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Liquid Chromatograph Mass Spectrometer LCMS-8045

Application News

Highly Sensitive LC-MS/MS Method for Quantification of Beclomethasone in Human Plasma

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User Benefits

- Rapid, simple and sensitive method with LLOQ of 5.0 pg/mL
- An extended Calibration Curve reduces reanalysis of samples outside range
- Quick and single step sample extraction method increased sample productivity

1. Introduction

Beclomethasone dipropionate (BDP) is an inhaled corticosteroid used as maintenance treatment in the prophylaxis of asthma attacks[1]. Beclomethasone is a 17 alpha-hydroxy steroid that is prednisolone in which the hydrogens at the 9 alpha and 16 beta positions are substituted by a chlorine and a methyl group, respectively[2]. Structure of the prodrug of BDP is provided in Fig. 1.

Formulations for oral inhalation, intranasal, and topical use are available for BDP. Compared to earlier corticosteroids such as dexamethasone and prednisolone, BDP is reported to be less irritating to the nasal mucosa with a longer duration of action when administered intranasally [1]. BDP administered in the form of oral inhalation results in very low systemic bioavailability. This translates into significant challenges to develop a sensitive and reproducible bioanalytical method that can reliably measure plasma levels of BDP at very low expected levels.

The required LLOQ for most inhalation products is typically in the range of pg/mL to sub pg/mL. Very few analytical methods have been developed to determine BDP in biological samples using HPLC with tandem mass spectrometric detection (LC–MS/MS) and the lowest reported LLOQ of 25.0 pg/mL[3–4]. These methods fall short of the ideal target sensitivity required by the pharmaceutical research, and this motivated us for the current study. The main aim of this work is to develop a LC-MS/MS method at picogram level (LLOQ –5.0 pg/mL) using BDP-D10 as internal standard to support regulatory studies. Structure of BDP-D10 (BDP-D10) is presented in Fig. 2.

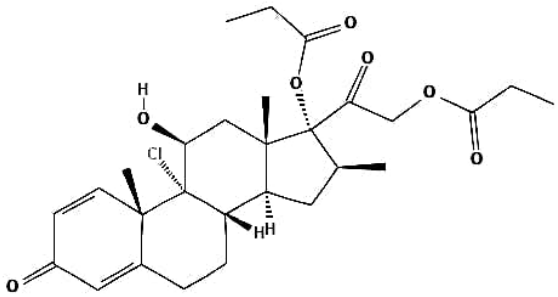

Fig. 1 Structure of BDP

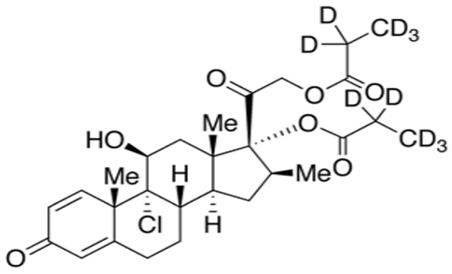

Fig. 2 Structure of BDP-D10

2. Salient Features

- Quantitative method for estimation of BPD in human plasma was developed and partially validated as per USFDA Guidelines [5] on LCMS-8045
- Effective throughput for quantitative assessment is increased by use of a single step extraction procedure
- Heated ESI along with New UF-Qarray ion guide technology contributes by increasing ion production and enhancing transmission respectively. This ensures sensitive and selective quantification of BDP at 5.0 pg/mL
- Optimum plasma (500 µL) volume avoided unnecessary wastage of plasma samples and at the same time increased the life of the mass spectrometer
- Customized gradient elution satisfied the peak shape, retention time and interference
- Method was partially validated as per US major guidelines for
  - ✓ Selectivity
  - ✓ Linearity
  - ✓ Inter-day and intra-day precision and accuracy (PA)
  - ✓ Recovery
  - ✓ Matrix effect
  - ✓ Carry over effect

Table 1 Method Validation Summary

Calibration curve range	5.00 pg/mL to 2000.00 pg/mL	
Intraday precision and accuracy (For LLOQ-QC)	Accuracy (%Nominal)	100.99
	Precision (% RSD)	11.97
Intraday precision and accuracy (For LQC, MQC, HQC)	Accuracy (%Nominal)	99.92 to 109.14
	Precision (% RSD)	2.97 to 6.86
Global precision and accuracy (For LLOQ-QC)	Accuracy (%Nominal)	112.14
	Precision (% RSD)	7.57
Global precision and accuracy (For LQC, MQC, HQC)	Accuracy (% Nominal)	96.99 to 101.89
	Precision (% RSD)	4.36 to 8.23
Global % recovery	Recovery (%)	60.14
	Precision (% RSD)	2.70
Matrix effect	LQC	1.05
	HQC	0.96

Note: LLOQ QC- Lower Limit of Quantification Quality Control
LQC- Lower Quality Control, MQC- Middle Quality Control
HQC- Higher Quality Control

3. Experimental

3.1. Sample preparation and analytical conditions

To a 450 µL aliquot of human plasma, 50 µL BDP – D10 and 400 µL of buffer was added followed by SPE purification. Samples were loaded on the preconditioned reversed-phase SPE cartridge. The loaded samples were washed with water, acetonitrile and eluted in 100% acetonitrile. Eluent was diluted with water in the ratio of 1:1 v/v and directly injected on LC-MS/MS for analysis.

3.2. Instrument parameters on LCMS-8045

Refer Table 2 for analytical conditions and instrument parameters. BDP produced two intense product ions (refer Table 3 for MRM transition) and the sum of both product ions was used for quantification of BDP as presented in Table 3.

Table 2 Analytical conditions and instrument parameters

Parameter	HPLC
Column	Shim-pack™ GIST C18 column 50 x 2.1 mm, 3.0 µm, (P/N: 227-30008-03)
Mobile Phase	A: 2 mM Ammonium Trifluoro acetate in Water B: Acetonitrile
Flow Rate	1.0 mL/min
Oven Temp	40 °C
Injection volume	25 µL
Parameter	**MS**
Interface	ESI
Interface Voltage and Temp	2 KV and 350 ˚C
MS Mode	MRM, Positive
Heat Block Temp	300 ˚C
DL Temp	120 ˚C
CID Gas	230 kPa
Nebulizing Gas	3 L/min
Drying Gas	18 L/min
Heating Gas	10 L/min

Table 3 MRM transition and parameters of BDP on LC-MS/MS

Compound	Precursor (*m/z*)	Product (*m/z*)	CE (V)
BDP	521.40	411.30	-9.0
BDP	521.40	319.10	-18.0
BDP-D10	531.30	416.00	-11.6

Fig. 3 Nexera™ X2 with LCMS-8045 system

4. Result and Discussion

4.1. Method Development

Both BDP and BDP-D10 produced higher signals by ESI than by APCI. In the mass spectrum of BDP, we observed that the summation of product ions at *m/z* 411.3 and *m/z* 319.1 resulted in achieving LLOQ of 5.0 pg/mL successfully. Several commercially available reversed phase HPLC columns were evaluated for BDP, but separation was achieved best on Shim-pack GIST C18 column with satisfactory chromatography, low back pressure and minimal background noise (refer Fig. 4).

Protein precipitation, LLE and SPE are the three commonly used sample preparation techniques which were evaluated to isolate BDP from biological matrices. In comparison to protein precipitation and LLE, SPE contributed to good recovery, reproducibility and consistent results. No interference and matrix effect were observed at the retention time and MRM transition of BDP and BDP-D10.

4.2. Method Validation

The method was validated for linearity, accuracy, precision, selectivity, matrix effect, recovery and stability according to the FDA guidelines for the validation of bioanalytical methods (for summary of results refer Table 1). Three independent standard curves were prepared on each of three separate days. Linearity was assessed by weighted linear regression (1/x2) of analyte-internal standard peak area ratios. Accuracy and precision were determined using QC samples at 5.0, 25.0, 250.0 and 1000.0 pg/mL in plasma. Intra- and inter-day precisions were found <15%, and the accuracy within ±15% (refer Tables 5 & 6). Precision at LLOQ level was found <20%, the accuracy was found within ±20%. Lower limit of quantification sample showed signal-to-noise ratio of 15:1 (refer Fig. 4).

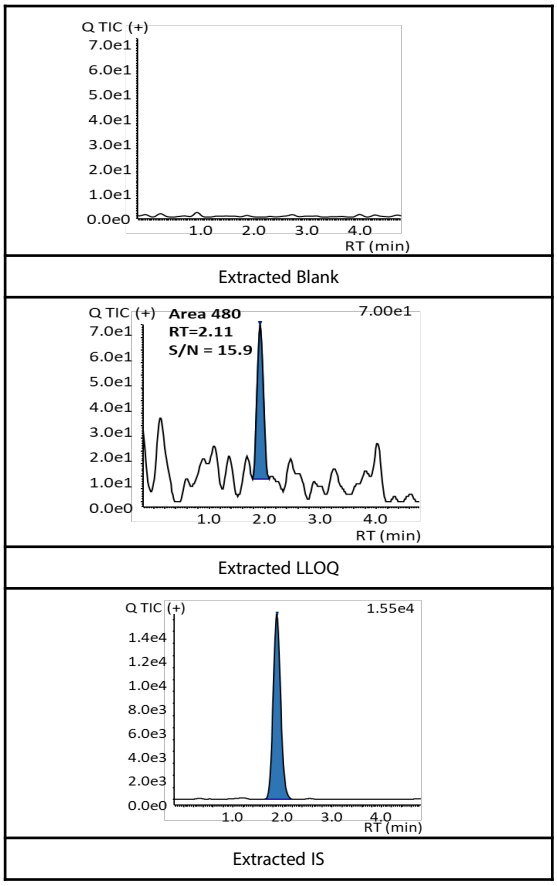

Fig. 4 Chromatograms of BDP (Ext Blank, Extracted LLOQ) and Extracted IS

- **Selectivity**

Selectivity of the method was assessed in different lots of blank human plasma. Interference from blank plasma was assessed for both BDP and BDP-D10. No significant interference was observed at the retention time and MRM transition of BDP and BDP-D10 as shown in Table 4 below.

Table 4 Selectivity

Plasma lot no.	BDP		
	Blank Plasma	LLOQ area	% Interference
V3071	12	454	2.64
V1889	21	472	4.45
V1789	6	637	0.94
V1166	18	457	3.94
V3074	15	684	2.19
V3077	23	422	5.45

Plasma lot no.	BDP-D10		
	Blank Plasma	LLOQ area	% Interference
V3071	68	936920	0.01
V1889	81	866110	0.01
V1789	213	892170	0.02
V1166	230	898380	0.03
V3074	191	913970	0.02
V3077	288	898650	0.03

- **Linearity**

Calibration curve was found linear from 5.0-2000.0 pg/mL (refer Fig. 5) The goodness of fit was consistently greater than 0.99 during the course of validation. Signal to noise ratio (s/n) at LLOQ level was found to be more than 10:1, across 3 PA batches. Calibration Curve is shown in Fig. 5.

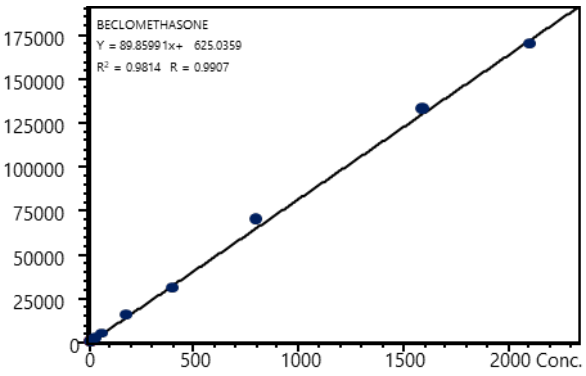

Fig. 5 Calibration curve

- **Intra-day precision and accuracy**

Intraday precision and accuracy was evaluated by processing 6 replicates of LLOQQC, LQC, MQC and HQC in one PA batch. Quantitative data is summarized in Table 5.

Table 5 Intra-day precision and accuracy

Intra-day (n=6)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (% RSD)
LLOQ QC (5.03 pg/mL)	5.08	100.99	11.97
LQC (25.09 pg/mL)	25.99	103.6	5.75
MQC (250.89 pg/mL)	250.7	99.92	6.86
HQC (1005.56 pg/mL)	1097.45	109.14	2.97

- **Global precision and accuracy**

Precision and accuracy experiments were evaluated at LLOQ, LQC, MQC and HQC level in 3 batches. Excellent accuracy and repeatability was observed with % CV < 8.23 % and % accuracy between 96.99 to 101.89% at LQC, MQC and HQC level. At LLOQQC level, the % CV was found to be 7.57 % and % accuracy as 112.14 %. The results are presented in Table 6.

Table 6 Global precision and accuracy

Intra-day (n=18)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (% RSD)
LLOQ QC (5.03 pg/mL)	5.64	112.14	7.57
LQC (25.09 pg/mL)	25.37	101.12	8.23
MQC (250.89 pg/mL)	243.35	96.99	4.36
HQC (1005.56 pg/mL)	1024.54	101.89	6.07

- **Recovery**

Recovery experiments were conducted to evaluate precision, reproducibility and consistency of the analyte(s) at LQC, MQC and HQC level. Recover of BDP was calculated by comparing the peak area of the plasma that is spiked with the analyte and extracted, with the peak area of blank plasma that is extracted and then spiked with the analyte. Mean recovery for BDP was found to be 60.14%, respectively. Recovery of BDP was found to be precise, consistent and reproducible at all QC levels (refer Table 7).

Table 7 Statistics of Recovery

QC level	Recovery
LQC (n=6)	61.99
MQC (n=6)	59.46
HQC (n=6)	58.96
Mean	**60.14**
SD	**1.63**
% RSD	**2.70**

- **Matrix effect**

Matrix effect was studied for both BDP and deuterated internal standard at LQC and HQC samples. IS normalized matrix factor was found to be 1.05 & 0.96 at LQC & HQC concentration respectively and meets the acceptance criteria (refer Table 8).

Table 8 Matrix factor

BDP	Response ratio of Aqueous sample	Response ratio of Post extracted sample	Matrix factor
LQC	0.0021	0.0019	1.11
	0.0019	0.0018	1.06
	0.0019	0.0020	0.95
	0.0020	0.0019	1.05
	0.0020	0.0019	1.05
	0.0021	0.0019	1.11
Mean			**1.05**
SD			**0.06**
% RSD			**5.38**

BDP-D10	Response ratio of Aqueous sample	Response ratio of Post extracted sample	Matrix factor
HQC	0.0957	0.1007	0.95
	0.1038	0.1044	0.99
	0.0943	0.1013	0.93
	0.1032	0.1006	1.03
	0.0951	0.1111	0.86
	0.1082	0.1097	0.99
Mean			**0.96**
SD			**0.06**
% RSD			**6.21**

- **Carry-over effect**

Carryover was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carryover was present/observed at the retention time and MRM transition of the analyte in the extracted blank sample following the highest standard calibrator.

5. Conclusion

LCMS-8045, along with special sample preparation, optimized chromatography provides a very selective and sensitive method for bioanalytical assay of BDP. Ultra-high speed and high-separation analysis was achieved on Nexera X2 UHPLC by using a simple mobile phase at a minimal gradient flow rate of 1.0 mL/min. These applications serve as important tools for detecting extremely low plasma concentrations during bioequivalence and pharmacokinetic studies of generic drug molecules. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavors.

6. References

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