Chapter 3
Analytical Method

Dermal Delivery of Protein/Peptide Based Antimicrobial to Treat Secondary Infection in Psoriasis and Eczema

3.1 Introduction

Analytical method development and its validation are the basics of any pharmaceutical development process. It is the process of selecting an accurate and precise assay procedure to quantify the desired component(s) from the developed formulation at different stages of product development. The key parameters that may be assessed during the analytical method development are accuracy, precision, linearity, range, specificity, limit of detection (LOD) and limit of quantification (LOQ) [1].

The present investigation required the analytical methods to measure the critical formulation characteristics, i.e., % entrapment, % loading efficacy, *in-vitro* drug release, *ex-vivo* skin deposition/permeation, and assay (drug retention) during stability studies. Hence, the high-performance liquid chromatography (HPLC) method was selected (offers high sensitivity) and developed to quantify the amount of Omiganan and DPK-060.

3.2 List of Material and Instruments

Table 3.1 List of materials

Materials	**Manufacturer**
Omiganan	S-Biochem, Kerala, India (Custom synthesis)
DPK-060	S-Biochem, Kerala, India (Custom synthesis)
Methanol (A.R. & HPLC Grade)	Spectrochem Pvt. Ltd., Mumbai
Acetonitrile (A.R. & HPLC Grade)	Spectrochem Pvt. Ltd., Mumbai
Tri-Fluro acetic acid (HPLC Grade)	Rankem, Vadodara
Double distilled filtered water	Prepared in-house

Table 3.2 List of instruments

Equipment /Instrument	**Manufacturer**
pH meter	Lab India Pvt. Ltd., Ambala, India
Digital analytical balance	Shimadzu, Japan
RP-HPLC with UV Detector (gradient)	Agilent OpenLab CDS EZChrom, India
Bath Sonicator	Sartorius, Mumbai

3.3 HPLC method development

3.3.1 HPLC method development for Omiganan:

❖ **Gradient HPLC method:** The HPLC method was provided by the S-Biochem.

Instrument: Agilent gradient HPLC

Solvents: Acetonitrile, Trifluoracetic acid, Filtered DDW

HPLC grade water was prepared by filtering DDW (DDW) with nylon filter paper having a pore size of 0.22µm diameter (Pall Life sciences, Mumbai, India)

Gradient chromatographic conditions:

Parameters	Chromatographic Conditions
Mobile Phase	**Mobile Phase A:** Acetonitrile with 0.1% Tri-Fluro Acetic acid (TFA)
	Mobile Phase B: Filtered DDW water with 0.1% TFA
HPLC column	C18 Column, 4.6 µm,250 mm (Thermo Scientific)
UV wavelength	220 nm
Injection volume	20µl
Flow Rate	1 ml/min
Run Time	30 min

Gradient program for Omiganan:

Time (Min)	Pump A	Pump B	Flow Rate (ml/min)
0.0 – 25.0	32%	68%	1
25.0 – 25.1	57%	43%	1
25.1 – 30.0	100%	0%	1
30.0		Stop	

3.3.1.1 Preparation of calibration plot of Omiganan

Accurately weighed 10.0 mg Omiganan was transferred to a 10 ml volumetric flask and dissolved in filtered DDW and the volume was made up to 10 ml (1 mg/ml) by filtered DDW. This solution was further diluted with filtered DDW to make a 100 µg/ml (standard stock solution) concentration of Omiganan. Subsequently, the calibration plot of the Omiganan was carried out in 1-5 µg/ml range. In Brief, 0.1, 0.2, 0.3, 0.4-, and 0.5-ml aliquots of Omiganan standard stock solutions were transferred to 10 ml calibrated volumetric flasks and diluted with filtered DDW up to the mark to obtain standard Omiganan solutions. The chromatograms were recorded at 220 nm detection wavelength for a run time of 30 minutes. The measurements (n=3) were recorded at initial and after 24 h to determine the stability of the peptide solution.

3.3.1.2 Validation of HPLC method for Omiganan

For analytical method validation, accuracy and intra-day & inter-day precision [2]. Accuracy, also referred to as recovery, is a tool for analyzing the trueness of test measurements with the standard. The mean % recovery values close to 100% represent the high accuracy of a method. While, precision is a measure of the consistency and reproducibility of a method.

3.3.1.2.1 Accuracy

The accuracy of the method was ascertained by showing recovery studies by the standard addition method. Briefly, known quantity of standard Omiganan was added at 3 different levels i.e., 80%, 100%, and 120%. Accuracy was assessed as the mean % recovery [3]. The % recovery of the added pure drug was calculated according to the following formula:

$$\%\ \boldsymbol{recovery} = \frac{\boldsymbol{Ct - Cs}}{\boldsymbol{Cs}}\ \boldsymbol{x}\ \mathbf{100}$$

Where Ct = Total concentration of drug determined from the method, Cs = Known or expected drug concentration.

3.3.1.2.2 Precision

The intraday and interday precision of the method was evaluated by assessing the corresponding responses three times on the same day and 3 consecutive days, respectively [3]. 3 different concentrations of Omiganan (1, 3, and 5 µg/ml) were chosen for the precision study of the developed HPLC method. The experiments were executed in triplicates, and the mean and % RSD was calculated to assess the suitability of the method.

3.3.1.2.3 LOD and LOQ

The LOD and LOQ of Omiganan were determined by the calibration standard method. LOD and LOQ were calculated as 3.3 σ/s and 10 σ/s respectively, where S is the slope of the calibration curve, and σ is the standard deviation of the y-intercept of the regression equation.

3.3.2 HPLC method development for DPK-060:

- **Gradient HPLC method:** The HPLC method was provided by the S-Biochem.

Instrument: Agilent gradient HPLC, **Solvents:** Acetonitrile, TFA, Filtered DDW.

Gradient Chromatographic conditions:

Parameters	Chromatographic Conditions
Mobile Phase	**Mobile Phase A:** Acetonitrile with 0.1% TFA
	Mobile Phase B: Filtered DDW with 0.1% TFA
HPLC column	C18 Column, 4.6 µm,250 mm (Thermo Scientific)
UV wavelength	220 nm
Injection volume	20µl
Flow Rate	1 ml/min
Run Time	30 min

Gradient Program for DPK-060:

Time (Min)	Pump A	Pump B	Flow Rate (ml/min)
0.0 – 25.0	25%	75%	1
25.0 – 25.1	50%	50%	1
25.1 – 30.0	100%	0%	1
30.0		Stop	

3.3.2.1 Preparation of calibration plot of DPK-060

Accurately weighed 10.0 mg DPK-060 into the 10 ml capacity volumetric flask and dissolved in a mixture of methanol and filtered DDW (2:8), make up the volume to 10 ml (1 mg/ml). This solution was further diluted with filtered DDW to make a 100 µg/ml (standard stock solution) concentration of DPK-060. Subsequently, the calibration plot of the DPK-060 was carried out in the range of 0.5-5 µg/ml. Briefly, 0.05, 0.1, 0.2, 0.3, 0.4-, and 0.5-ml aliquots of DPK 060 standard stock solutions were transferred to 10 ml calibrated volumetric flasks and diluted with filtered DDW up to the mark to obtain standard DPK 060 solutions. The chromatograms were recorded at 220 nm detection wavelength for a run time of 30 minutes. The measurements (n=3) were recorded at initial and after 24 h to determine the stability of the peptide solution.

3.3.2.2 Validation of HPLC method for DPK-060

The HPLC method for DPK-060 was validated for accuracy, precision, and sensitivity, in a similar way as described in section 3.3.1.2.

3.4 Results and Discussion

The observations and results obtained have been discussed below.

3.4.1 HPLC method for Omiganan

A typical chromatogram obtained from RP-HPLC analysis using the C18 column is given in Fig. 3.1. Sharp, symmetric peaks (Avg. tailing factor < 1) were observed with an average retention time of 14.6 min at 220 nm with a flow rate of 1 ml/min.

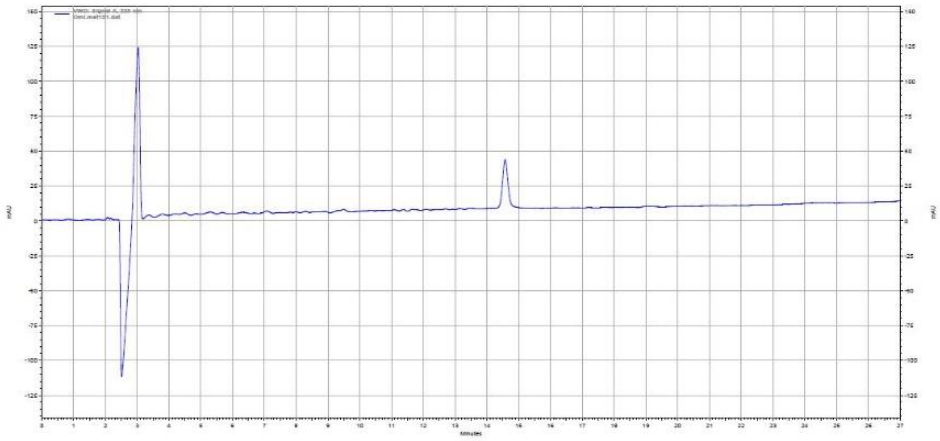

Figure 3.1 Typical HPLC chromatogram of Omiganan (3 µg/ml)

The peak area values corresponding to the selected concentration of Omiganan are given in Table 3.3. The overlay plot and calibration plot for the same are illustrated in Fig. 3.2 and 3.3.

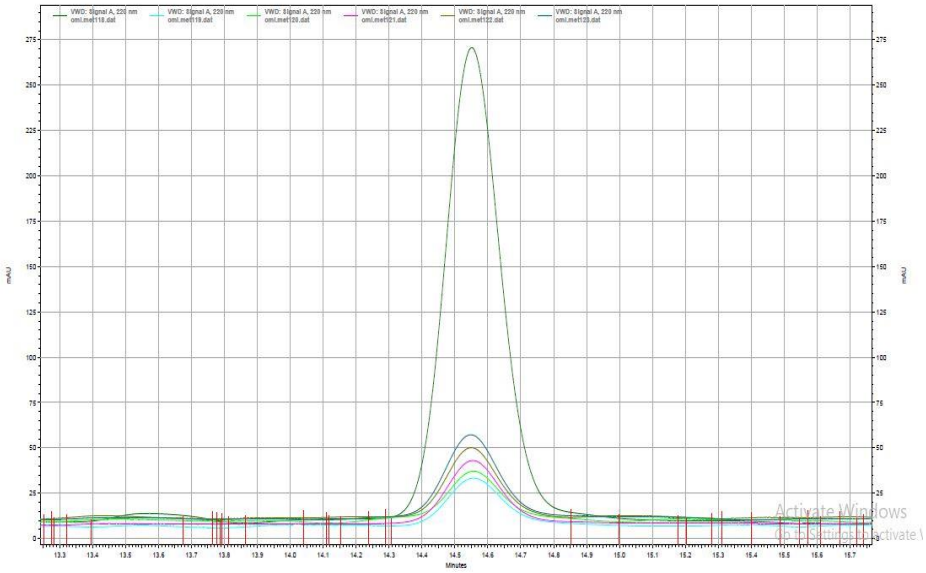

Figure 3.2 Overlay plot of Omiganan by HPLC method

The regression analysis of calibration data showed a positive correlation between the concentration of Omiganan and peak area values with good linearity (R^2 = 0.9991). The result reflected that Beer's law was obeyed for the selected Omiganan concentration range of 1-5 µg/ml by the developed HPLC method.

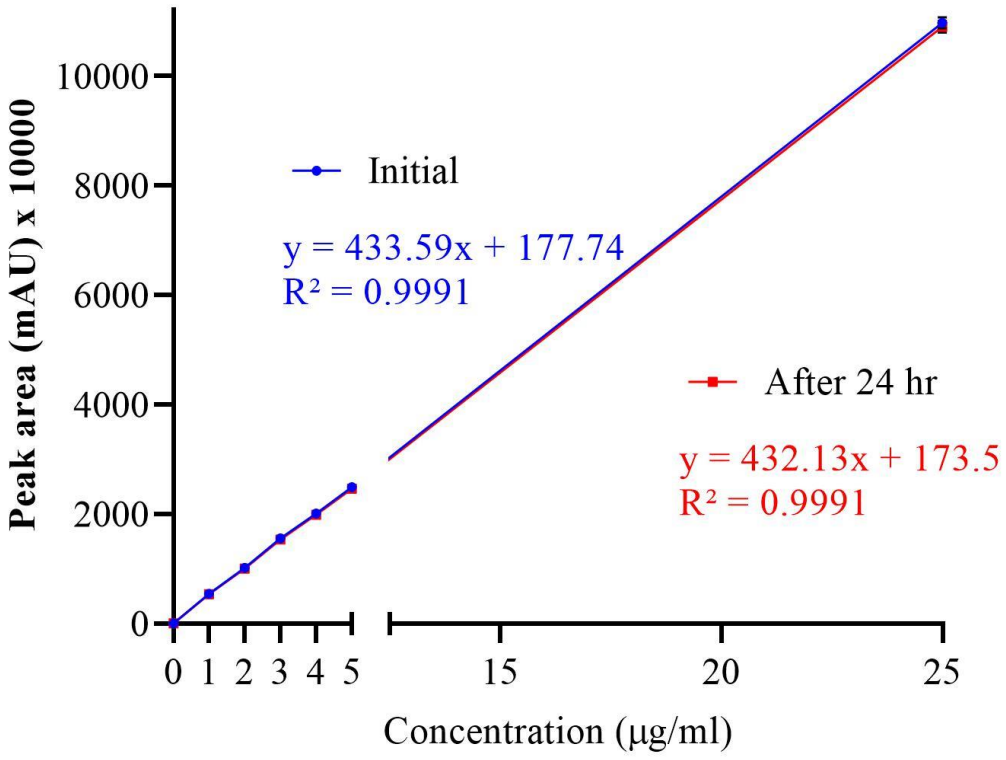

Figure 3.3 Calibration plot of Omiganan by HPLC method

Table 3.3 Peak area data of Omiganan at initial and 24 h for calibration and stability

Concentration (µg/ml)	Peak area (mAU * 10000)	
	Initial	After 24 hr
0	0	0
1	542 ± 6	540 ± 7
2	1016 ± 14	1011 ± 16
3	1558 ± 20	1549 ± 22
4	2009 ± 24	1997 ± 27
5	2494 ± 35	2472 ± 38
25	10967 ± 61	10929 ± 69

Mean ± SD (n=3)

Measurement of same standard Omiganan solutions after storage for 24 hours at room temperature did not show any significant change in the peak area values (Table 3.3, Fig. 3.3), indicating the stability of standard Omiganan solutions throughout the analysis.

3.4.2 Validation of HPLC method for Omiganan

3.4.2.1 Accuracy

The mean % recovery value for lower, intermediate, and higher concentrations, i.e., 80,100 and 120%, was 99.20%, 99.66%, and 99.48%, respectively. The mean % recovery values, close to 100% with low relative standard deviation (% RSD < 2 %), represented the high accuracy of the developed HPLC method. The results of this study are shown in Table 3.4.

Table 3.4 Accuracy of the HPLC method for Omiganan

% Drug spiked	Concentration (µg/ml)	Recovered Concentration (µg/ml)	% Recovery	% RSD
80%	1	0.992	99.20 %	0.51
100%	3	2.990	99.66 %	0.39
120%	5	4.974	99.48 %	0.62

3.4.2.2 Precision

Intraday and interday precision data under the same operating conditions are summarized in Table 3.5. The results were precise over the selected time interval as the % RSD values obtained for the HPLC method were within the acceptable range (< 2%).

Table 3.5 Intraday & interday precision analysis of HPLC method of Omiganan

Intraday precision						
Concentration (µg/ml)	**Peak area at different time slot (mAU * 10000)**			**Mean peak area (mAU * 10000)**	**SD**	**% RSD**
	Slot 1	**Slot 2**	**Slot 3**			
1	545	542	540	542.33	2.52	0.46
3	1565	1556	1549	1556.66	8.02	0.52
5	2497	2482	2471	2483.33	13.05	0.53
Interday precision						
Concentration (µg/ml)	**Peak area at different days (mAU * 10000)**			**Mean peak area (mAU * 10000)**	**SD**	**%RSD**
	Day 1	**Day 2**	**Day 3**			
1	545	539	535	539.67	5.03	0.93
3	1565	1547	1539	1550.33	13.32	0.85
5	2497	2472	2462	2476.67	18.45	0.74

Table 3.6 summarizes the LOD and LOQ values of the HPLC method for Omiganan estimation in analytical samples. The LOD values were found well below the concentration range selected for calibration, indicating the sensitivity of methods for accurate detection of Omiganan present in standard solutions.

Table 3.6 Sensitivity evaluation of HPLC method of Omiganan

HPLC method of Omiganan	**Slope of line**	**SD of line**	**LOD (µg/ml)**	**LOQ (µg/ml)**
	433.586	9.033	0.062	0.208

3.4.3 HPLC method for DPK-060

A typical chromatogram obtained from RP-HPLC analysis using the C18 column is given in Fig. 3.4. Sharp, symmetric peaks (Avg. tailing factor < 1.2) were observed with an average retention time of 8.5 min at 220 nm with a 1 ml/min flow rate.

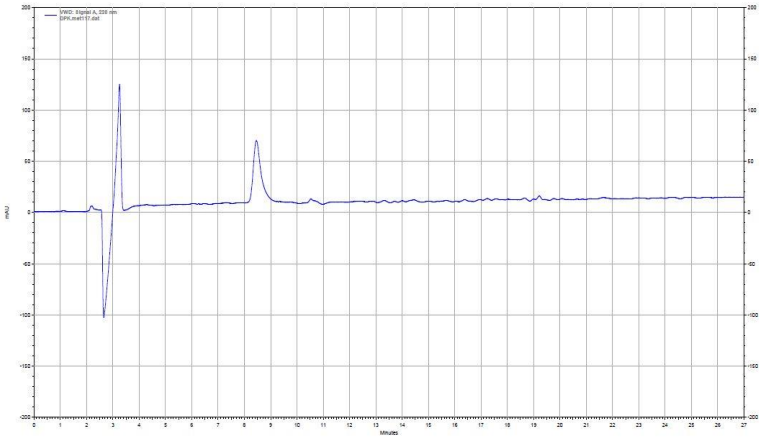

Figure 3.4 Typical HPLC chromatogram of DPK-060 (1 µg/ml)

The peak area values corresponding to the selected concentration of DPK-060 are given in Table 3.7. The overlay plot and calibration plot for the same are illustrated in Fig. 3.5 and 3.6.

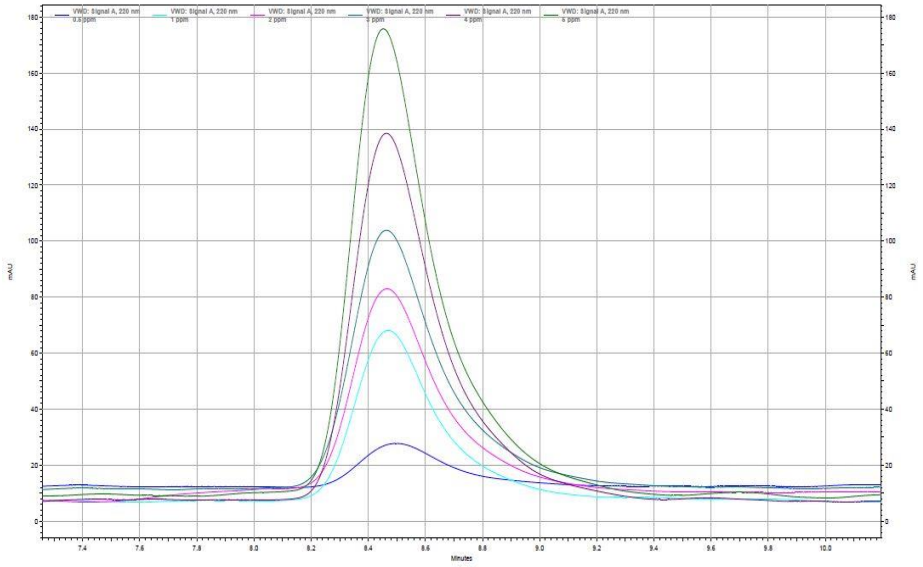

Figure 3.5 Overlay plot of DPK-060 by HPLC method

The regression analysis of calibration data showed a positive correlation between the concentration of DPK-060 and peak area values with good linearity (R^2 = 0.999). The result reflected that Beer's law was obeyed for the selected DPK-060 concentration range of 0.5-5 µg/ml by the developed HPLC method.

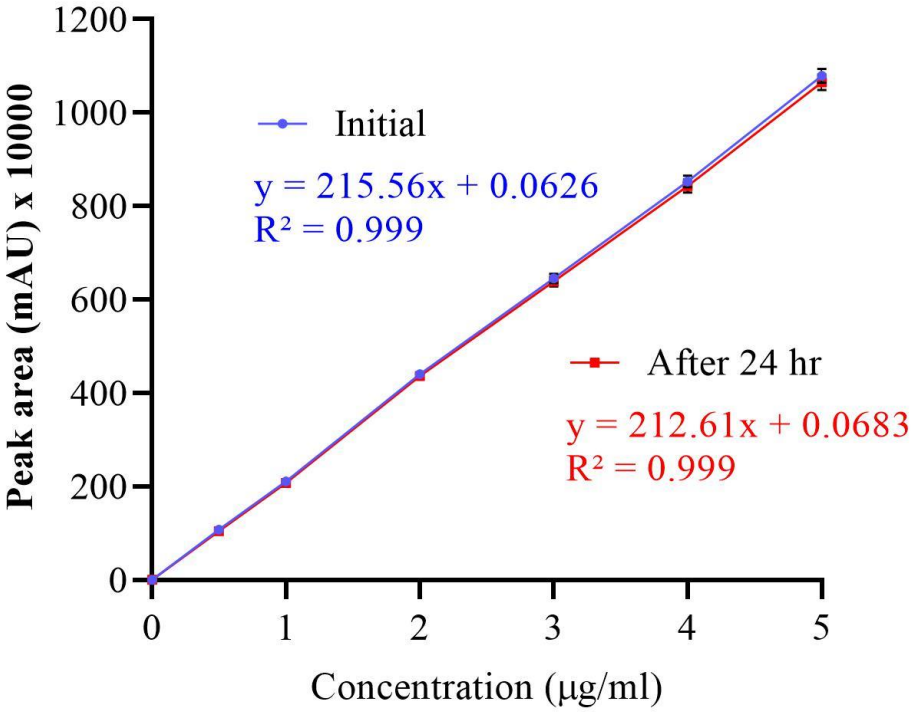

Figure 3.6 Calibration plot of DPK-060 by HPLC method

Table 3.7 Peak area data of DPK-060 at initial and 24 h for calibration and stability

Concentration (µg/ml)	Peak area (mAU * 10000)	
	Initial	After 24 hr
0	0	0
0.5	107 ± 1.5	104 ± 1.6
1	210 ± 3.1	207 ± 3.3
2	440 ± 5.0	436 ± 4.8
3	645 ± 8.5	639 ± 9.1
4	852 ± 11.0	843 ± 11.8
5	1079 ± 11.1	1065 ± 12.2

Mean ± SD (n=3)

Measurement of same standard DPK-060 solutions after storage for 24 hours at room temperature did not show any significant change in the peak area values (Table 3.7, Fig. 3.5), indicating the stability of standard DPK-060 solutions throughout the analysis.

3.4.4 Validation of HPLC method for DPK-060

3.4.4.1 Accuracy

The mean % recovery value for lower, intermediate, and higher concentrations, i.e., 80,100 and 120%, was 99.40%, 99.53%, and 99.60%, respectively. The mean % recovery values, close to 100% with low relative standard deviation (% RSD < 2 %), represented the high accuracy of the developed HPLC method. The results of this study are summarized in Table 3.8.

Table 3.8 Accuracy of the HPLC method for DPK-060

% Drug spiked	Concentration (µg/ml)	Recovered Concentration (µg/ml)	% Recovery	% RSD
80%	1	0.994	99.40 %	0.42
100%	3	2.986	99.53 %	0.55
120%	5	4.980	99.60 %	0.49

3.4.4.2 Precision

Intraday and interday precision data under the same operating conditions are summarized in Table 3.9. The results were precise over the selected time interval as the % RSD values obtained for the HPLC method were within the acceptable range (< 2%).

Table 3.9 Intraday and interday precision analysis of HPLC method of DPK-060

Intraday precision						
Concentration (µg/ml)	Peak area at different time slot (mAU * 10000)			Mean peak area (mAU * 10000)	SD	% RSD
	Slot 1	Slot 2	Slot 3			
1	208	206	205	206.33	1.53	0.74
3	649	646	641	645.33	4.04	0.63
5	1072	1068	1059	1066.66	6.11	0.57

Interday precision						
Concentration (µg/ml)	Peak area at different days (mAU * 10000)			Mean peak area (mAU * 10000)	SD	%RSD
	Day 1	Day 2	Day 3			
1	208	205	204	205.66	2.08	1.01
3	649	642	638	643.00	5.57	0.86
5	1072	1065	1053	1063.33	9.61	0.90

Table 3.10 summarizes the LOD and LOQ values of the HPLC method for DPK-060 estimation in analytical samples. The LOD values were found well below the concentration range selected for calibration, indicating the sensitivity of methods for accurate detection of DPK-060 present in standard solutions.

Table 3.10 Sensitivity evaluation of HPLC method of DPK-060

HPLC method of DPK-060	Slope of line	SD of line	LOD (µg/ml)	LOQ (µg/ml)
	215.096	2.398	0.033	0.111

3.5 Conclusion

HPLC methods for quantification of Omiganan and DPK-060 were successfully established. Validation revealed that all the developed HPLC methods were linear, precise, accurate, sensitive, and specific.

3.6 References

1. Friedrich, C.L., et al., *Antibacterial action of structurally diverse cationic peptides on gram-positive bacteria.* Antimicrobial agents and chemotherapy, 2000. **44**(8): p. 2086-2092.
2. Guideline, I.H.T., *Validation of analytical procedures: text and methodology.* Q2 (R1), 2005. **1**.
3. Swartz, M. and I. Krull, *HPLC method development and optimization with validation in mind.* Handbook of analytical validation, 1st edn. Taylor & Francis Group, Boca Raton, FL, 2012: p. 37-60.