Chapter 5: Analytical method development

5.0 Analytical Method development

5.1 Introduction

The current study entailed the incorporation of two drugs in a single liposomal nanocarrier. The quantitative estimation of encapsulation of both drugs, VCR and DOX in the nanoliposomes were estimated using reverse phase High performance liquid chromatography (RP-HPLC). The simultaneous estimation of assay and free drug content of the drugs in the formulation were performed using this versatile chromatographic technique (1). Importantly, suitable analytical methods need to be developed for the selection of efficient separation of the two drugs. Such development would require a careful examination of the physico-chemical properties of the drugs and selection of chromatographic conditions including diluent selection and its properties, pH of the buffer phase, wavelength of detection and type of detector, choice of stationary phase, phase column temperature, injection rate and volume, selection of solvent in mobile phase, flow rate of mobile phases and elution technique among others (2). Additionally, the stability of the chromatographic conditions, precision and repeatability need to be ascertained while determining the appropriate analytical method (3). The following sections detail out the method development for the assay and free drug determination of the drugs.

5.2 Materials

The various materials used in the method development for the simultaneous estimation of the drugs vincristine sulphate and doxorubicin hydrochloride are mentioned in Table 7.

Sl. No	Material	Manufacturer	Grade
1	Diethyl amine	Sigma Aldrich, USA	HPLC grade
2	Ortho-Phosphoric acid 85%	Sigma Aldrich, USA	HPLC grade
3	Methanol	Merck Millipore, Germany	HPLC grade
4	Milli-Q water	Merck Millipore, Germany	HPLC grade
5	Ammonium Formate	Sigma Aldrich, USA	HPLC grade
6	Acetonitrile	Sigma Aldrich, USA	HPLC grade
7	Ammonia Solution	Sigma Aldrich, USA	HPLC grade
8	Ammonium acetate	Sigma Aldrich, USA	HPLC grade
9	Formic acid	Sigma Aldrich, USA	HPLC grade

Sl. No	Material	Manufacturer	Grade
10	Superdex 75	GE healthcare, Singapore	HPLC grade
11	Ammonium Sulphate	Sigma Aldrich, USA	HPLC grade
12	Tetrahydrofuran	Rankem chemicals, India	HPLC grade

Table 7: Materials used in the method development of simultaneous estimation of the drugs and the assay of lipids

5.3 Method development for simultaneous estimation of Assay of both drugs

5.3.1 Adaptation of USP based assay methods

Preparation of Buffer: The buffer was prepared by adding 33 ml of Diethyl amine to 2000 ml water, pH adjusted to 7.5 using orthophosphoric acid and filtered through 0.45µ nylon membrane filter. The buffer solution was sonicated for 10 minutes and degassed prior to further usage.

Preparation of Mobile phase: The mobile phase was prepared by mixing the buffer solution and methanol in 30:70 (%v/v) ratio. The mobile phase was mixed well prior to further usage.

Instrumental conditions: High Performance Liquid Chromatograph (HPLC) equipped with UV-visible detector (Shimadzu, Japan) and Empower Chromatography Data System (Waters, USA) was used with the following conditions.

- Column: Zorbax C18, 150 x 4.6 mm, 3.5 µ, FD-581 (Agilent USA)
- Flow rate: 1.5 ml/min.
- Detection UV wavelength: 297 nm for Vincristine sulphate, 254 nm for Doxorubicin hydrochloride
- Injection volume: 10 µl
- Run time: 30 minutes
- Column oven temp: 25°C
- Sample cooler temp: 10°C

Standard solution preparation (Doxorubicin hydrochloride): Accurately weighed working standards of 20.0 mg Doxorubicin Hydrochloride USP was transferred to a 100 ml volumetric flask. The drug was dissolved using 50 ml of methanol using sonication (Bath sonicator, Oscar

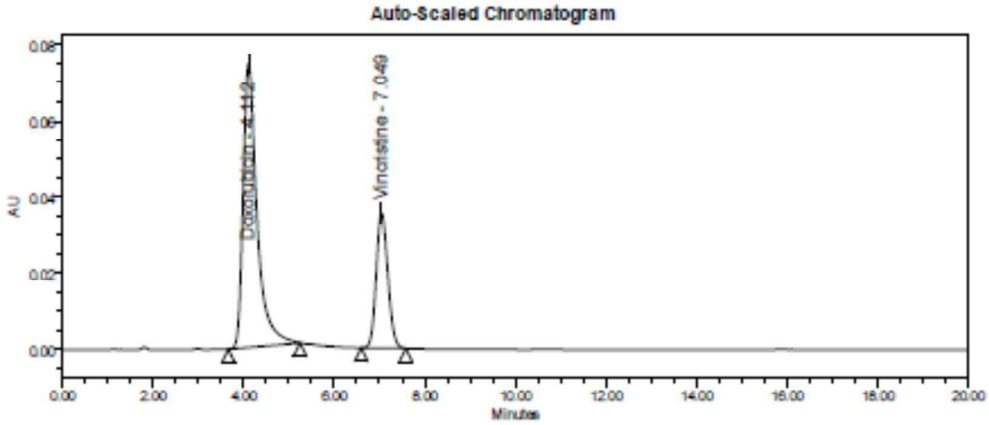
Ultrasonic, India) and volume was made up to 100 ml using the methanol. The resultant solution was cooled to room temperature. The prepared standard solution as shaken well prior to usage (Solution A).

Standard solution preparation (Vincristine sulphate): Accurately weighed working standards of 20 mg Vincristine Sulphate USP was transferred to a 100 ml volumetric flask. The drug was dissolved using 50 ml of methanol using sonication (Bath sonicator, Oscar Ultrasonic, India) and volume was made up to 100 ml using the methanol. The resultant solution was cooled to room temperature. The prepared standard solution as shaken well prior to usage (Solution B).

Preparation of mixed standard solution (100 ppm each): The mixed standard was prepared by transferring 5 m1 of Doxorubicin Hydrochloride (Solution A) and 5m1 of Vincristine Sulphate (Solution B) to 25 ml volumetric flask. The solution was mixed properly prior to usage. The prepared mixed standard solution was injected into chromatographic system. Single standards of similar concentration were injected into the system for identification of the peaks.

Observation: The peak shape of doxorubicin hydrochloride peak (found at the retention time of 4.1 minute) was found to be distorted while the peak shape of vincristine sulphate (retention time of 7.0 minute) was found to be sharp. Further, the resolution between each peak was not found to be satisfactory while high column back pressure was observed. The chromatogram using mixed standard solution as obtained with this experiment was presented in Figure 14.

Conclusion: The resolution and the peak shape of doxorubicin hydrochloride was found to be non-satisfactory while that of vincristine sulphate were proper. A change in the buffer system or change in elution technique from isocratic to gradient elution may be needed to get better resolution and peak shape.



	Retention Time (min)	Name	Calculated_Area	USP Resolution	USP Tailing	USP Plate Count
1	4.11	Doxorubicin	1582.68		1.6	981.3
2	7.05	Vincristine	629.99	5.7	1.1	3596.0
Sum			2212.67			

Figure 14: Chromatogram of Adaptation of USP method of assay for both drugs

5.3.2 Change in buffer system and elution technique

Preparation of Buffer: The buffer was prepared by dissolving 3.15 gm ammonium formate in 1000 ml water, pH adjusted to 7.5 with dilute ammonia solution and filtered through 0.45µ nylon membrane filter. The buffer solution was sonicated for 10 minutes and degassed prior to further usage.

Mobile phases: Gradient elution technique was used for HPLC analysis. Three mobile phases were prepared

- Mobile phase-A: Acetonitrile
- Mobile phase-B: Buffer pH-7.5
- Mobile phase-C: Methanol

Instrumental conditions: High Performance Liquid Chromatograph (HPLC) equipped with UV-visible detector (Shimadzu, Japan) and Empower Chromatography Data System (Waters, USA) was used with the following conditions.

- Column: Symmetry shield RP C-18 150 x 4.6 mm, 3.5 µ, FD-581 (Waters, USA)
- Flow rate: 1.0 ml/min.
- Detection UV wavelength: 297 nm for Vincristine sulphate and 254 nm for Doxorubicin

hydrochloride

- Injection volume: 10 µl
- Run time: 30 minutes
- Column oven temperature: 25°C
- Sample cooler temperature: 10°C

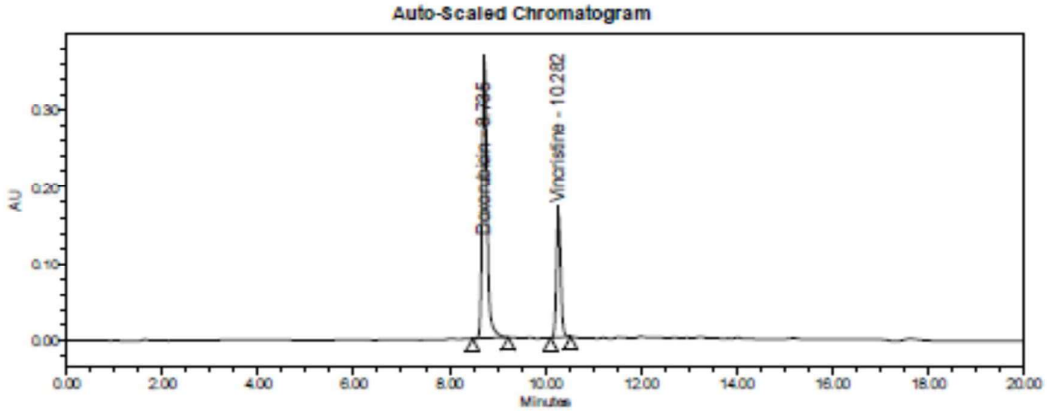
Gradient Program: The gradient program using various % volumes of the three mobile phases was prepared and followed (Table 8).

Time (Minutes)	% Mobile Phase A	% Mobile Phase B	% Mobile Phase C
0	8	80	12
5	20	50	30
10	30	20	50
15	30	20	50
15.1	8	80	12
20	8	80	12

Table 8: Gradient elution program change in buffer system and elution technique

Procedure: The prepared mixed standard solution was injected into chromatographic system. Single standards of similar concentration were injected into the system for identification of the peaks (preparation as detailed in 5.3.1).

Observation: The peak shape of doxorubicin hydrochloride peak (found at the retention time of 8.7 minutes) was found to be distorted while the peak shape of vincristine sulphate (retention time of 10.28 minute) was found to be sharp. Further, resolution between each peak was not satisfactory and the chromatogram using mixed standard solution as obtained with this experiment was presented in Figure 15.



	Retention Time (min)	Name	Calculated_Area	USP Resolution	USP Tailing	USP Plate Count
1	8.74	Doxorubicin	2495.23		1.3	40764.3
2	10.28	Vincristine	982.99	9.0	1.1	64318.6
Sum			3478.22			

Figure 15: Chromatogram of experiment with change in buffer system and elution technique

Conclusion: The resolution and the peak shape of doxorubicin hydrochloride was found to be non-satisfactory while that of vincristine sulphate were proper. A change in the buffer system pH or change in the column may be needed to get better resolution and peak shape.

5.3.3 Change in pH of buffer system and column

Preparation of Buffer: The buffer was prepared by dissolving 7.76 gm ammonium acetate in 1000 ml water, pH adjusted to 5.04 with dilute ortho phosphoric acid and filtered through 0.45µ nylon membrane filter. The buffer solution was sonicated for 10 minutes and degassed prior to further usage.

Mobile phases: Gradient elution technique was used for HPLC analysis. Two mobile phases were prepared

- Mobile phase-A: Buffer pH-5.0
- Mobile phase-B: Mobile phase B was prepared by mixing the prepared methanol and acetonitrile in the ratio of 80:20 (% v/v).

The prepared mobile phases were sonicated for 10 minutes and degassed prior to further usage.

Instrumental conditions: High Performance Liquid Chromatograph (HPLC) equipped with UV-visible detector (Shimadzu, Japan) and Empower Chromatography Data System (Waters, USA) was used with the following conditions.

- Column: YMC Pack pro C-18 150 x 4.6 mm, 3.5 µ (YMC, Japan)
- Flow rate: 1.0 ml/min.
- Detection UV wavelength: 297 nm for Vincristine sulphate and 254 nm for Doxorubicin hydrochloride
- Injection volume: 10 µl
- Run time: 30 minutes
- Column oven temperature: 30°C
- Sample cooler temperature: 10°C

Gradient program: The gradient program using various % volumes of the three mobile phases was prepared and followed (Table 9).

Time (Minutes)	% Mobile Phase A	% Mobile Phase B
0	50	50
15	20	80
20	20	80
20.1	50	50
25	50	50

Table 9: Gradient elution program for change in buffer pH and column

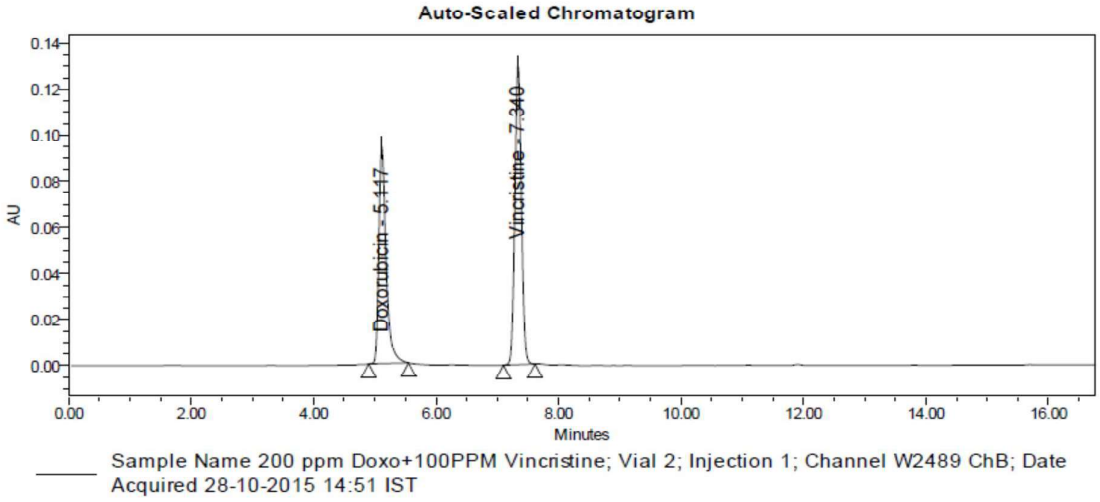
Standard solution preparation (Doxorubicin hydrochloride): Accurately weighed working standards of 20.0 mg Doxorubicin Hydrochloride USP was transferred to a 50 ml volumetric flask. The drug was dissolved using 30 ml of methanol using sonication (Bath sonicator, Oscar Ultrasonic, India) and volume was made up to 50 ml using the methanol. The resultant solution was cooled to room temperature. The prepared standard solution as shaken well prior to usage (Solution A).

Standard solution preparation (Vincristine sulphate): Accurately weighed working standards of 20 mg Vincristine Sulphate USP was transferred to a 100 ml volumetric flask. The drug was dissolved using 50 ml of methanol using sonication (Bath sonicator, Oscar Ultrasonic, India) and volume was made up to 100 ml using the methanol. The resultant solution was

cooled to room temperature. The prepared standard solution as shaken well prior to usage (Solution B).

Preparation of mixed standard solution (200 ppm doxorubicin + 100 ppm vincrisitine): The mixed standard was prepared by transferring 5 m1 of Doxorubicin Hydrochloride (Solution A) and 5m1 of Vincristine Sulphate (Solution B) to 25 ml volumetric flask. The solution was mixed properly prior to usage. The prepared mixed standard solution was injected into chromatographic system. Single standards of similar concentration were injected into the system for identification of the peaks.

Observation: The peak shape of doxorubicin hydrochloride peak (found at the retention time of 5.1 minutes) was found to be distorted while the peak shape of vincristine sulphate (retention time of 7.3 minute) was found to be sharp. Further, resolution between each peak was not satisfactory (reduced to 10.79 from 28.3) and the chromatogram using mixed standard solution as obtained with this experiment was presented in Figure 16.



	Retention Time (min)	Name	Calculated_Area	USP Resolution	USP Tailing	USP Plate Count
1	5.12	Doxorubicin	771.93		1.48	9626.7
2	7.34	Vincristine	963.82	10.75	1.06	22729.7
Sum			1735.75			

Figure 16: Chromatogram of experiment with change in pH of buffer system and column

Conclusion: The peak shape of doxorubicin hydrochloride was found to be non-satisfactory while that of vincristine sulphate were proper. A change in the pH of the buffer resulted in reduced resolution between the peaks. Change in polarity of mobile phase B with buffer ammonium formate solution pH adjusted to 7.5 may be needed to get better resolution and peak shape.

5.3.4 Change in polarity of mobile phase B

Preparation of Buffer: The buffer was prepared by dissolving 6.30 gm ammonium formate in 2000 ml water, pH adjusted to 7.5 with dilute ammonia solution and filtered through 0.45µ nylon membrane filter. The buffer solution was sonicated for 10 minutes and degassed prior to further usage.

Mobile phases: Gradient elution technique was used for HPLC analysis. Two mobile phases were prepared

- Mobile phase-A: Buffer pH-7.5
- Mobile phase-B: Mobile phase B was prepared by mixing buffer: methanol: acetonitrile in ratio of 20:50:30 (% v/v)

Instrumental conditions: High Performance Liquid Chromatograph (HPLC) equipped with UV-visible detector (Shimadzu, Japan) and Empower Chromatography Data System (Waters, USA) was used with the following conditions.

- Column: YMC Pack pro C-18 150 x 4.6 mm, 3.5 µ (YMC, Japan)
- Flow rate: 1.0 ml/min.
- Detection UV wavelength: 297 nm for Vincristine sulphate and 254 nm for Doxorubicin hydrochloride
- Injection volume: 10 µl
- Run time: 30 minutes
- Column oven temperature: 30°C
- Sample cooler temperature: 10°C

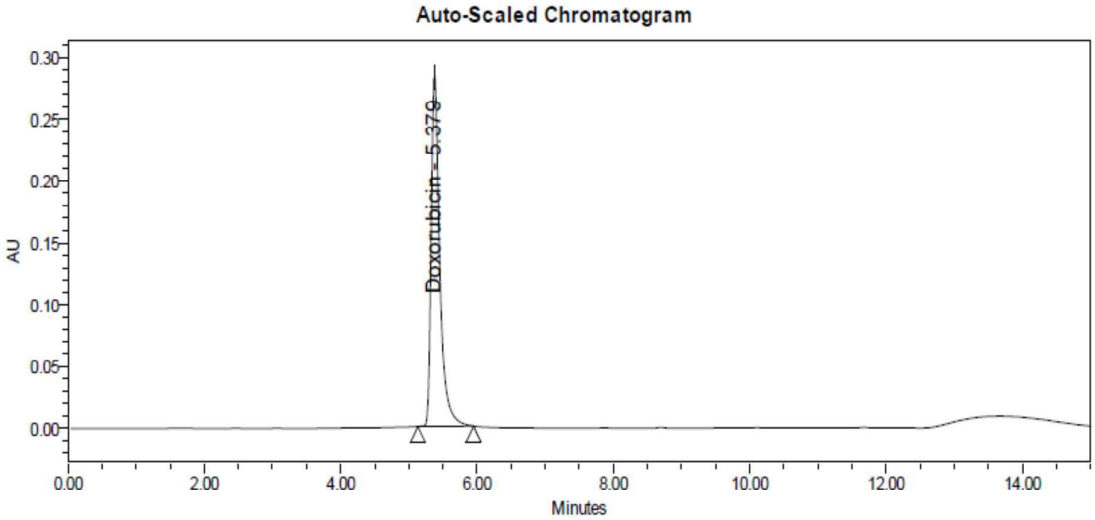
Gradient program: The gradient program using various % volumes of the three mobile phases was prepared and followed (Table 10).

Time (Minutes)	% Mobile Phase A	% Mobile Phase B
0	50	50
4	40	60
7	40	60
7.1	24	76
10.0	24	76
10.1	50	50

Table 10: Gradient elution program for change in polarity of mobile phase B

Procedure: The prepared mixed standard solution was injected into chromatographic system. Single standards of similar concentration were injected into the system for identification of the peaks (preparation as detailed in 5.3.3).

Observation: The peak shape of doxorubicin hydrochloride peak (found at the retention time of 5.37 minutes) was found to be distorted with tailing factor of 1.6 while the peak of vincristine sulphate was found to have not eluted till 15 minutes. The chromatogram using mixed standard solution as obtained with this experiment was presented in Figure 17.



	Retention Time (min)	Name	Calculated_Area	USP Tailing	USP Plate Count
1	5.38	Doxorubicin	2468.39	1.60	9867.8
Sum			2468.39		

Figure 17: Chromatogram of experiment with change in composition of mobile phase B

Conclusion:

The resolution and peak shape of doxorubicin hydrochloride was found to be non-satisfactory with absence of peak of vincristine sulphate till 15 minutes. A change in gradient elution program or the mobile phase composition may be needed may be needed to get better resolution and peak shape.

5.3.5 Change in gradient elution program and mobile phase composition

Preparation of Buffer: The buffer was prepared by dissolving 3.15 grams of ammonium formate in 1000 ml water, pH adjusted to pH 4.5 using formic acid and filtered through 0.45µ nylon membrane filter. The buffer solution was sonicated for 10 minutes and degassed prior to further usage.

Mobile phases: Gradient elution technique was used for HPLC analysis. Two mobile phases were prepared using different volume/volume ratios of buffer.

- Mobile phase-A: Mobile phase A was prepared by mixing the prepared buffer and methanol in the ratio of 80:20 (% v/v). The mobile phase was sonicated for 10 minutes and degassed prior to further usage.
- Mobile phase-B: Mobile phase B was prepared by mixing the prepared buffer and acetonitrile in the ratio of 20:80 (% v/v). The mobile phase was sonicated for 10 minutes and degassed prior to further usage.

Instrumental conditions: High Performance Liquid Chromatograph (HPLC) equipped with UV-visible detector (Shimadzu, Japan) and Empower Chromatography Data System (Waters, USA) was used with the following conditions.

- Column: YMC Pack pro C-18 150 x 4.6 mm, 3.5 µ (YMC, Japan)
- Flow rate: 1.0 ml/min.
- Detection UV wavelength: 297 nm for Vincristine sulphate and 254 nm for Doxorubicin hydrochloride
- Injection volume: 10 µl
- Run time: 30 minutes
- Column oven temperature: 30°C

- Sample cooler temperature: 10°C

Gradient program: The gradient program using various % volumes of the three mobile phases was prepared and followed (Table 11).

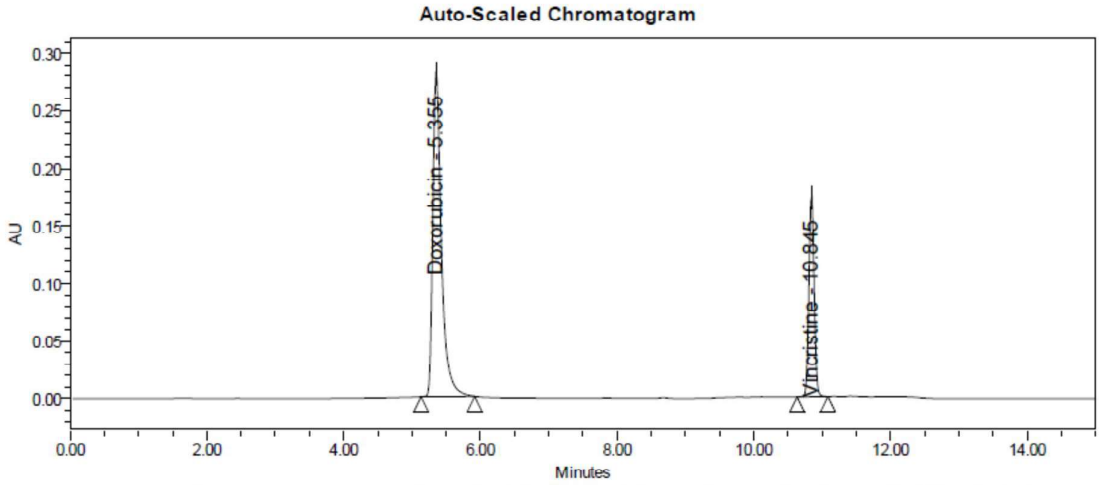
Time (Minutes)	% Mobile Phase A	% Mobile Phase B
0	50	50
4	40	60
7	40	60
7.1	5	95
10.0	5	95
10.1	50	50
15.0	50	50

Table 11: Change in gradient elution program

Procedure: The prepared mixed standard solution was injected into chromatographic system. Single standards of similar concentration were injected into the system for identification of the peaks (preparation as detailed in 5.3.3).

Observation: The peak shape of doxorubicin hydrochloride peak was not good (found at the retention time of 5.3 minutes) with tailing factor of 1.6 while the peak of vincristine sulphate (found at the retention time of 10.57 minutes) was found to have be sharp. The chromatogram using mixed standard solution as obtained with this experiment was presented in Figure 18.

Conclusion: The resolution and peak shape of doxorubicin hydrochloride was found to be non-satisfactory while that of vincristine sulphate was found to be satisfactory. Since, the intended peak resolution was not attained for doxorubicin hydrochloride, there is further need to modify the gradient elution program. Additionally, change in the chromatographic conditions may help in the attainment of proper resolution and peak shape.



	Retention Time (min)	Name	Calculated_Area	USP Resolution	USP Tailing	USP Plate Count
1	5.36	Doxorubicin	2454.02		1.62	10083.6
2	10.84	Vincristine	942.28	29.71	1.05	93778.7
Sum			3396.30			

Figure 18: Chromatogram of experiment with change in gradient elution program and mobile phase composition

5.3.6 Modulation of chromatographic conditions

Preparation of Buffer: The buffer was prepared by dissolving 3.15 grams of ammonium formate in 1000 ml water, pH adjusted to pH 4.5 using formic acid and filtered through 0.45µ nylon membrane filter. The buffer solution was sonicated for 10 minutes and degassed prior to further usage.

Mobile phases: Gradient elution technique was used for HPLC analysis. Two mobile phases were prepared using different volume/volume ratios of buffer.

- Mobile phase-A: Mobile phase A was prepared by mixing the prepared buffer and methanol in the ratio of 80:20 (% v/v). The mobile phase was sonicated for 10 minutes and degassed prior to further usage.
- Mobile phase-B: Mobile phase B was prepared by mixing the prepared buffer and acetonitrile in the ratio of 20:80 (% v/v). The mobile phase was sonicated for 10 minutes and degassed prior to further usage.

Instrumental conditions: High Performance Liquid Chromatograph (HPLC) equipped with UV-visible detector (Shimadzu, Japan) and Empower Chromatography Data System (Waters, USA) was used with the following conditions.

- Column: Symmetry shield RP C-18 150 x 4.6 mm, 3.5 µ, FD-581 (Waters, USA)
- Flow rate: 1.0 ml/min.
- Detection UV wavelength: 254 nm for both Vincristine sulphate and Doxorubicin hydrochloride
- Injection volume: 20 µl
- Run time: 30 minutes
- Column oven temperature: 40°C
- Sample cooler temperature: 10°C

Gradient program: The gradient program using various % volumes of the three mobile phases was prepared and followed (Table 12).

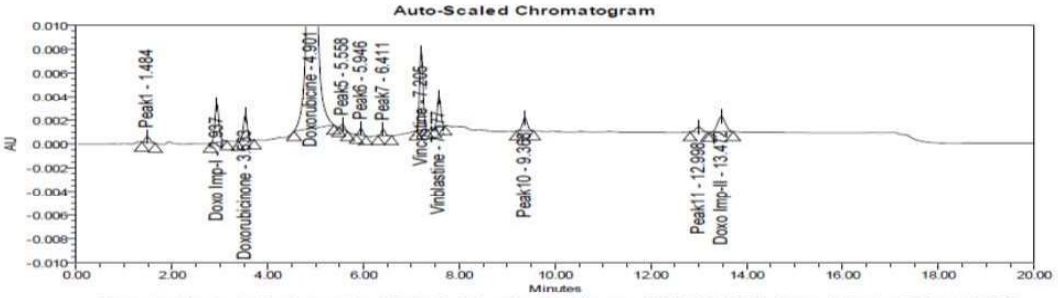
Time (Minutes)	% Mobile Phase A	% Mobile Phase B
0	65	35
4	30	70
5	5	95
15	5	95
15.1	65	35
20	65	35

Table 12: Gradient elution program for modulations in chromatographic conditions

Procedure: The prepared mixed standard solution was injected into chromatographic system. Single standards of similar concentration were injected into the system for identification of the peaks (preparation as detailed in 5.3.3). Additionally, placebo of drug and lipids, lipid free buffer system, drug free liposomes, doxorubicin hydrochloride and vincristine sulphate spiked in drug free liposomes, individual impurities of doxorubicin hydrochloride (doxorubicin impurity-I, doxorubicin impurity-II) and individual impurity of vincristine sulphate (vinblastine) were injected into chromatographic system to access the interference of the other components of the formulation in the chromatographic system.

Observation: No interference in the chromatography of the formulations was found from buffer system and placebo in the retention time of Doxorubicin hydrochloride and Vincristine

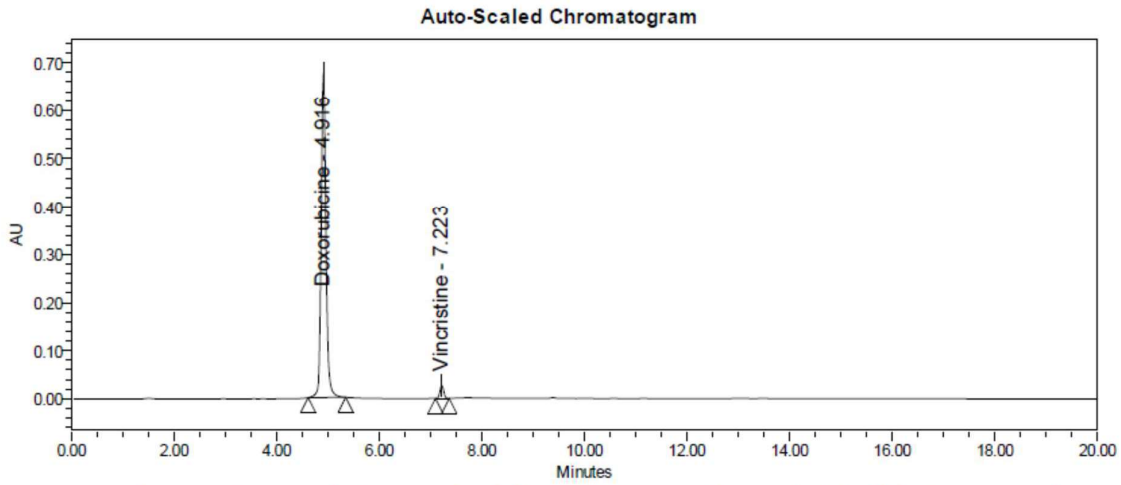
sulphate peaks. The peak shape of doxorubicin hydrochloride (found at retention time of 4.9 minutes) was found to be sharp with low tailing factor of 1.2. The peak shape of Vincristine sulphate (found at retention time of 5.49 minutes) was sharp with low tailing factor of 1.03. The retention times of injected impurities were found to be separated in chromatogram as individual peaks separate from the drug peaks. The chromatogram using mixed standard solutions and individual impurities as obtained with this experiment was presented in Figure 19.



	Retention Time (min)	Name	Calculated_Area	USP Resolution	USP Tailing	USP Plate Count
1	1.48	Peak1	3.70		1.28	850.3
2	2.94	Doxo Imp-I	19.82	7.85	1.11	5446.8
3	3.53	Doxorubicinone	12.43	3.78	0.94	8494.8
4	4.90	Doxorubicine	1657.54	8.46	1.17	13971.4
5	5.56	Peak5	2.27	4.04	1.26	20907.3
6	5.95	Peak6	3.02	2.60	1.08	26821.7
7	6.41	Peak7	3.10	3.14	1.04	30488.8
8	7.21	Vincristine	33.87	5.49	1.03	44607.9
9	7.58	Vinblastine	13.63	2.64	1.03	46764.1
10	9.37	Peak10	7.80	10.64	1.05	40262.4
11	13.00	Peak11	3.85	16.24	1.03	43462.3
12	13.47	Doxo Imp-II	14.51	1.69	1.06	31683.3
Sum			1775.55			

Figure 19: Chromatogram of experiment with modulations in chromatographic conditions: study samples

The retention time of the placebo spiked samples of doxorubicin hydrochloride and vincristine sulphate was found to be similar to that of the standard solutions. The chromatogram of this preparations as obtained with this experiment was presented in Figure 20.



	Retention Time (min)	Name	Calculated_Area	USP Resolution	USP Tailing	USP Plate Count
1	4.92	Doxorubicine	4396.60		1.2	14214
2	7.22	Vincristine	134.60	15.1	1.0	45449
Sum			4531.20			

Figure 20: Chromatogram of experiment with modulations in chromatographic conditions: standard solutions

Conclusion: The resolution and the peak shape of doxorubicin hydrochloride as well as vincristine sulphate were found to be satisfactory. No interference in the drug peaks were observed due to the presence of impurities, drug free liposomes and buffer system of liposomal formulations. Further, slight modifications to sample preparation for liposomal were warranted to improve the suitability of the method.

5.4 Finalised Method of analysis of assay and free drug

5.4.1 Method of analysis of assay of both drugs from liposomes

Preparation of Buffer: The buffer was prepared by dissolving 3.15 grams of ammonium formate in 1000 ml water, pH adjusted to pH 4.5 using formic acid and filtered through 0.45µ nylon membrane filter. The buffer solution was sonicated for 10 minutes and degassed prior to further usage.

Mobile phases: Gradient elution technique was used for HPLC analysis. Two mobile phases were prepared using different volume/volume ratios of buffer.

- Mobile phase-A: Mobile phase A was prepared by mixing the prepared buffer and methanol in the ratio of 80:20 (% v/v). The mobile phase was sonicated for 10 minutes and degassed prior to further usage.
- Mobile phase-B: Mobile phase B was prepared by mixing the prepared buffer and acetonitrile in the ratio of 20:80 (% v/v). The mobile phase was sonicated for 10 minutes and degassed prior to further usage.

Instrumental conditions: High Performance Liquid Chromatograph (HPLC) equipped with UV-visible detector (Shimadzu, Japan/ Agilent USA) and Empower Chromatography Data System (Waters, USA) was used with the following conditions.

- Column: Symmetry shield RP C-18 150 x 4.6 mm, 3.5 µ, FD-581 (Waters, USA)
- Flow rate: 1.0 ml/min.
- Detection UV wavelength: 254 nm for both Vincristine sulphate and Doxorubicin hydrochloride
- Injection volume: 20 µl
- Run time: 20 minutes
- Column oven temperature: 30°C
- Sample cooler temperature: 10°C
- Diluent: Methanol

Gradient program: The gradient program used during HPLC analysis is mentioned in table 13.

Time (Minutes)	% Mobile Phase A	% Mobile Phase B
0.0	75	25
4.0	60	40
7.0	30	70
7.1	5	95
15.0	5	95
15.1	75	25
20	75	25

Table 13: Gradient elution program for simultaneous determination of assay of both drugs

Standard preparation (Doxorubicin hydrochloride): Accurately weighed working standards of 27.0 ± 3.0 mg Doxorubicin Hydrochloride USP was transferred to a 50 ml volumetric flask. The drug was dissolved using 30 ml of methanol using sonication (Bath sonicator, Oscar Ultrasonic, India) and volume was made up to 50 ml using the methanol. The resultant solution was cooled to room temperature. The prepared standard solution as shaken well prior to usage (Solution A).

Standard preparation (Vincristine sulphate): Accurately weighed working standards of 13.0 ± 1.0 mg Vincristine Sulphate USP was transferred to a 20 ml volumetric flask. The drug was dissolved using 10 ml of methanol using sonication (Bath sonicator, Oscar Ultrasonic, India) and volume was made up to 20 ml using the methanol. The resultant solution was cooled to room temperature. 5 ml of the prepared solution was transferred to 20 ml volumetric flask and diluted to 20 ml using methanol. The prepared standard solution as shaken well prior to usage (Solution B).

Mixed standard: The mixed standard was prepared by transferring 3m1 of Doxorubicin Hydrochloride (Solution A) and 5m1 of Vincristine Sulphate (Solution B) to 25 ml volumetric flask. The volume was made up to 25 ml using with elution solution and mixing the solution well prior to usage.

Preparation of sample: The sample was prepared by transferring 2 ml of sample to a 50 ml volumetric flask, added with 30 ml diluent and sonicated for about 10 minutes. The prepared solution was cooled at room temperature with volume being made up to 50 ml using the diluent.

Procedure:

- The chromatographic system was set up as described under instrumental conditions.
- 20 µ1 of diluent as blank and standard preparation (five replicates) was injected into the chromatographic system for system precision determination. The retention time of Doxorubicin was found to be about 4 to 6 minutes. The retention time of Vincristine was found to be about 6 to 8 minutes.
- The relative standard deviation for area of Doxorubicin and Vincristine peak in the five replicate injections of standard should not be more than 2.0%. Tailing factor of both peaks should not be more than 2.

- 20 µl of prepared sample preparation was injected in duplicate into the chromatographic system and mean area response of Doxorubicin and Vincristine peak was calculated.

The percentage assay of Doxorubicin hydrochloride was calculated using the formula

$$\% \text{ Doxorubicin of Label claim} = \frac{AT \times WS \times 3 \times 50 \times AV \times P}{AS \times 50 \times 20 \times VL \times LC}$$

Where: AT = Average area count of Doxorubicin peak in sample preparation; AS = Average area count of Doxorubicin peak in standard preparation; WS = Taken weight of Doxorubicin working standard (mg); VL = Taken volume of sample (ml); P = Percentage potency of Doxorubicin working standard; AV-Average weight of sample; LC = Label claim of Doxorubicin (mg/ml)

The percentage assay of Vincristine sulphate was calculated using the formula

$$\% \text{ Vincristine of Label claim} = \frac{AT \times WS \times 5 \times 20 \times 50 \times AV \times P}{AS \times 20 \times 20X \times 5 \times VL \times LC}$$

Where: AT = Average area count of Vincristine peak in sample preparation; AS = Average area count of Vincristine peak in standard preparation; WS = Taken weight of Vincristine working standard (mg); VL = Taken volume of sample (ml); P = Percentage potency of Vincristine working standard; AV-Average weight of sample; LC = Label claim of Vincristine (mg/ml).

The representative chromatogram for assay of both drugs is presented in Figure 21.

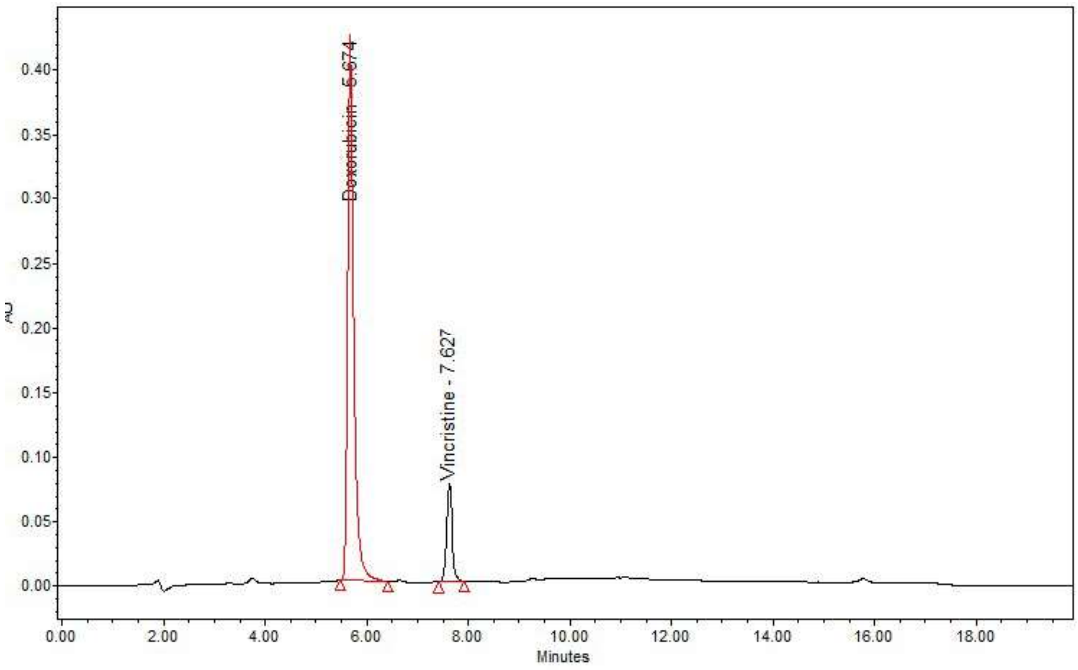


Figure 21: Chromatogram of method of assay of both drugs as acquired during analysis of optimised dual drug liposomal batch DV6 detailed in (chapter 7 and 8).

5.4.2 Method of analysis of free drug content of both drugs from liposomes

Preparation of Buffer: The buffer was prepared by dissolving 3.15 grams of ammonium formate in 1000 ml water, pH adjusted to pH 4.5 using formic acid and filtered through 0.45µ nylon membrane filter. The buffer solution was sonicated for 10 minutes and degassed prior to further usage.

Mobile phases: Gradient elution technique was used for HPLC analysis. Two mobile phases were prepared using different volume/volume ratios of buffer.

- Mobile phase-A: Mobile phase A was prepared by mixing the prepared buffer and methanol in the ratio of 80:20 (% v/v). The mobile phase was sonicated for 10 minutes and degassed prior to further usage.
- Mobile phase-B: Mobile phase B was prepared by mixing the prepared buffer and acetonitrile in the ratio of 20:80 (% v/v). The mobile phase was sonicated for 10 minutes and degassed prior to further usage.

Elution Solution: The buffer was prepared by dissolving 3.15 grams of ammonium formate in 1000 ml water, pH adjusted to pH 2.5 using formic acid and filtered through 0.45µ nylon membrane filter. The buffer solution was sonicated for 10 minutes and degassed prior to further usage.

Instrumental conditions: High Performance Liquid Chromatograph (HPLC) equipped with UV-visible detector (Shimadzu, Japan/ Agilent USA) and Empower Chromatography Data System (Waters, USA) was used with the following conditions.

- Column: Symmetry shield RP C-18 150 x 4.6 mm, 3.5 µ, FD-581 (Waters, USA)
- Flow rate: 1.0 ml/min.
- Detection UV wavelength: 254 nm for both Vincristine sulphate and Doxorubicin hydrochloride
- Injection volume: 20 µl
- Run time: 20 minutes
- Column oven temperature: 30°C
- Sample cooler temperature: 10°C

Gradient program: The gradient program used during HPLC analysis is mentioned in table 14.

Time (Minutes)	% Mobile Phase A	% Mobile Phase B
0.0	75	25
4.0	60	40
7.0	30	70
7.1	5	95
15.0	5	95
15.1	75	25
20	75	25

Table 14: Gradient elution program for simultaneous determination of free drug content of both drugs

Standard preparation (Doxorubicin hydrochloride): Accurately weighed working standards of 27.0 ± 3.0 mg Doxorubicin Hydrochloride USP was transferred to a 50 ml volumetric flask. The drug was dissolved using 30 ml of methanol using sonication (Bath sonicator, Oscar Ultrasonic, India) and volume was made up to 50 ml using the methanol. The resultant solution was cooled to room temperature. The prepared standard solution as shaken well prior to usage (Solution A).

Standard preparation (Vincristine sulphate): Accurately weighed working standards of 13.0 ± 1.0 mg Vincristine Sulphate USP was transferred to a 20 ml volumetric flask. The drug was dissolved using 10 ml of methanol using sonication (Bath sonicator, Oscar Ultrasonic, India) and volume was made up to 20 ml using the methanol. The resultant solution was cooled to room temperature. 5 ml of the prepared solution was transferred to 20 ml volumetric flask and diluted to 20 ml using methanol. The prepared standard solution as shaken well prior to usage (Solution B).

Mixed standard: The mixed standard was prepared by transferring 3m1 of Doxorubicin Hydrochloride (Solution A) and 5m1 of Vincristine Sulphate (Solution B) to 25 ml volumetric flask. The volume was made up to 25 ml using with elution solution and mixing the solution well prior to usage.

Preconditioning of cartridge: The cartridges were preconditioned by washing the 1 ml cartridge (Oasis, Make-Waters) with l ml water by application of 2 bar positive pressure using Ezypress-48. 1ml of Superdex 75 was percolated at concentration of 30 mg/ml and the prepared cartridge was conditioned by repeated passage of 0.5m1 of water (for 5 times) using Ezypress-48.

Sample preparation: 500 µl of sample solution was added using 1 ml micropipette on the preconditioned cartridge and percolated by application of 2-bar pressure by using Ezypress-48 positive pressure processor. The resultant cartridge was washed five times with 0.5m1 of water and the solution was collected in separate container. The free drug content from cartridge was washed six times with 0.5 m1 of elution solution and collected in a clean dry test tube.

Procedure:

- The chromatographic system was set up as described under instrumental conditions.
- 20 µ1 of diluent as blank and standard preparation (five replicates) was injected into the chromatographic system for system precision determination. The retention time of Doxorubicin was found to be about 4 to 6 minutes. The retention time of Vincristine was found to be about 6 to 8 minutes.

- The relative standard deviation for area of Doxorubicin and Vincristine peak in the five replicate injections of standard should not be more than 2.0%. Tailing factor of both peaks should not be more than 2.
- 20 µl of prepared sample preparation was injected in duplicate into the chromatographic system and mean area response of Doxorubicin and Vincristine peak was calculated.

The percentage free drug of Doxorubicin hydrochloride was calculated using the formula

$$\% \text{ Doxorubicin of Label claim} = \frac{AT \times WS \times 3 \times 3 \times P}{AS \times 50 \times 25 \times 0.5 \times LC}$$

Where: AT = Average area count of Doxorubicin peak in sample preparation; AS = Average area count of Doxorubicin peak in standard preparation; WS = Taken weight of Doxorubicin working standard (mg); P = Percentage potency of Doxorubicin working standard; LC = Label claim of Doxorubicin (mg/ml)

The percentage free drug of Vincristine sulphate was calculated using the formula

$$\% \text{ Vincristine of Label claim} = \frac{AT \times WS \times 5 \times 5 \times 3 \times P}{AS \times 20 \times 20 \times 25 \times 0.5 \times LC}$$

Where: AT = Average area count of Vincristine peak in sample preparation; AS = Average area count of Vincristine peak in standard preparation; WS = Taken weight of Vincristine working standard (mg); P = Percentage potency of Vincristine working standard; LC = Label claim of Vincristine (mg/ml).

The representative chromatogram for free drug content of both drugs is presented in Figure 22.

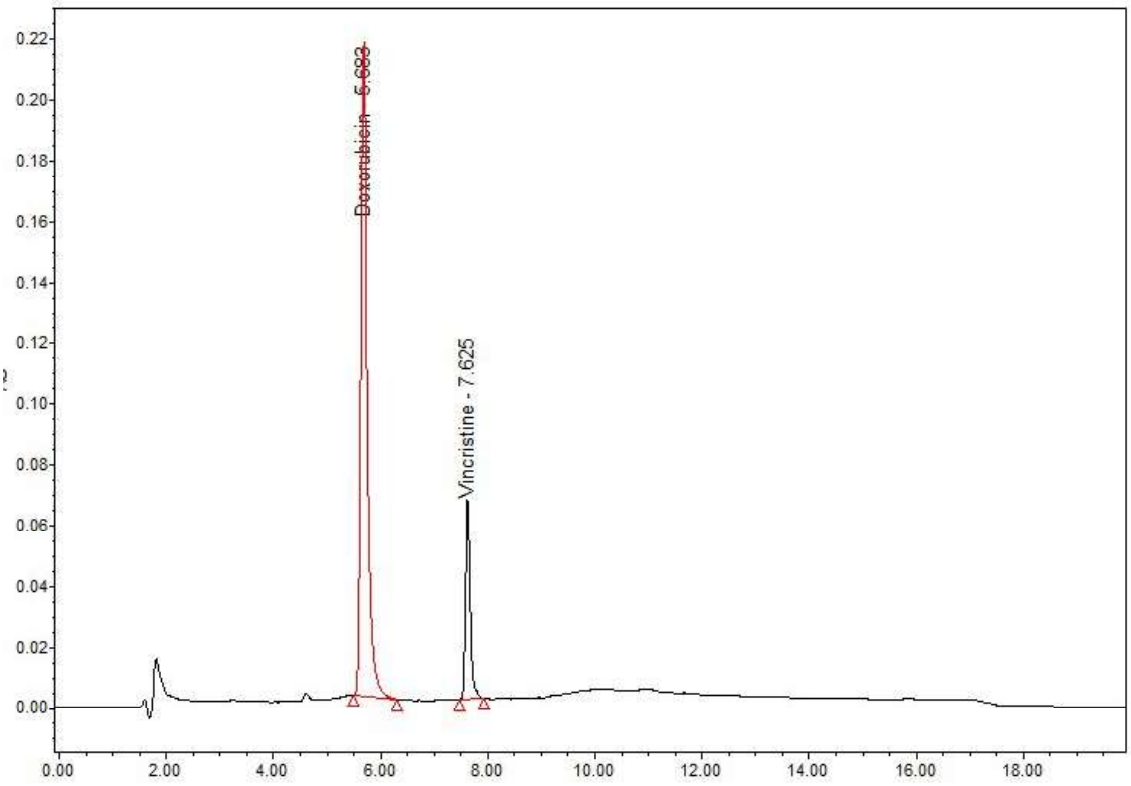


Figure 22: Chromatogram of method of free drug content for both drugs as acquired during analysis of optimised dual drug liposome batch DV6 (detailed in chapter 7 and 8).

5.5 Method of analysis of assay of lipids

5.5.1 Method of analysis of assay of HSPC and Cholesterol

Preparation of mobile phase and diluent: The mobile phase and the diluent were prepared by mixing methanol and water in 1000: 20 (volume/volume ratio) and filtered through 0.45µ nylon membrane filter.

Standard preparation: Accurately weighed working standards of 60.0±6.0 mg Hydrogenated soy phosphatidyl choline (HSPC) and 20.0± 2.0 mg cholesterol were transferred to a 50 ml volumetric flask. The lipids were dissolved using 30 ml of diluent using sonication (Bath sonicator, Oscar Ultrasonic, India) and volume was made up to 50 ml using the diluent. The resultant solution was further diluted by transferring 4 ml of this solution to 25 ml volumetric

flask and making up the volume to 25 ml. The prepared standard solution as shaken well prior to usage.

Sample preparation: 1 ml of the liposomal suspension was taken in 50 ml volumetric flask and volume was made up to 50 ml using the prepared diluent. The resultant solution was mixed well and checked for the clarity (absence of any lipid precipitates).

Instrumental conditions: High Performance Liquid Chromatograph (HPLC) equipped with UV-visible detector (Shimadzu, Japan/ Agilent USA) and Empower Chromatography Data System (Waters, USA) was used with the following conditions.

- Column: Inertsil ODS 3V, 250 x 4.6 mm, 5µ (GL Sciences, Japan)
- Flow rate: 2.0 ml/min.
- Detector: Refractive Index detector (RID, Waters, USA) with attenuation 1024.
- Injection volume: 100 µl
- Run time: 30 minutes
- Column temperature: 45°C
- Detector cell temperature: 45°C

Procedure:

- The chromatographic system was set up as described under instrumental conditions.
- 100 µ1 of diluent as blank and standard preparation (5 replicates) were injected into the chromatographic system to determine the system precision. The retention time of HSPC was about 12.2 minutes (HSPC peak 1) and 16.6 minutes (HSPC peak 2). The retention time of cholesterol was about 9.1 minutes. The relative standard deviation of HSPC and cholesterol should not be more than 2.0%. The experimental values of theoretical plates and the tailing factor should not be less than 3000 and not more than 2.0 respectively for all peaks.
- 100 µl of the prepared sample (in duplicate) was injected into the chromatographic system and the mean area count was calculated for the samples.

The assay of HSPC was calculated using the below mentioned formula

$$\% \text{ HSPC of Label claim} = \frac{AT \times WS \times 4 \times 50 \times P}{AS \times 50 \times 25 \times VT \times LC}$$

Where: AT = Average area count of sum of HSPC peaks in test preparation (i.e., HSPC-1 and HSPC-2 peaks); AS = Average area count of sum of HSPC peaks in standard preparation. (i.e., HSPC-1 and HSPC-2 peaks); WS = Weight of HSPC WS (in mg); VT= Volume of test sample taken (in ml); P = Percentage potency of HSPC working standard; LC = Label claim of HSPC (mg/ml).

The assay of Cholesterol was calculated using the below mentioned formula

$$\% \text{ Cholesterol of Label claim} = \frac{AT \times WS \times 4 \times 50 \times P}{AS \times 50 \times 25 \times VT \times LC}$$

Where: AT = Average area count of cholesterol peak in test preparation; AS = Average area count of cholesterol peak in standard preparation; WS = Weight of cholesterol WS (in mg); VT= Volume of test sample taken (in ml); P = Percentage potency of Cholesterol working standard; LC = Label claim of Cholesterol (mg/ml).

5.5.2 Method of analysis of assay of mpeg-2000-DSPE

Preparation of buffer: The buffer was prepared by dissolving 1.54 grams of ammonium acetate in 1000 ml water and filtered through 0.45µ nylon membrane filter. The buffer solution was sonicated for 10 minutes and degassed prior to further usage.

Preparation of solvent mixture: The organic solvents methanol and tetrahydrofuran were individually filtered using 0.45µ Polytetrafluoroethylene (PTFE) membrane filter and mixed in the ratio of 90:10 (volume/volume).

Preparation of mobile phase and diluent: The mobile phase was prepared using a mixture of buffer and diluent in ratio of 130:870 (volume/volume). The mobile phase was sonicated for 10 minutes and degassed prior to further usage. The solvent mixture was used as the diluent.

Preparation of ammonium sulphate solution: The buffer was prepared by dissolving 200 mg of ammonium sulphate in 50 ml water (in 100 ml volumetric flask) by shaking for 10 minutes and volume was made up to 100 ml using water.

Standard preparation: Accurately weighed working standard of 16.0±1.6 mg mpeg-2000-DSPE was transferred to a 50 ml volumetric flask. The lipid was dissolved using 15 ml of diluent using sonication (Bath sonicator, Oscar Ultrasonic, India). 5 ml of ammonium sulphate solution was added to the lipid solution and volume was made up to 50 ml using the diluent. The prepared standard solution as shaken well prior to usage.

Sample preparation: 2 ml of the liposomal suspension was taken in 20 ml volumetric flask and volume was made up to 20 ml using the prepared diluent. The resultant solution was mixed well and checked for the clarity (absence of any lipid precipitates).

Instrumental conditions: High Performance Liquid Chromatograph (HPLC) equipped with UV-visible detector (Shimadzu, Japan/ Agilent USA) and Empower Chromatography Data System (Waters, USA) was used with the following conditions.

- Column: Zorbax C8, 150 x 4.6 mm, 5m (Agilent, Technonologies, USA.)
- Flow rate: 2.0 ml/min.
- Detector: Refractive Index detector (RID, Waters, USA) with attenuation 1024.
- Injection volume: 200 µl
- Run time: 75 minutes
- Column temperature: 35°C
- Detector cell temperature: 35°C

Procedure:

- The chromatographic system was set up as described under instrumental conditions.
- 200 µl of diluent as blank with standard preparation (5 replicates) was injected into the chromatographic system to determine the system precision. The retention time of mpeg-2000-DSPE was about 6.8 minutes and the relative standard deviation of mpeg-2000-DSPE should not be more than 2.0%.
- 100 µl of the prepared sample (in duplicate) was injected into the chromatographic system and mean area count for the samples was calculated.

The assay of mpeg-2000-DSPE was calculated using the below mentioned formula

$$\% \text{ mpeg-2000-DSPE of Label claim} = \frac{AT \times WS \times 20 \times P}{AS \times 50 \times VT \times LC}$$

Where: AT = Average area count of mpeg-2000-DSPE peak in test preparation; AS = Average area count of mpeg-2000-DSPE peak in standard preparation; WS = Weight of mpeg-2000-DSPE WS (in mg); VT= Volume of test sample taken (in ml); P = Percentage potency of mpeg-2000-DSPE working standard; LC = Label claim of mpeg-2000-DSPE (mg/ml).

5.6 References

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