 1.580 and 2.633 ng/mL, respectively.				

Table 3: Stability data for budesonide in plasma (*n*=6).

Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Precision (%)	Accuracy/ Stability (%)
Auto-sampler (66 h)	0.260	0.255 ± 0.01	7.45	98.21
	2.633	2.606 ± 0.22	8.81	99.00
Wet extract stability (64 h)	0.260	0.257 ± 0.02	9.78	99.87
	2.633	2.751 ± 0.19	7.05	104.51
Bench top (16 h)	0.260	0.248 ± 0.01	5.71	95.71
	2.633	2.463 ± 0.09	3.82	93.56
Freeze and Thaw (4 Cycles)	0.260	0.260 ± 0.03	11.5	100.32
	2.633	2.838 ± 0.21	7.62	107.80
Re-injection (45 h)	0.260	0.262 ± 0.02	7.69	99.56
	2.633	2.822 ± 0.22	7.88	97.94
Long–term (55 days)	0.260	0.267 ± 0.02	10.3	97.36
	2.633	2.579 ± 0.27	10.7	92.83

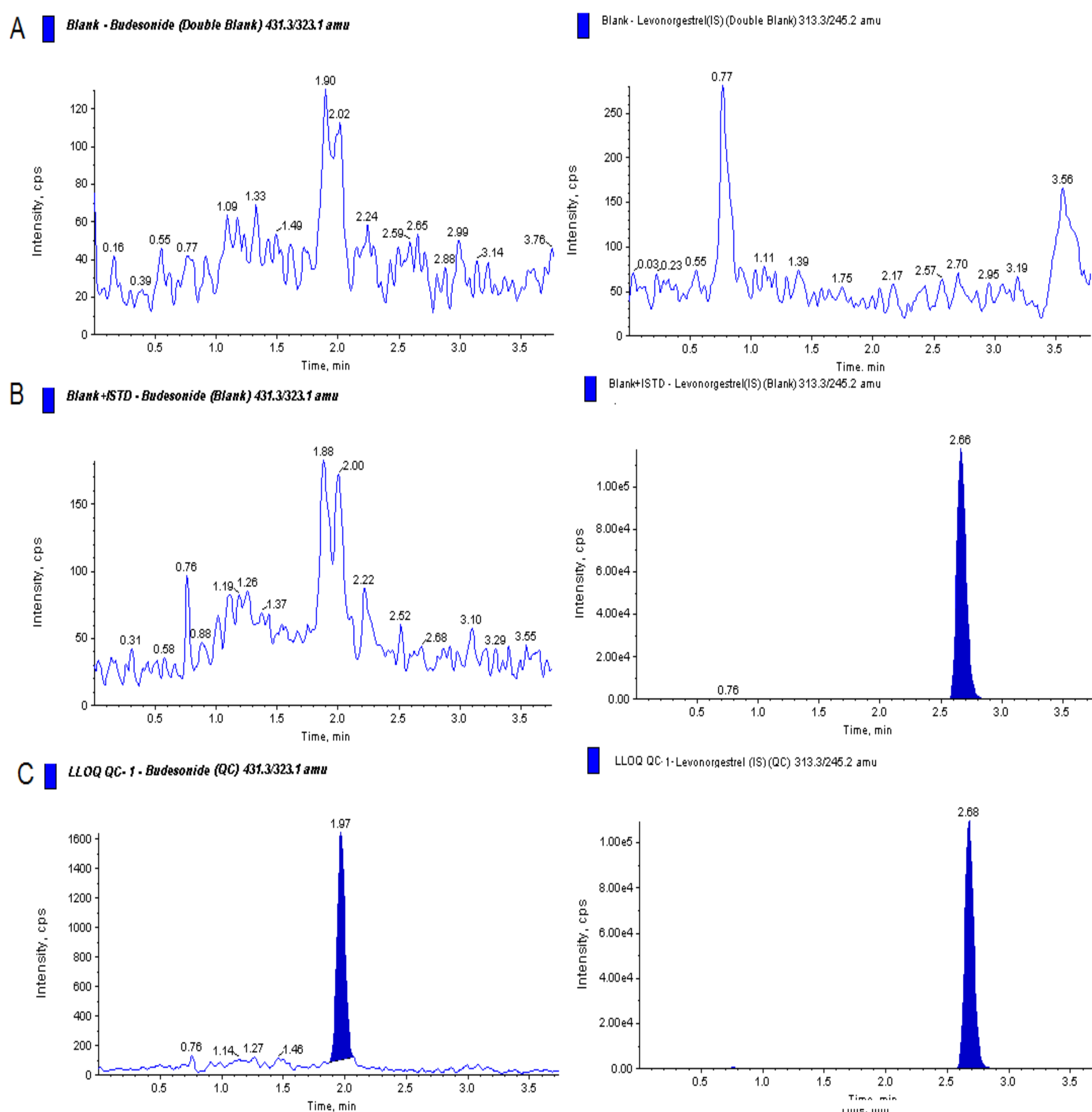

Figure 1: A model MRM chromatogram of budesonide (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS (C).

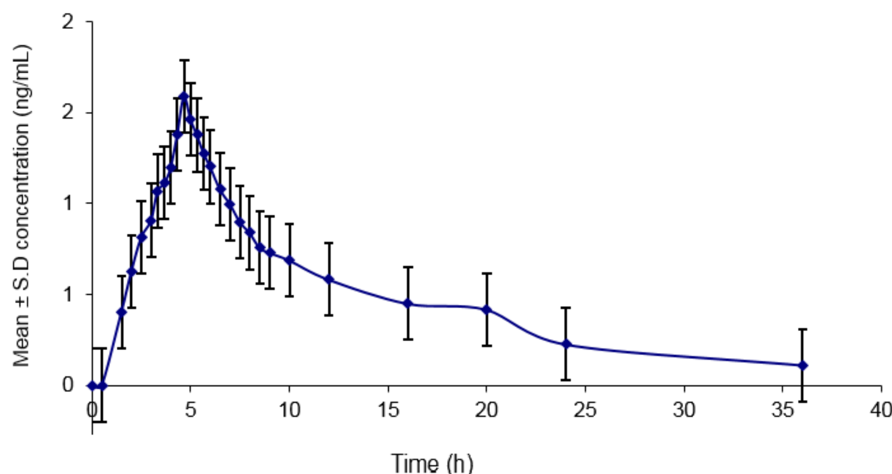

Figure 2: Mean concentration-time profile of budesonide in human plasma following oral administration of budesonide 3 mg capsules to healthy volunteers under fed condition (n=7).

VI. CONCLUSIONS

In conclusion, the proposed LC-MS/MS method is simple, rapid and sensitive for the determination of budesonide in human plasma. Use of structurally similar compound levonorgestrel as internal standard helped us to obtain the reproducible and consistent results. The current method has shown acceptable precision and adequate sensitivity for the quantification of budesonide in human plasma samples obtained for pharmacokinetic studies. There is no matrix effect in recovery between the budesonide and internal standard. The sample preparation with LLE gave high recovery for the analyte. The analyte and IS was stable under various conditions. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

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