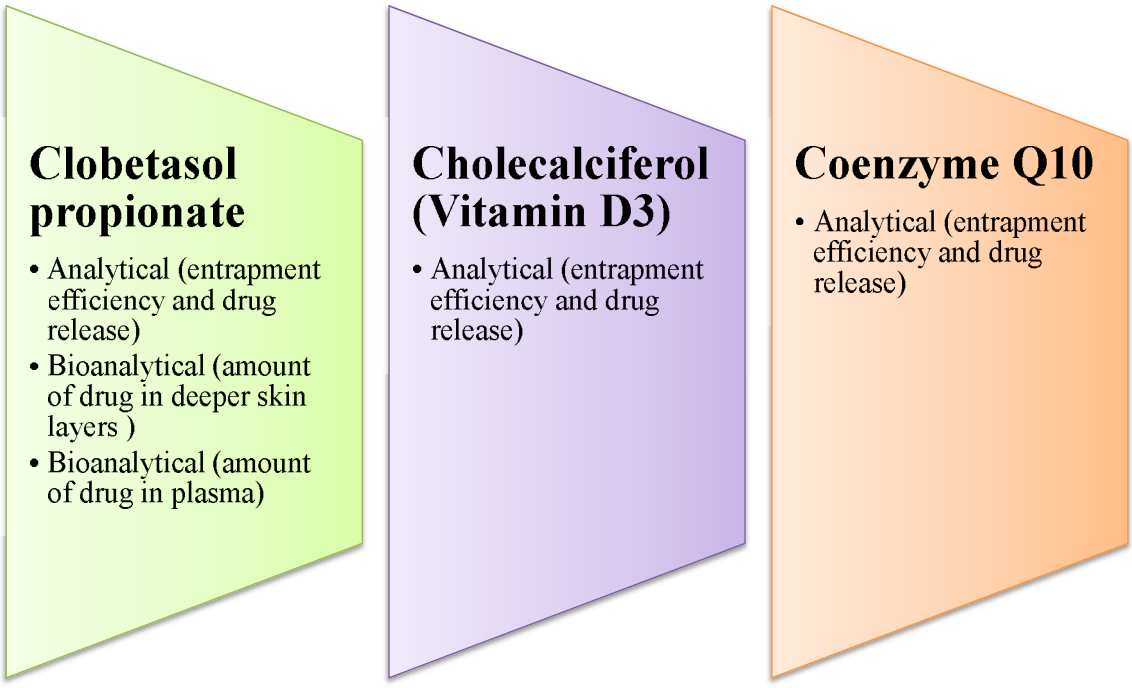
CHAPTER 2

ANALYTICAL AND BIOANALYTICAL METHOD VALIDATION



- Analytical and bioanalytical method validation of Clobetasol propionate
- Analytical method validation of Cholecalciferol (Vitamin D3)
- Analytical method validation of Coenzyme Q10

Chapter 2 is divided into two sections i.e., 2.1 and 2.2. In sections 2.1, various methods for analysis of clobetasol propionate including analytical and bioanalytical (for determining the quantitites of drug permeated in deeper skin layers and plasma, respectively) were given while in section 2.2 the analysis of cholecalciferol (vitamin D3) and coenzyme Q10 were provided.

2. Materials

Clobetasol propionate (CP) and cholecalciferol (vitamin D3) were obtained as a generous gift sample from Orbicular Pharmaceutical Technology Pvt. Ltd. (Hyderabad, India). Docetaxel (internal standard) was obtained as a generous gift sample from Fresenius Kabi (Bad Homburg, Germany). Coenzyme Q10 (CoQ10) was purchased from Sisco Research Laboratories Pvt. Ltd (Mumbai, India). Acetonitrile (ACN), methanol and isopropyl alcohol (IPA) of HPLC grade was procured from Merck, Limited (Mumbai, India). Skin was collected from the pre-shaved *Swiss albino* mice (females; 8–12 weeks, 25–30 g) that were procured from the Central Animal Facility, BITS-Pilani (Pilani, Rajasthan India) and was stored at -80 ± 15 °C until further use. Plasma was collected from the *Swiss albino* mice (females; 8–12 weeks, 25–30 g) were procured from the Central Animal Facility, BITS-Pilani (Pilani, Rajasthan India) and was stored at -80 ± 15 °C. The Institutional Animal Ethics Committee (IAEC) approved the animal protocols, BITS-Pilani (protocol no: IAEC/RES/25/10, IAEC/RES/25/11 and IAEC/RES/26/05) and experiments were conducted as per CPCSEA guidelines. Acetonitrile (ACN) and methanol of HPLC grade was procured from Merck, Limited (Mumbai, India). Purified water was obtained from *in house* Millipore Direct-Q ultra-pure water system (Millipore, Bedford, USA).

2.1. Analytical and bioanalytical methods for the analysis of clobetasol propionate

2.1.1. Chromatographic Conditions

A sensitive, accurate and reliable method for CP was developed using Shimadzu HPLC system coupled with a photodiode array (PDA) detector (SPD-M20A), binary pump (LC-20AD) and autosampler (SIL-HTC, Shimadzu, Japan). Chromatographic separation was carried out on Inertsil ODS-3V C18 column (5 μm, 4.6 × 250 mm). The chromatographic conditions for all the methods (analytical, bioanalytical method for determining the quantities of drug permeated in deeper skin layers and plasma) are mentioned in below Table 2.1. The analysis was performed using an LC solution software version 1.22 SP1. The HPLC system was primarily equilibrated for 30 min followed by analysis of the samples [1-9].

Table 2.1. Chromatographic conditions for the CP methods

Parameters	**Analytical method**	**Bioanaytical method (Skin)**	**Bioanaytical method (Plasma)**
Mobile Phase (ACN: Water)	80:20	60:40	55:45
Flow Rate (mL/min)	1	1	1
Injection volume (µL)	20	60	60
Run Time (min)	10	20	30
Wavelength (nm)	240	240	240

2.1.2. Preparation of stock solution, calibration standards and quality control samples

For analytical method, a primary stock solution of CP of 100 µg/mL concentration was prepared by dissolving 10 mg of CP in 100 mL of ACN**.** The stock solution (100 µg/mL) was

serially diluted to obtain calibration standards. In brief, seven different concentrations for CP were prepared over the linearity range of 0.25 µg/mL to 100 µg/mL. Three quality control (QC) samples were also prepared directly from primary stock solution i.e. 0.92 µg/mL as LQC, 45 µg/mL MQC and 90 µg/mL as HQC.

For bioanalytical method (skin), a primary stock solution of CP of 100 µg/mL concentration was prepared by dissolving 10 mg of CP in 100 mL of methanol. The secondary stock solutions of various concentrations i.e. 0.25, 0.5, 2.5, 10 µg/mL were prepared by appropriately diluting the primary stock solution with methanol. These secondary stock solutions were used to spike skin extract and prepare calibration curve samples (working solution) over the linearity range of 25-1000 ng/mL. DTX was employed as the internal standard (IS) and primary stock solution was prepared by dissolving 10 mg of DTX in 100 mL of methanol to achieve a concentration of 100 µg/mL. The secondary stock solution of IS was prepared at a concentration of 10 µg/mL which was later spiked in calibration standards. Likewise, four quality control (QC) samples were also prepared directly from secondary stock solution i.e. 25 ng/mL as LLOQ, 60 ng/mL as LQC, 600 ng/mL MQC and 900 ng/mL as HQC.

For bioanalytical method (plasma), a primary stock solution of CP of 100 µg/mL concentration was prepared by dissolving 10 mg of CP in 100 mL of ACN. The secondary stock solutions of various concentrations i.e. 0.25, 0.5, 0.75, 1, 2.5, 5, 7.5, 10 µg/mL were prepared by appropriately diluting the primary stock solution with ACN. These secondary stock solutions were used to prepare plasma calibration curve samples. The DTX (IS) primary stock solution was prepared by dissolving 10 mg of DTX in 100 mL of acetonitrile to achieve a concentration of 100 µg/mL from which the secondary stock solution of IS was prepared at a concentration of 10 µg/mL, which was later used for spiking plasma samples (working solutions) containing the

drug. In brief, eight different concentrations for CP were prepared over a linearity range of 25-1000 ng/mL and each sample was spiked with 10 µL IS (10 µg/mL). Four quality control (QC) samples were also prepared directly from secondary stock solution i.e. 25 ng/mL as LLOQ, 200 ng/mL as LQC, 600 ng/mL MQC and 900 ng/mL as HQC.

Linearity was analyzed for the concentration range with the help of least square linear regression analysis. For analytical method, plot of area of drug (CP) versus concentration and for both bioanalytical methods, plots of area ratio of drug to IS against concentration were plotted and the regression equation was used to calculate the drug concentration in quality control samples containing CP.

2.1.3. Skin sample preparation and extraction procedure

Skin isolated from *Swiss albino* mice was thoroughly washed using phosphate buffer saline (pH 7.4) to remove any adhered fat or blood debris. The stratum corneum layer was removed from the remaining skin (RS) by tape-stripping technique (skin was tape-stripped 15 times with 19 mm Scotch (3M, USA) cellophane tape). The RS was finely chopped and placed in 50 ml falcon tubes and extracted using MeOH by subjecting to bath sonication for 2 h. Further, the contents were centrifuged at 10,000 RPM for 20 min at 4°C and supernatant containing skin extract was isolated and used for analysis [6-8].

2.1.4. Plasma extraction procedure and sample analysis

The protein-precipitation method using acetonitrile (organic solvent) was employed for the extraction of drug and IS from the plasma. Briefly, 10 µL CP secondary stock solutions (0.25-10 µg/mL) and 10 µL of IS (10 µg/mL) were spiked in 90 µL fresh plasma in a 2 mL microcentrifuge tube and vortexed for 1 min on a cyclomixer (Tarsons, India). Further, 200 µL sodium hydroxide solution (2mM) was added to the tube and vortexing was further continued for

5 min. To this, 1.5 mL chilled acetonitrile was added and vortexing was carried out for another 4.5 min. Contents were centrifuged at 17500 RPM for 15 min at 4°C and the supernatant was transferred to another 2 mL microcentrifuge tube and was kept for overnight drying. Further, the dried product was reconstituted with solution containing 10 µL external spiking solution (CP; 100 ng and IS; 200 ng) and 90 µL mobile phase by vortexing for 4 min. Contents were centrifuged at 17500 RPM for 15 min at 4°C and the supernatant was transferred to micro-inserts, and samples were analyzed by RP-HPLC using a developed bioanalytical method. Blank plasma samples were processed in a similar way and were externally spiked with solution containing 10 µL external spiking solution (CP; 100 ng and IS; 200 ng) and 90 µL mobile phase and analyzed and their values (areas) obtained were subtracted from the areas of calibration standards samples and the areas ratio of drug to IS was calculated and plotted against concentrations. Furthermore, blank plasma and zero samples were analyzed for comparison [9].

2.1.4. Validation of the method

The analytical and bioanalytical methods were validated as per the ICH Q2R1 and USFDA guidelines. The following parameters were determined.

2.1.4.1. Specificity

Specificity of the developed analytical method was studied using QC samples with different excipients that were used in the preparation of LPH nanoparticles.

2.1.4.2. Selectivity

The selectivity of the bioanalytical method was checked to identify the major chromatographic interferences from the skin and plasma constituents with that of the drug. For

this, skin and plasma from six different sources was collected and employed for the analysis by keeping other parameters constant.

2.1.4.3. Linearity and Range

The linearity of the developed method for CP were determined with a linearity range of 0.25-100 µg/mL (analytical), 0.025-1 µg/mL (bioanalytical (skin)), 25-1000 ng/mL (bioanalytical (plasma)). The calibration curve for analytical method was constructed by plotting concentration on X-axis versus peak area (mAU) on Y-axis, whereas for bioanalytical methods, concentration on X-axis was plotted versus areas ratios of drug to IS on Y-axis expressed by the equation y = mx + c, where m is slope and c is the intercept.

2.1.4.4. Accuracy and precision

For accuracy and precision study, intra-day and inter-day samples were analyzed with three different QC samples i.e. LQC, MQC and HQC for analytical and four different quality control samples i.e. LLOQ, LQC, MQC and HQC for bioanalytical (skin and plasma) in triplicate. Further, accuracy and precision were expressed in terms of % bias and % RSD, respectively. According to ICH Q2R1 guidelines, both % bias and % RSD of the quality control samples should fall within the range of ±2%. According to USFDA guidelines, both % bias and %RSD of the quality control samples should fall within the range of ±15% except for LLOQ samples where both % bias and % RSD should not exceed ±20%.

2.1.4.5. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were determined for all the methods on the basis of signal to noise (S/N) ratio method. According to guidelines LOD and LOQ must have signal to noise ratio > 3 and > 10 times, respectively.

2.1.4.6. System suitability

It was performed six times at MQC level and system performance was verified from various system parameters, including tailing factor, retention time, theoretical plate numbers and HETP.

2.1.4.7. Robustness

The robustness of the developed analytical methods were studied using two parameters *viz.* flow rate and mobile phase composition. For flow rate, samples were run at three different flow rates i.e., at 1.1, 1.0, and 0.9 mL/min at mobile phase composition ACN:Water of 80:20 (% v/v) whereas, for changing another parameter i.e., mobile phase ratio, samples were analyzed at three different mobile phase composition (ACN:Water (% v/v)) i.e., 85:15, 80:20, and 75:25, respectively at a flow rate of 1.0 mL/min. According to the ICH guidelines both % bias and % RSD must fall within ±2 % for samples.

2.1.4.8. Recovery (%) and Carry-over effect

The percentage recoveries of CP were determined by comparing the areas of all four QC levels (LLOQ, LQC, MQC and HQC) in the skin extract and plasma samples with corresponding standard concentrations. The percentage recovery of IS was also calculated at 250 and 300 ng/mL for both bioanalytical methods. The carry-over effect was analyzed in blank samples (n=3) after injecting the HQC and LLOQ samples. As per the guidelines, the response of blank samples should not exceed 20% LLOQ.

2.1.5. Results and discussion

2.1.5.1. Assay validation

Validation of the method was performed on the basis of various parameters, including accuracy, precision, specificity, system suitability and stability as per the guidelines.

2.1.5.2. Specificity

For specificity, the developed method for CP was verified with a sample prepared by spiking the mobile phase with different excipients (Precirol®ATO5, linoleic acid, mPEG-PLA and tween 80) that were used for the preparation of lipid polymer hybrid nanoparticles. It was observed that the developed method was specific for CP with purity profile of 0.9999, suggesting a pure peak. The representative pictorials of chromatograms are shown in Figures 2.1.

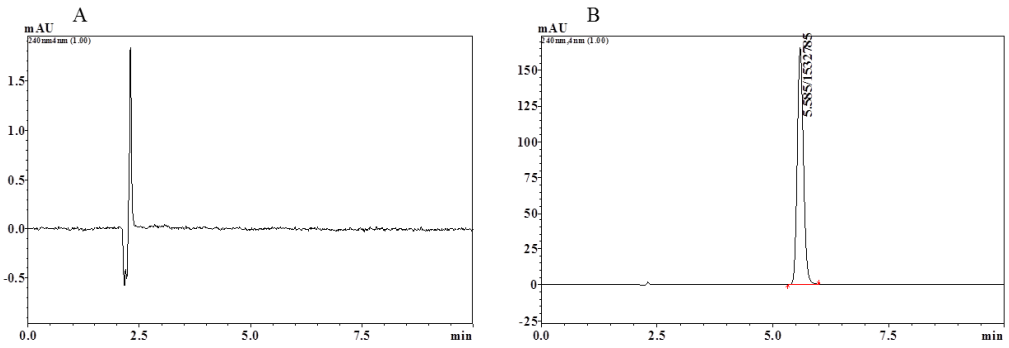


Figure 2.1. Representative chromatogram of (A) mobile phase spiked with formulation excipients and (B) analytical sample containing CP dissolved in ACN (45µg/mL).

2.1.5.3. Selectivity

For selectivity, unspiked skin extract and plasma (blank samples) were run using the developed bioanalytical method to check whether there are any interferences from skin and

plasma constituents with peak of drug wherein the results suggested no such interferences from the skin matrix near the retention time (Rt) of drug and IS, as shown in Figure 2.2.

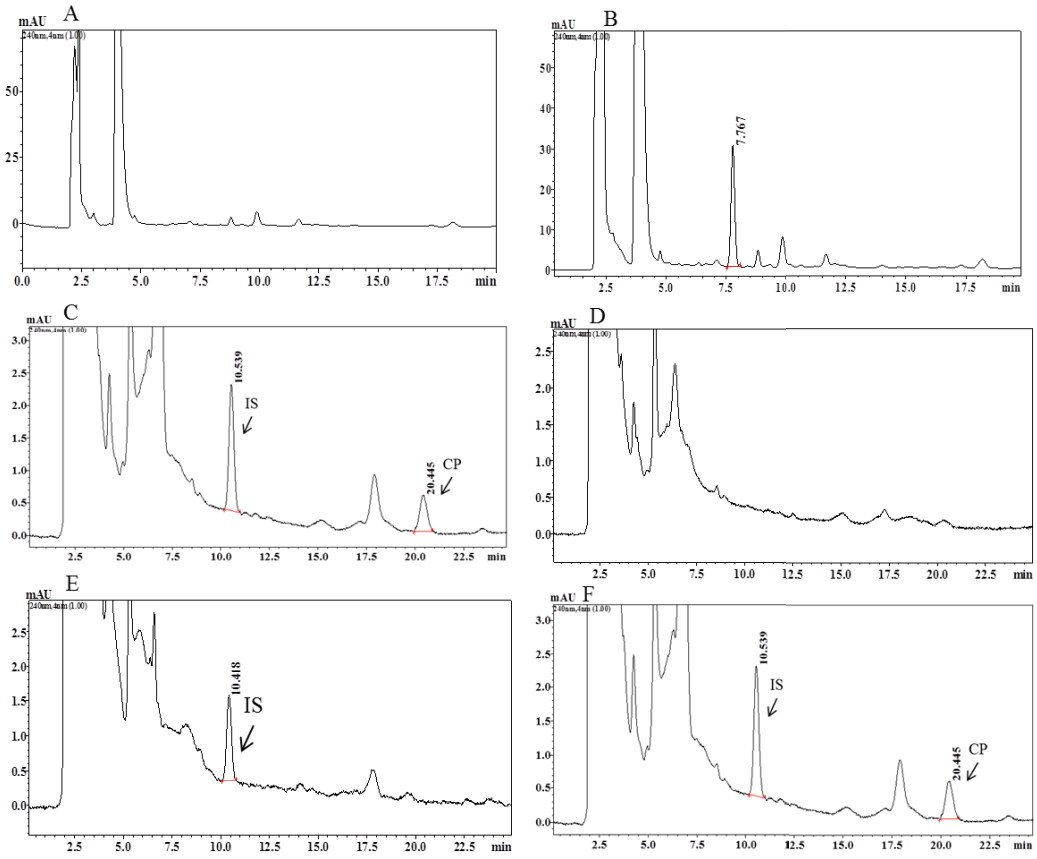


Figure 2.2. Representative chromatogram of (A) blank skin extract, (B) zero i.e. IS (DTX) (Rt:7.77 min) spiked in the skin extract, (C) CP (Rt: 14.03 min) and DTX (Rt: 7.78 min) spiked in the skin extract at the concentration of 500 ng/mL and 250 ng/mL respectively, (D) blank plasma sample, (E) zero i.e. plasma sample spiked with IS and (F) plasma sample spiked with IS (Rt: 10.539 min) and CP (Rt: 20.445 min) at concentration of 300 ng/mL and 75 ng/mL respectively.

2.1.5.4. Skin and plasma sample preparation and extraction procedure

The sample processing and extractions were found to be simple and robust. The optimum percentage recovery (> 94% and > 78%) of CP and IS with better resolution (R_s 13.988 ± 0.18 and R_s 17.31 ± 0.12) without any interference from the skin and plasma constituents with peak purity of 0.9923 and 0.9970 were achieved.

2.1.5.5. Linearity and Range

For linearity and range, the developed method was subjected to analysis of different concentrations from 0.25-100 µg/mL (analytical), 0.025-1 µg/mL (bioanalytical (skin)) and 25-1000 ng/mL (bioanalytical (plasma)). It was observed that linearity and range successfully fitted the respective calibration range with regression coefficient (R^2) of 0.9999 with equation of y = 34614x – 1069.7 (analytical), 0.9999 with equation of y = 39.606x + 0.066 (bioanalytical (skin)) and 0.9996 with equation of y = 0.0042x - 0.0179 (bioanalytical (plasma)), respectively. The respective calibration curve with the equation is shown in Figure 2.3).

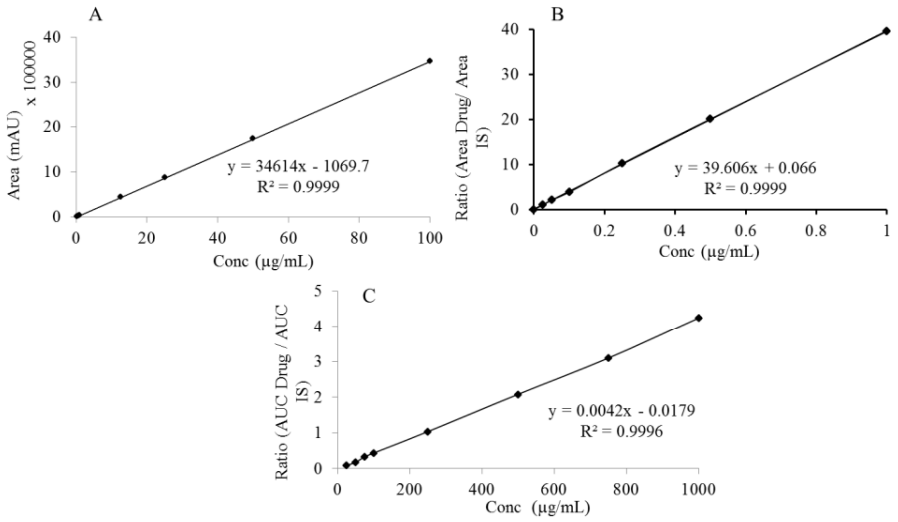


Figure 2.3. Representative calibration curve of (A) CP in analytical samples, (B) skin extract samples spiked with CP and (C) plasma samples spiked with CP. .

2.1.5.6. Accuracy and precision

Intra-day and inter-day samples of accuracy and precision of all the methods were analyzed in triplicates. Further, accuracy and precision were expressed in terms of % bias and % RSD, respectively. From the results, both % bias and % RSD of the quality control samples were within the range of ±2% and ±15%, for analytical method and bioanalytical methods respectively. Representative data for accuracy and precision are shown in Table 2.2, 2.3 and 2.4, respectively.

Table 2.2. Intra-day and Inter-day precision and accuracy of analytical samples of CP.

		Intra-day			Inter-day		
Level	**Nominal Conc (µg/mL)**	**Observed Conc (µg/mL) Mean ± SD**	**% RSD**	**% Bias**	**Observed Conc (µg/mL) Mean ± SD**	**% RSD**	**% Bias**
LQC	0.92	0.92±0.005	0.501	0.020	0.94±0.019	1.979	1.712
MQC	45	44.26±0.103	0.233	-1.653	44.26±0.108	0.244	-1.640
HQC	90	89.72±0.085	0.095	-0.316	89.44±0.221	0.247	-0.619

Table 2.3. Intra-day and Inter-day precision and accuracy of CP spiked in skin extract.

		Intra-day			Inter-day		
Level	**Nominal Conc (ng/mL)**	**Observed Conc (µg/mL) Mean ± SD**	**% RSD**	**% Bias**	**Observed Conc (µg/mL) Mean ± SD**	**% RSD**	**% Bias**
LLOQ	25	23±0.001	3.656	-7.756	23±0.001	5.894	-9.095
LQC	60	57±0.003	4.578	-4.570	56±0.001	1.356	-6.283
MQC	600	604±0.008	1.385	0.650	601±0.007	1.201	0.208
HQC	900	922±0.006	0.641	2.439	945±0.008	0.848	4.977

Table 2.4. Intra-day and Inter-day precision and accuracy of CP in mice plasma.

		Intra-day			Inter-day		
Level	**Nominal Conc (ng/mL)**	**Observed Conc (ng/mL) Mean ± SD**	**% RSD**	**% Bias**	**Observed Conc (ng/mL) Mean ± SD**	**% RSD**	**% Bias**
LLOQ	25	26.42±3.4	12.759	5.666	27.79±3.9	14.053	11.159
LQC	200	171.05±15.6	9.141	-14.477	173.13±16.0	9.225	-13.433
MQC	600	540.70±59.4	10.990	-9.883	634.19±38.8	6.111	5.698
HQC	900	782.33±73.9	9.447	-13.075	888.56±75.2	8.465	-1.272

2.1.5.7. LOD and LOQ

On the basis of signal to noise ratio, LOD and LOQ values were found to be 65.35 ng/mL and 201.60 ng/mL (analytical), 7.91 ng/mL and 23.75 ng/mL (bioanalytical (skin)) and 7.81 ng/mL and 23.60 ng/mL (bioanalytical (plasma)), respectively.

2.1.5.8. System suitability

System suitability of developed methods were performed using six replicates of the QC sample at MQC (45 μg/mL and 600 ng/mL for both analytical and bioanalytical methods respectively). All the system performance parameters were obtained from the system suitability samples and indicated that the developed method was suitable for the analysis of CP. The representative values of the different system performances have been mentioned in Table 2.5.

Table 2.5. System suitability parameters for various methods.

Parameters	**Acceptance limit**	**Analytical**	**Bioanalytical (skin)**	**Bioanalytical (plasma)**
Tailing factor	<1.5	1.13	1.01	1.049

Theoretical plate	>2000	8489.76	9795.3	14021.2
HETP	-	17.69	13.988	17.305
Retention time; Rt (min)	-	5.517	15.314	10.698

2.1.5.9. Robustness

The robustness of the developed methods were studied using two parameters viz. flow rate and mobile phase composition. The data for robustness is shown in Table 2.9. On considering the data, both % bias and % RSD were within the acceptance limit ±2 %. Thus, it can be concluded that the developed analytical method is robust and could be used for routine analysis.

Table 2.6. Robustness studies of CP.

Parameters	**Level**	**Nominal Conc. (µg/mL)**	**Observed Conc (µg/mL) Mean ± SD**	**% RSD**	**% Bias**
Mobile phase ratio ACN:Water (%v/v) at flow rate of 1 mL/min	85:15	45	44.41±0.139	0.313	-1.319
	80:20	45	44.26±0.108	0.233	-1.653
	75:25	45	45.41±0.049	0.109	0.906
Flow rate (mL/min) at mobile phase ratio MeOH:ACN (%v/v) of 80:20	1.1	45	44.82±0.103	0.230	-0.410
	1	45	44.26±0.103	0.244	-1.640
	0.9	45	44.72±0.863	1.929	-0.624

2.1.5.11. Conclusion

A reverse-phase HPLC based analytical and bioanalytical method for the analysis of CP were developed and validated as per ICH Q2R1 and USFDA guidelines. Results supported the

specificity, selectivity and sensitivity of the developed method. The calibration curve depicted the better correlation coefficient and linearity in the concentration range: 0.25-100 µg/mL (analytical), 0.025-1 µg/mL (bioanalytical (skin)) and 25-1000 ng/mL (bioanalytical (plasma)). The developed method could be employed for the determination of CP in samples of drug loading, entrapment efficiency, drug release studies, skin permeation studies and plasma samples.

2.2. Analytical method for the analysis of cholecalciferol (Vitamin D3 (VD3)) and coenzyme Q10 (CoQ10)

2.2.1. Chromatographic Conditions

A sensitive, accurate and reliable method for Vitamin D3 and coenzyme Q10 was developed using Shimadzu HPLC system equipped with an autosampler (SIL-HTC, fixed with a 100µL loop, Shimadzu, Japan) and photodiode array (PDA) detector (SPD-M20A) was used for the analysis and chromatographic separation was performed on WATERS Symmetry C18 column (4.6 x 75 mm). The chromatographic conditions of analytical methods for VD3 and CoQ10 are mentioned in below Table 2.10. The data interpretation and recording was carried out using LC solution software (version 1.22 SP1). The HPLC system was primarily equilibrated for 30 min followed by the analysis of the samples [10-14].

Table 2.7. Chromatographic conditions for analytical methods

Parameters	**VD3**	**CoQ10**
Mobile Phase	MeOH:ACN (80:20)	MeOH:IPA (95:05)
Flow Rate (mL/min)	1	2
Injection volume (µL)	20	20

Run Time (min)	10	20
Wavelength (nm)	265	273

2.2.2. Preparation of stock solution, calibration standards and quality control samples

A primary stock solution of both VD3 and CoQ10 were prepared at concentration of 100 µg/mL by dissolving 2.5 mg of respective drug separately in 25 mL of methanol**.** The calibration standards were prepared by appropriately diluting the stock solution with methanol. In brief, nine different concentrations for VD3 were prepared (0.05-100 µg/mL) along with three quality control (QC) samples directly from primary stock solution i.e. 0.6 µg/mL as LQC, 45 µg/mL MQC and 90 µg/mL as HQC. For CoQ10, ten different concentrations were prepared over the linearity range (0.5-100 µg/mL) with four quality control (QC) samples directly from primary stock solution i.e. 0.5 µg/mL as LLOQ, 3.5 µg/mL as LQC, 45 µg/mL MQC and 90 µg/mL as HQC. Linearity was analyzed for the concentration range with the help of least square linear regression analysis. This regression equation was used to calculate the drug concentration in quality control samples of both drugs.

2.2.3. Validation

The method was validated as per the ICH Q2 R1 guidelines for analytical method validation. The following parameters were determined.

2.2.3.1. Specificity

Specificity of developed method was studied using quality control samples with different excipients that were used in the preparation of nano-formulation.

2.2.3.2. Linearity and Range

The linearity of developed method for VD3 and CoQ10 were determined with a linearity range of 0.05-100 µg/mL and 0.5-100 µg/mL, respectively. The calibration curve was constructed by plotting concentration (µg/mL) on X-axis versus peak area (mAU) on Y-axis, expressed by the equation y = mx + c, where m is slope and c is intercept.

2.2.3.3. Accuracy and precision

For accuracy and precision study, intra-day and inter-day samples were analyzed with four and five different quality control samples for VD3 and CoQ10 respectively, in triplicate. Further, accuracy and precision were expressed in terms of % bias and % RSD, respectively. According to ICH Q2R1 guidelines, both % bias and % RSD of the quality control samples should fall within the range of ±2%.

2.2.3.4. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ was determined as per ICH Q2R1 guidelines on the basis of signal to noise (S/N) ratio method. According to guidelines LOD and LOQ must be 3 and 10 times higher than signal to noise ratio, respectively.

2.2.3.5. System suitability

System suitability was performed for 6 times at MQC level and system performance was verified from various system parameters including tailing factor, theoretical plate numbers and HETP.

2.2.3.6. Robustness

The robustness of the developed method was studied using two parameters viz. flow rate and mobile phase composition. For VD3, in the flow rate, samples were run at three different flow rates i.e. at 1.2, 1.0, and 0.8 mL/min at mobile phase composition MeOH:ACN of 80:20 (% v/v) whereas, for changing another parameter i.e. mobile phase ratio, samples were analysed at three different mobile phase composition (MeOH:ACN (% v/v)) i.e. 85:15, 80:20, and 75:25, respectively at flow rate of 1.0 mL/min. For CoQ10, in the flow rate, samples were run at three different flow rates i.e. at 1.8, 2.0, and 2.2 mL/min at mobile phase composition MeOH:IPA of 1.9:0.1 (% v/v) whereas, for mobile phase ratio, samples were analysed at three different mobile phase composition (MeOH:IPA (% v/v)) i.e. 1.95:0.5, 1.9:0.1, and 1.85:0.15, respectively at flow rate of 2.0 mL/min. According to the ICH guidelines both % bias and % RSD must fall within ±2 % for samples.

2.2.4. Results and discussion

2.2.4.1. Validation

Assay validation of the method was performed on the basis of various parameters including accuracy, precision, specificity, system suitability and stability as per the guidelines.

2.2.4.2. Specificity

For specificity, the developed methods for both VD3 and CoQ10 were verified with sample prepared by spiking the mobile phase with different excipients (Precirol®ATO5, linoleic acid, mPEG-PLA and tween 80) that were used for preparation of nano-formulation. From the results it was observed that the developed methods were highly specific for both drugs with

purity profile of 0.9997 and 0.9949 respectively, suggesting pure peaks. The representative pictorials of chromatograms are shown in Figure 2.4.

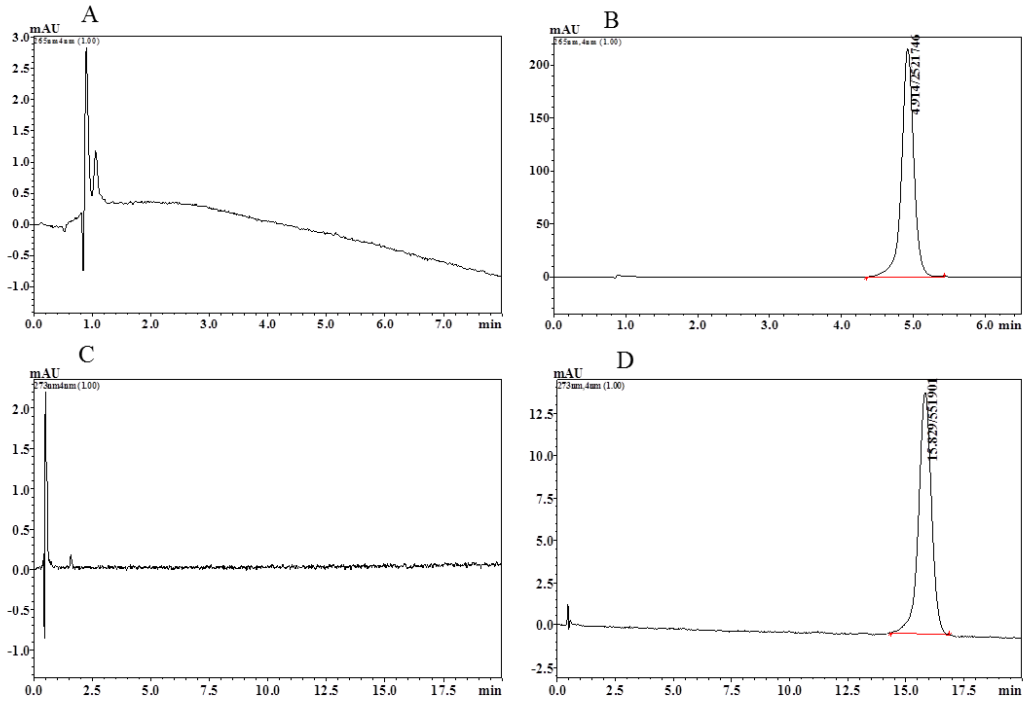


Figure 2.4. Representative chromatogram of (A) mobile phase of VD3 spiked with formulation excipients, (B) analytical sample containing vitamin D3 dissolved in methanol (45µg/mL), (C) mobile phase of CoQ10 spiked with formulation excipients and (D) analytical sample containing CoQ10 dissolved in methanol (45µg/mL).

2.2.4.3. Linearity and Range

For linearity and range study, the developed method was subjected to analysis of different concentrations of VD3 and CoQ10 over the linearity range of 0.05-100 µg/mL and 0.5-100 µg/mL, respectively. It was observed that linearity and range successfully fitted calibration range with regression co-efficient (R^2) of 0.9998 (equation y = 56513x – 3571.3) and 0.9996 (equation

y = 12124x - 1377.7), respectively. The respective calibration curves with equation are shown in Figure 2.5.

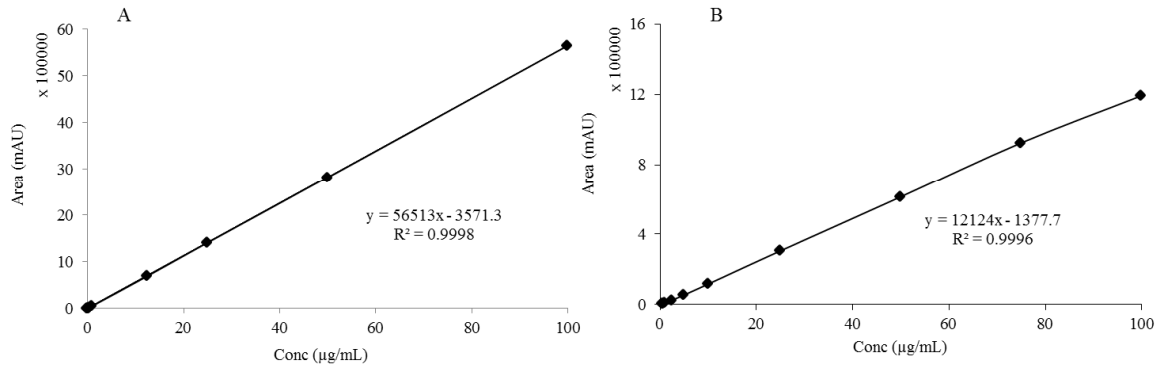


Figure 2.5. Calibration curve for (A) VD3 and (B) CoQ10 in methanol.

2.2.4.4. Accuracy and precision

Intra-day and inter-day samples of accuracy and precision of developed method were analysed with three different quality control samples i.e. LOQ, MQC and HQC (for VD3) and four different quality control samples i.e. LLOQ, LOQ, MQC and HQC (for CoQ10), respectively in triplicates. Further, accuracy and precision were expressed in terms of % bias and %RSD, respectively. From the results, both % bias and % RSD of the quality control samples was within the range of ±2%. Representative data for accuracy and precision were shown in Tables below.

Table 2.8. Intra-day and Inter-day precision and accuracy of analytical samples of VD3

		Intra-day			**Inter-day**		
Level	**Nominal Conc (µg/mL)**	**Observed Conc (µg/mL) Mean ± SD**	**% RSD**	**% Bias**	**Observed Conc (µg/mL) Mean ± SD**	**% RSD**	**% Bias**
LQC	0.6	0.60±0.011	1.840	-0.636	0.60±0.005	0.762	-0.438
MQC	45	44.71±0.060	0.133	-0.637	45.38±0.696	1.535	0.835
HQC	90	89.57±1.575	1.758	-0.480	89.96±0.244	0.271	-0.045

Table 2.9. Intra-day and Inter-day precision and accuracy of analytical samples of CoQ10.

		Intra-day			Inter-day		
Level	**Nominal Conc (µg/mL**	**Observed Conc (µg/mL) Mean ± SD**	**% RSD**	**% Bias**	**Observed Conc (µg/mL) Mean ± SD**	**% RSD**	**% Bias**
LLOQ	0.5	0.51±0.004	0.827	1.975	0.51±0.005	1.050	1.436
LQC	3.5	3.53±0.026	0.740	0.793	3.53±0.036	1.019	0.751
MQC	45	45.86±0.282	0.616	1.914	45.86±0.099	0.216	1.921
HQC	92	92.70±0.218	0.235	0.760	93.22±0.520	0.558	1.323

2.2.4.5. LOD and LOQ

On the basis of signal to noise ratio, LOD and LOQ values were found to be 16.17 ng/mL and 48.5 ng/mL (for VD3) and 150.2 ng/mL and 495.66 ng/mL (for CoQ10), respectively.

2.2.4.6. System suitability

System suitability of developed method was performed using six replicates of the QC sample (MQC). All the system performance parameters were obtained from the system suitability samples and indicated that the developed method was highly sensitive. The representative values of the different system performances for both methods have been mentioned in table 2.13.

Table 2.10. System suitability parameters.

Parameters	**Acceptance limit**	**VD3**	**CoQ10**
Tailing factor	<1.5	1.030	0.987
Theoretical plate	>2000	4220.0	4132.5
HETP	-	35.55	15.829

2.2.4.7. Robustness

Robustness of the developed methods were studied using two parameters viz. flow rate and mobile phase composition. The data for robustness were shown in Table 2.16 and 2.17. On considering the data, both % bias and % RSD were within the acceptance limit (±2%). Thus, it can be concluded that these developed methods are robust and could be used for routine analysis.

Table 2.11. Robustness of analytical samples of VD3.

Parameters	**Level**	**Nominal Conc. (µg/mL)**	**Observed Conc (µg/mL) Mean ± SD**	**% RSD**	**% Bias**
Mobile phase ratio MeOH:ACN (%v/v) at flow rate of 1 mL/min	85:15	45	44.66±0.114	0.256	-0.745
	80:20	45	44.71±0.133	0.133	-0.637
	75:25	45	45.72±0.255	0.557	1.597
Flow rate (mL/min) at mobile phase ratio MeOH:ACN (%v/v) of 80:20	1.2	45	45.37±0.240	0.529	0.827
	1	45	45.38±0.696	1.535	0.835
	0.8	45	45.80±0.397	0.867	1.778

Table 2.12. Robustness of analytical samples of CoQ10.

Parameters	**Level**	**Nominal Conc. (µg/mL)**	**Observed Conc (µg/mL) Mean ± SD**	**% RSD**	**% Bias**
Mobile phase ratio MeOH:IPA (%v/v) at flow rate of 2 mL/min	1.85:0.15	45	45.65±0.108	0.237	1.444
	1.90:0.10	45	45.86±0.099	0.616	1.914
	1.95:0.05	45	45.67±0.420	0.920	1.486
Flow rate (mL/min) at mobile phase ratio MeOH:IPA (%v/v) of 95:5	1.8	45	45.81±0.304	0.665	1.810
	2	45	45.86±0.282	0.216	1.921
	2.2	45	44.12±0.050	0.114	-1.962

2.2.5. Conclusions

A reverse-phase HPLC based analytical methods for the analysis of VD3 and CoQ10 were developed and validated as per ICH Q2R1 guidelines. Results supported the selectivity, specificity and sensitivity of the developed method. The calibration curve depicted the better correlation coefficient and linearity in the concentration range (0.05-100 µg/mL) for VD3 and (0.5-100 µg/mL) for CoQ10 in analytical samples. The developed methods could be employed for determination of VD3 and CoQ10 in the samples of drug loading, entrapment efficiency and drug release studies.

References

[1] A. Bana, M.A. Sathe, S.J. Rajput, Analytical method development and validation for simultaneous estimation of halobetasol propionate and mupirocin in the ratio 1: 40 by uv spectroscopy and RP-HPLC method, Int J Pharm Sci Res, 10 (3), (2019), 1392-1401..

[2] H. Barange, S. Asghar, P. Gour, Development of analytical method and validation for simultaneous estimation of clobetasol propionate and ketoconazole in pharmaceutical cream formulation by RP-HPLC method, Indo Am J Pharm Sci, 4(10) (2017).

[3] M.H.U. Bhuyian, H.A. Rashid, A.A. Islam, M.I. Tareque, Development and validation of method for determination of clobetasol propionate and salicylic acid from pharmaceutical dosage form by HPLC, J Pharm Res Int, (2015), 375-385.

[4] N.N.M. Nagulakonda, K.K.P. Gadde, N. Krishnamurthy Tatikonda, R. Gollapalli, Identification and quantitation of related substances of super potent steroid in its topical combination drug product with vitamin D3 analogue, J Appl Pharm Sci, 9(08) (2019), 071-078.

[5] N. Patel, P. Patel, D. Meshram, A Development and Validation of RP-HPLC Method For Simultaneous Estimation of Nadifloxacin and Clobetasol Propionate In Its Pharmaceutical Dosage Form, Am J PharmTech Res, 6 (5) (2016).

[6] T. Ângelo, M.S. Cunha-Filho, G.M. Gelfuso, T. Gratieri, Chromatographic method for clobetasol propionate determination in hair follicles and in different skin layers, Biomed Chromatogr, 31(2) (2017), e3804.

[7] L. Maione-Silva, K.A.D. Rocha, L.C. de Oliveira, S.F. Taveira, E.M. Lima, Development and validation of a simple and rapid liquid chromatography method for the determination of genistein in skin permeation studies, Biol Pharm Bull, 35(11) (2012), 1986-1990.

[8] L.A.D. Silva, S.F. Taveira, E.M. Lima, R.N. Marreto, In vitro skin penetration of clobetasol from lipid nanoparticles: drug extraction and quantitation in different skin layers, Braz J Pharm Sci, 48(4) (2012), 811-817.

[9] P.B. Musmade, P.B. Deshpande, S.J. Dengle, K.B. Talole, S.M. Pathak, M. Adiga, K. Bhat, N. Udupa, K.L. Bairy, High performance liquid chromatographic method for determination of clobetasol in rat plasma and its application to skin penetration, J Bioanal Biomed, 2(1) (2010), 1-7.

[10] S. Lunetta, M. Roman, Determination of coenzyme Q10 content in raw materials and dietary supplements by high-performance liquid chromatography-UV: collaborative study, J AOAC Int, 91(4) (2008), 702-708.

[11] S. Kumar, D. Chawla, A. Tripathi, An improved and sensitive method for vitamin D3 estimation by RP-HPLC, Pharm Anal Acta, 6(8) (2015), 1-6.

[12] D. Suryawanshi, D.K. Jha, U. Shinde, P.D. Amin, Development and validation of a stability-indicating RP-HPLC method of cholecalciferol in bulk and pharmaceutical formulations: Analytical quality by design approach, J Appl Pharm Sci, 9(06) (2019), 021-032.

[13] Ž. Temova, R. Roškar, Stability-indicating HPLC–UV method for vitamin D3 determination in solutions, nutritional supplements and pharmaceuticals, J Chromatogr Sci, 54(7) (2016), 1180-1186.

[14] A.C. Grace, T. Prabha, M. Jagadeeswaran, K. Srinivasan, T. Sivakumar, Analytical Method Development For Simultaneous Determination Of Ubidecarenone And Vitamin E Acetate In Capsule Dosage Form By HPLC, Int J Pharm Pharm Sci, 11(1) (2019).