CHAPTER- 4 PREPARATION AND EVALUATION OF SMEDDS FOR VALSARTAN

4.1 ANALYTICAL METHODS DEVELOPMENT AND VALIDATION

Different analytical methods like ultra-violet spectrophotometric, spectrofluorimetric and high performance liquid chromatography were developed and validated to estimate valsartan in various *in vitro* and *in vivo* studies.

4.1.1 Spectroscopic Methods:

The modern methods of choice for quantitative analysis are HPLC, GC, GLC, HPTLC etc., which require high degree of sophistication and are somewhat more expensive, so in order to analyze the drug with required accuracy, precision and sensitivity at laboratory scale and small scale industries, absorption spectrophotometric and spectrofluorimetric techniques are preferred. They are very simple and do not involve high cost. The wide acceptance of absorption spectrophotometry over other analytical techniques has largely due to factors such as speed, simplicity, selectivity, sensitivity, and specificity (1).

4.1.1.1 Simple UV spectroscopy

Instrument

Shimadzu UV – 1700UV visible spectrophotometer with 10 mm quartz cells was used for spectral measurements.

Reagents

Methanol (analytical reagent grade) was used to prepare the primary stock solution and subsequent dilutions for the estimation of valsartan.

1. Experimental

Preparation of Primary stock solution

Valsartan was accurately weighed 10 mg and transferred to 100 mL volumetric flask. About 70 mL of the methanol was added to volumetric flask. The solution was vortex mixed for 2 min to allow the dissolution of suspended drug molecules at ambient temperature. The final dilution was made to 100 mL (i.e. 100 µg/mL) using methanol.

Determination of UV Absorbance Maxima of Valsartan

Valsartan test solution of concentration 10 µg/mL was scanned for determination of absorbance maxima (λ_{max}) on a spectrophotometer. The scanning was carried out in a

range of 200-400 nm.

Method Validation: (2)

1. Linearity:

The linearity of an analytical method is its ability to elicit, test results that are directly, or by well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range. Lambert's law states that the absorbance is proportional to the thickness of the solution; Beer's law states that the absorbance is proportional to the concentration of the absorbing species. Beer-Lambert's law can be mathematically expressed as:

$$A = log \frac{Intensity\ of\ incident\ light}{Intensity\ of\ transmitted\ light} = abc$$

The absorptivity "a" is proportionality constant and is independent of concentration, path length and intensity of the incident radiation but dependent upon wavelength and solvent. The absorptivity has the units of L gm^{-1} cm^{-1} when "b" is path length expressed in cms and "c" is the concentration in gm/liter. If "c" is a molar concentration. The absorptivity is called molar absorptivity (ε) and expressed in terms of L mol gm^{-1} cm^{-1}.

The linearity of the assay was determined by preparing calibration curve.

Preparation of Calibration Curve

Secondary stock solution with concentration of 50 µg/mL was prepared by diluting 5 ml of primary stock solution (100 µg/mL) to 10 mL with methanol. Aliquots of the secondary stock solutions of valsartan ranging from 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 were transferred into separate 10mL volumetric flasks and volumes were made up to 10 mL using methanol to obtain final concentrations of 7.5, 10, 12.5, 15, 17.5, 20 and 22.5 µg/mL. Six different sets of primary stock solutions were prepared and final dilutions were made using methanol. The absorbance of samples was measured on three consecutive days against methanol as a blank. Calibration curves were obtained by plotting mean absorbance vs. concentration. Linear least-square regression analyses of the calibration graphs were performed and the values are noted in Table 4.1.1. Calibration curve is obtained by plotting mean absorbance vs. concentration (Figure

4.1.1.)

2. Accuracy and precision

2.1. Accuracy

The accuracy of an analytical method is defined as the degree to which the determined value of analyte in a sample corresponds to the true value. Accuracy may be measured in different ways and the method should be appropriate to the matrix. The accuracy of an analytical method may be determined by any of the following methods:

- Analysing a sample of known concentration and comparing the measured value to the 'true' value. However, a well characterized sample (e.g., reference standard) must be used. Accuracy can be calculated in terms of relative error.

$$\%Relative\ error = \frac{Observed\ value - True\ value}{True\ value} \times 100\ (Equation\ 4.1)$$

- Spiked – placebo (product matrix) recovery method. In the spiked – placebo recovery method, a known amount of pure active constituent is added to formulation blank [sample that contains all other ingredients except the active(s), the resulting mixture is assayed, and the results obtained are compared with the expected result.
- Standard addition method. In the standard addition method, a known amount of pure active constituent is added to a previously analyzed sample and the sample is again assayed. The difference between the results of the two assays is compared with the expected answer.

In both methods (spiked – placebo recovery and standard addition method), recovery is defined as the ratio of the observed result to the expected result expressed as a percentage.

The accuracy of a method may vary across the range of possible assay values and therefore must be determined at several different fortification levels. The accuracy should cover at least 3 concentrations (80, 100 and 120%) in the expected range. Accuracy may also be determined by comparing test results with those obtained using another validated test method.

Acceptance criteria: the expected recovery depends on the sample matrix, the sample processing procedure and on the analyte concentration. The mean % recovery should be within the following ranges:

% Active/impurity content	Acceptable mean recovery
≥10	98 –102%
≥ 1	90 –110%
0.1 – 1	80 – 120%
< 0.1	75 – 125%

2.1.1. Intra-day Accuracy of the Assay:

Primary stock solutions are appropriately diluted using methanol to obtain final concentrations of 7.5 (LQC), 15 (MQC) and 22.5 µg/mL (HQC). Six different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance of samples was measured at λ_{max} 248 nm three times on the same day. The solutions were prepared freshly on each time. Methanol was used as a blank. The amount of drug found was calculated as percent of true value of drug analyzed and the results are recorded in Table 4.1.3.

2.1.2. Inter-day Accuracy of the Assay:

Primary stock solutions are appropriately diluted using methanol to obtain final concentrations of 7.5 (LQC), 15 (MQC) and 22.5 µg/mL (HQC). Six different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance of samples was measured at λ_{max} 248 nm on three consecutive days. The solutions were prepared freshly on each day. Methanol was used as a blank. The amount of drug found was calculated as percent of true value of drug analyzed and the results are recorded in Table 4.1.4.

2.2. Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. For these guidelines, a simple assessment of repeatability will be acceptable. The precision of an analytical procedure is usually expressed as the variance, standard

deviation or coefficient of variation of a series of measurements. A minimum of 5 replicate sample determinations should be made together with a simple statistical assessment of the results, including the percent relative standard deviation.

The precision of an analytical method is usually expressed as the Standard Deviation (*S.D.*) or Relative Standard Deviation (RSD). The standard deviation is calculated from following formula given in equation below

$$S.D. = \sqrt{\sum(X_i - X)^2/(N-1)} \quad \textit{(Equation 4.2)}$$

Where Xi is an individual measurement in a set,

X is the arithmetic mean of the set and,

N is the total number of replicated measurement taken in the set

Precision between different samples can be compared with RSD as follows:

$$\%\ RSD = \frac{S.D}{Mean} \times 100 \ (Equation\ 4.3)$$

The following levels of precision are recommended.

% Active content	Acceptable Limits
≥ 10.0%	≤ 2%
1.0 up to 10.0%	≤ 5%
0.1 up to 1.0%	≤ 10%
< 0.1%	≤ 20%

The intra- and inter day precisions of the assay were calculated by replicate analysis of the solutions of known concentrations of valsartan at three quality control concentration (LQC, MQC, and HQC) levels. The observed concentrations of the drug were calculated (from absorbance) using the equation of standard calibration curve. The variations between the observed concentrations were determined by calculating the percent Relative Standard Deviation (% RSD) using equation 4.3.

2.2.1. Intra-day Precision of the Assay: Aliquots from primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 7.5 µg/mL (LQC), 15 µg/mL (MQC) and 22.5 µg/mL (HQC). Six different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance of samples was measured at λ_{max} 248 nm three times on the same day. The solutions were

prepared freshly on each time. Methanol was used as a blank. The precision was calculated as % relative standard deviation and the results are recorded in Table 4.1.3.

2.2.2. Inter-day Precision of the Assay:

Aliquots from primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 7.5 µg/mL (LQC), 15 µg/mL (MQC) and 22.5 µg/mL (HQC). Six different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance of samples was measured at λ_{max} 248 nm on three consecutive days. The solutions were prepared freshly on each day. Methanol was used as a blank. The precision was calculated as % relative standard deviation and the results are recorded in Table 4.1.4.

3. Robustness and Ruggedness:

Robustness and ruggedness of the method was evaluated by changing solvents, analyzing samples using different spectrophotometer and different analyst. To check this parameter solution having intermediate concentrations (MQC) of 15 µg/mL was prepared and absorbance was measured at 248 nm. The concentration was back-calculated from the linearity curve (mean of n=6) against observed absorbance of the sample.

4. Stability:

The stability of WRS samples were checked at for 24 hrs. The absorbance was measured.

5. Limit of Detection and Limit of Quantification:

The Limit of Detection (LOD) is a qualitative parameter. It is the lowest concentration of the analyte in a sample that can be detected with acceptable precision and accuracy under stated experimental conditions, but not necessarily quantities as an exact value (2). It is expressed as the concentration of analyte (% ppm) in the sample. The limit is usually expressed in terms of µg/mL, ng/mL, pg/mL, etc. LOD values are always specific for a particular set of experimental conditions. Anything that changes the sensitivity of a method, including instrument, sample preparation etc will change detection limits.

Limit of Quantification (LOQ) is the lowest concentration of analyte in a sample that

may be measured in a sample matrix. Analyte signal is 10 times more than blank signal. Six random readings (absorbance) for analytical blank signal after "Auto Zero" were as follows 0.002, 0.001, 0.001, 0.001, 0.002 and 0.001.

LOD and LOQ were determined using the following equation.

$$LoD(or)LoQ = \frac{kS_B}{S} \quad (Equation\ 4.4)$$

$Where\ k\ =\ a\ constant\ (3\ for\ LoD\ and\ 10\ for\ LoQ),$

$S_B\ =\ the\ standard\ deviation\ of\ the\ analytical\ blank\ signal,$

$S\ =\ the\ slope\ of\ the\ concentration/response\ graph,$

The resulted are listed in Table 4.1.5.

2. Result and Discussion

Absorbance maxima of Valsartan

The maximum absorbance (λ_{max}) of valsartan standard solution was found at 248 nm.

Calibration Curve of Valsartan:

A linear calibration curve was plotted as absorbance against concentration with slope, intercept and correlation coefficient of 0.0366, 0.0242 and 0.9997.

Method Validation:

1. Linearity

Sr. No.	Concentration (µg/mL)	Absorbance ± SD (n=6)
1	7.5	0.253 ± 0.013
2	10	0.342 ± 0.015
3	12.5	0.431 ± 0.017
4	15	0.523 ± 0.012
5	17.5	0.609 ± 0.014
6	20	0.708 ± 0.011
7	22.5	0.803 ± 0.012

Table 4.1.1 Absorbance of valsartan at different concentrations

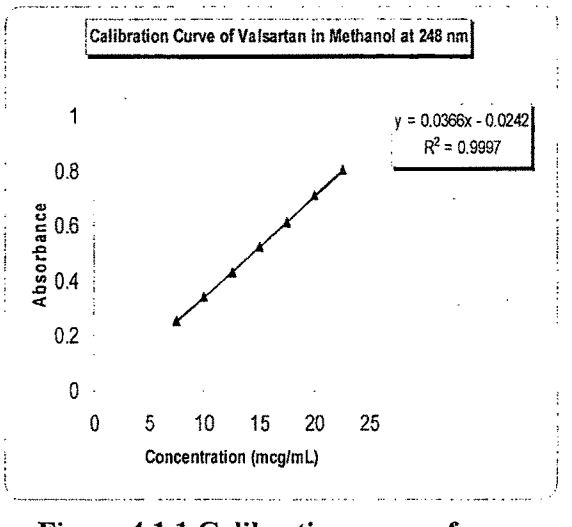


Figure 4.1.1 Calibration curve of Valsartan in Methanol at 248 nm

The calibration curve was found to be linear in the concentration range form 7.5 µg/mL to 22.5 µg/mL. The values of absorbance obtained at each concentration are listed in Table 4.1.1 and calibration graph is shown in Fig 4.1.1.

Calibration was done on 3 different days. The values of slope, intercept and linear least square are summarized in Table 4.1.2.

Day	Number of Runs (n)	Slope	Intercept	Linear Least Square Regression (r^2)
1	6	0.0366	0.0242	0.9997
2	6	0.0362	0.0201	0.9996
3	6	0.0362	0.0197	0.9997
Mean	**6**	**0.0363**	**0.0213**	**0.9997**

Table 4.1.2 Calibration curves of Valsartan in Methanol at 248 nm on different days

2. Accuracy and Precision

Intra day and inter day accuracy and precision are reported in Table no 4.1.3 and 4.1.4 at three concentration levels.

Parameters	Valsartan Concentration		
	Low QC, 7.5 µg/mL	Medium QC, 15 µg/mL	High QC, 22.5 µg/mL
Mean	7.36	14.95	22.12
SEM	0.039	0.058	0.104
Precision as % RSD	1.26	1.64	1.02
Accuracy (%)	98.11	99.69	98.30

Table 4.1.3 Intra day Accuracy and Precision for Valsartan determination

%RSD was less than 2 for all three concentrations hence intra day precision in the range of 2 ± 0.5% and accuracy is in the range of 100 ± 2% which complies with the

limit of ICH guideline. Developed method was accurate and precise for entire calibration range.

Parameters	Valsartan Concentrations		
	Low QC, 7.5 µg/mL	Medium QC, 15 µg/mL	High QC, 22.5 µg/mL
Mean	7.33	14.97	22.37
SEM	0.039	0.058	0.104
Precision as % RSD	1.82	1.73	1.88
Accuracy (%)	97.76	99.78	99.43

Table 4.1.4 Inter day Accuracy and Precision for Valsartan determination

The similar results are observed in case of inter day accuracy and precision. Theses results indicate that method is accurate and precise to give the correct results.

3. Robustness and Ruggedness

While repeating the method, it gave same results irrespective of instrument and person performing the analysis indicative robustness and ruggedness of method.

4. Stability

The samples were found to be stable at room temperature.

5. Limit of Detection and Limit of Quantification

Limit of detection and limit of quantification were determined based on blank reading of solvent. LOD and LOQ for valsartan at 248 nm are 0.042 and 0.141 respectively.

Sr. No.	Absorbance of blank
1	0.002
2	0.001
3	0.001
4	0.001
5	0.002
6	0.001
Mean	0.001333
SD	0.000516
LOD	0.042
LOQ	0.141

Table 4.1.5 Determination of LOD and LOQ

3. Conclusion

The developed method is accurate, precise, repeatable, reproducible, linear, simple and inexpensive. It can be used for estimation of valsartan in bulk drug and marketed formulations. Some critical parameters for the method are enumerated in Table 4.1.6.

PARAMETERS	RESULTS
λ_{max} (nm)	248
Beer's range (µg/ml)	7.5 to 22.5
Regression Equation y= $mx + c$ Slope (m) Intercept (c)	 0.0366 -0.0213
Limit of Detection (µg/ml)	0.042
Limit of Quantification (µg/ml)	0.141
Coefficient of determination	0.9997
Precision Intra day Inter day	 < 2% < 2%
Accuracy Intra day Inter day	 98-100% 97-100%

Table 4.1.6 Parameters of method validation for UV spectroscopy

4.1.1.2 Spectrofluorometric Method:

Instrument

A Shimadzu Spectrofluorophotometer (Model RF-540 with DR-3 data recorder), equipped with a 1 cm fluorescence free quartz cell having four transparent side was used for all spectral and fluorescence measurements.

Reagents

Methanol (analytical reagent grade) was used to prepare the primary stock solution and subsequent dilutions for the estimation of valsartan.

1. Experimental

Method Development

Preparation of Primary stock solution

10 mg of valsartan was accurately weighed and transferred to 10 mL volumetric flask. About 7 mL of the methanol was added to volumetric flask. The solution was vortex mixed for 2 min to allow the dissolution of suspended drug molecules at ambient temperature. The final dilution was made to 10 mL (i.e. 1 mg/mL) using methanol. The primary stock solution was stored at room temperature.

Preparation of Secondary stock solution and Test solution

Secondary stock solution of concentration 10 µg/mL was prepared by diluting 1 ml of primary stock solution (1 mg/mL) to 100 with methanol. Aliquots of the secondary stock solutions of valsartan ranging from 0.75, 1.0, 1.25, 1.50, 1.75, 2.0, 2.25 were transferred into different 10mL volumetric flasks using micropipette and volume were made up to 10 mL with methanol to obtain final concentrations of 0.75, 1.0, 1.25, 1.50, 1.75, 2.0, 2.25 µg/mL.

Determination of absorption and emission maxima of Valsartan

Valsartan standard solution (1 µg/mL) was scanned in the range of 200-350 nm for determination of excitation wavelength and it was found to be λ_{ex} = 286 nm. Same solution was scanned for determination of emission wavelength in the range of 310-450 nm taking 286 nm as excitation wavelength and it was found to be λ_{em} =373 nm. Prepared standard solutions were scanned in above emission range to measure their relative fluorescence intensity at 373 nm. The calibration curve was prepared by plotting concentration against fluorescence intensity.

Preparation of Calibration Curve

Measured absorbances of the standard solutions in range of 0.75-2.25 µg/mL were used to prepare calibration curve and linearity was determined by using straight line equation.

Method Validation

Developed method was validated by using analytical parameters such as precision, linearity, limit of detection (LOD), limit of quantification (LOQ) as per ICH guideline.

The procedure for these parameters is mentioned in section 4.1.1.1.

2. Results and Discussion

Method Development

It was found that the fluorescence of Valsartan is solvent dependant; as if water, acetonitrile or chloroform were used as solvent then the fluorescence intensity was quite reduced.

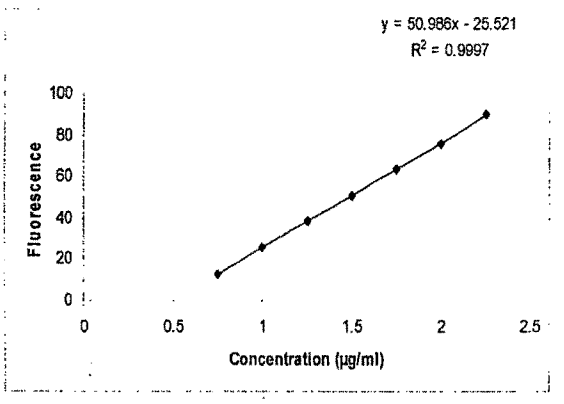


Fig 4.1.2 Calibration plot of Valsartan fluorescence

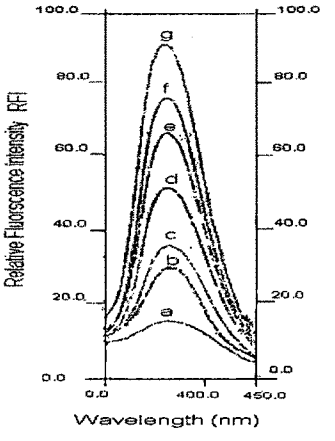


Fig 4.1.3 Fluorescence spectra of Valsartan

Calibration curve was repeated six times and RSD of each concentration level was found to be less than 2%. Fig 4.1.2 shows linearity curve and Fig 4.1.3 shows typical spectra of fluorescence.

Method Validation

Different validation parameters were measured for developed fluorimetric method and results are summarized in Table 4.1.6. The excitation wavelength was set at 286nm and emission was at 373 nm. The method was found linear for the range of 0.75 µg/mL-2.25µg/mL. The coefficient of determination was also lies in specified limit i.e 0.9996-0.9999. The LOD and LOQ were found as low as 0.02425µg/mL and 0.08125µg/mL respectively which shows that method is quite sensitive. The %RSD for Inter-day and Intra-day precision were found to be <1.49 % and <1.12 % respectively, ascertain repeatability of the developed method. The coefficient of regression establishes the linearity in the proposed method. Limit of detection and limit of quantification were calculated as suggested by Winefordner and Long (3),

taking into account the statistics involved in the difference between a given response and the blank signal and the uncertainty introduced by the presence of errors in both the slope and intercept of the calibration line (4). The intraday precision values suggested that standard, stock and working solutions of valsartan when wrapped in aluminum foil were stable up to 6 hrs can be a conclusion of intra day precision when wrapped in aluminum foil.

3. Conclusion

The developed method is accurate, precise, repeatable, reproducible, linear, simple and inexpensive. Some critical parameters for the method are enumerated in Table 4.1.7.

Parameters	Results
Excitation wavelength, λ_{ex} (nm)	286
Emission wavelength, λ_{em} (nm)	373
Linearity range (µg/mL)	0.75 – 2.25
Regression equation (Y^*)	Y = 0.051X - 25.521
Slope (b)	0.051
Intercept (a)	25.521
Coefficient of determination (r^2)	0.9997
Correlation coefficient (r)	0.9998
Limit of detection, LOD (µg/mL)	0.024
Limit of quantification, LOQ (µg/mL)	0.081
Inter-day % RSD	<1.49%
Intra-day % RSD	<1.12%
% Assay	102.96%

Table 4.1.7 Validation Parameter of Spectrofluorometric method of Valsartan

The developed method was applied to analyze the marketed formulation and the assay

was found 102.96% indicating that method can be applicable to formulation also.

4.1.2 Chromatographic Methods:

Many chromatographic methods like High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Ultra Performance Liquid Chromatography (UPLC) and Liquid Chromatography with Mass Spectroscopy (LCMS) are used as modern techniques for analysis of drug molecules. Among all these advanced methods, as HPLC is considered more economical and reproducible technique we have developed and validated HPLC methods for analysis of drug in formulation and *in vitro* studies.

4.1.2.1 High Performance Liquid Chromatography (HPLC)

Instrument

Shimadzu HPLC consists of UV-VIS detector (SPD-20A Prominence UV-VIS detector), solvent delivery pump (LC-20AT Liquid Chromatography) and manual injector (Rhododyne injector).

Reagents

Methanol, acetonitrile and distilled water used were of HPLC grade.

1. Experimental

Method Development

Development of Chromatographic conditions

The mobile phase was prepared by mixing acetonitrile and water in ratio of 50:50. To that 0.2% of triethyl amine was added. The pH of resultant mixture was set to 3.0 ± 0.5 with ortho-phosphoric acid. The mobile phase was filtered through a 0.45 µm membrane filter, degassed by ultra sonication for 5 min and pumped from the solvent reservoir to the column at a flow rate of 1.00 mL/min. The run time was set for 10 min. The volume of injection loop was 20 µL. The column used was RP C-18 250×4.6mm with 5µ particles size. Prior to injection of the drug solutions; the column was equilibrated for at least 30 min with the mobile phase flowing through the systems. The eluent peak was monitored at 215 nm and the data was acquired, stored and interpreted with the software Spinchrom CFR version (Shimadzu).

Optimization of Chromatographic conditions

The method was optimized using different composition of mobile phase and flow rate. Different peak parameters were observed like peak shape, tailing factor, theoretical plates which are summarized in Table 4.1.8.

Preparation of Primary Stock Solution

Exactly 100 mg of Valsartan was weighed in to 100 mL volumetric flask. Approximately 70 mL of Methanol was added and vortex mixed to allow the dissolution of drug into methanol. The final volume was adjusted up to the mark with methanol to prepare the primary stock solution of concentration 1 mg/mL. This primary stock solution was used as a standard for further dilutions.

Preparation of Secondary Stock Solution

5 mL of primary stock solution was transferred to 100 mL volumetric flask by using bulb pipette and diluted with diluent (water: methanol in ratio of 50:50) up to the mark to make a solution of 50µg /mL. This solution was used as secondary stock solution.

Determination of Detection Wavelength

Secondary stock solution was used to determine the absorbance maxima in UV detector. The detector was set at two different wavelength i.e. 215 nm followed by 248nm and injections of secondary stock solution (50µg /mL) were injected in HPLC pump for both the wavelength. The detector responses in terms of peak area at both wavelengths were compared. The peak response and area at wavelength 215 nm was found better than another one. Hence wavelength at 215 nm was set to prepare calibration curve.

Preparation of Working Reference Standards (WRS)

WRS solutions were prepared to plot a calibration graph. Different aliquots of secondary stock solutions like 1, 2, 5, 10, 20, 50 mL were taken out using micropipette and transferred to separate 10mL volumetric flask. The final volume was made up to the mark with diluent to make solutions of concentration range of 0.500, 1.0, 2.5, 5.0, 10.0, 25.0 µg/mL.

Method Validation (2)

Method validation was performed as per ICH guideline. Different parameters of

method validation like linearity, accuracy (inter day and intra day), precision, % recovery, limit of detection (LOD) and limit of quantification (LOQ) were determined.

1. System suitability

To determine system suitability 5 injections of secondary solution having concentration of 50 µg/mL were injected and different peak parameters were observed like retention time, tailing factor, theoretical plates and % relative standard deviation (% RSD) of area. These are summarized in Table 4.1.9.

2. Linearity

WRS solutions of concentrations 0.500, 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 µg/mL were injected in HPLC and detector responses were measured at 215 nm in terms of peak area. Six different sets of primary WRS solutions were prepared and absorbances of samples were measured on three consecutive days. Calibration curves were obtained by plotting mean area under curve vs. concentration. Linear least-square regression analyses of the calibration graphs were performed and the values are noted in Table 4.1.10.

3. Precision

3.1. Intra-day Precision

Three concentrations within linearity range of WRS i.e. 0.5 (LQC), 10.0 (MQC) and 50.0 µg/mL (HQC) were selected for determination of precision. Six different sets of primary stock solutions were prepared and diluted as per preparation of working standard. These solutions were analyzed by the proposed HPLC method. The areas obtained of samples were measured at detection wavelength 215 nm by repeating same experiment three times on the same day. The solutions were prepared freshly on each time. The precision and accuracy were calculated and the results are recorded in Table 4.1.11.

3.2. Inter-day Precision

Primary stock solutions are appropriately diluted using methanol to obtain final concentrations of 0.5 (LQC), 10.0 (MQC) and 50.0 µg/mL (HQC). Six different sets of primary stock solutions were prepared and diluted as per preparation of working standard. All three concentrations were injected in HPLC on three consecutive days.

The solutions were prepared freshly on each day. The precision and accuracy were calculated and the results are recorded in Table 4.1.12.

4. Accuracy

Accuracy was determined by spiking the preanalyzed sample solution with the aliquots of standard drug solution in the range of 80%, 100% and 120% of the claimed amount. Stock solution of standard drug was prepared 100 µg/mL in methanol. Suitable aliquots of 0.2, 0.4 and 0.6 mL were taken from stock solution of market formulation of concentration 100µg/mL in 10 mL volumetric flask, and in same flask 0.6 mL of standard drug solution of concentration 100 µg/mL was added, and volumes were made up with mobile phase to prepare series of solution in the range of 80%, 100% and 120% for recovery study. Detector response was noted at 215 nm. The % recovery was calculated on basis of drug found to drug added. The results are listed in Table 4.1.13.

5. Ruggedness

5.1. System to system variability, Column to Column Variability and Analyst to Analyst Variability:

The study was conducted for system to system, column to column and analyst to analyst variability study on two HPLC systems of different manufacturer by using different column and by different analyst. Six injections of standard drug solution having concentration of 50 µg/mL were injected as per the test method. Ruggedness was checked as precision in terms of % RSD and results are listed in Table 4.1.14.

5.2. Stability

Standard solutions having concentration of 10 µg/mL were prepared in duplicate and keep them on bench top for 24 hrs. The samples were withdrawn at regular intervals of 0, 1, 6, 8, 12 and 24 hrs and analyzed by proposed HPLC method. % assay values were calculated against a fresh standard each time. The area was measured and results are recorded in Table 4.1.15.

6. Limit of Detection and Limit of Quantification

The Limit of Detection (LOD) is a quantitative parameter. It is the lowest concentration of the analyte in a sample that can be detected with acceptable precision

and accuracy under stated experimental conditions, but not necessarily quantities as an exact value. It is expressed as the concentration of analyte (% ppm) in the sample. The limit is usually expressed in terms of µg/mL, ng/mL, pg/mL, etc. LOD values are always specific for a particular set of experimental conditions. Anything that changes the sensitivity of a method, including instrument, sample preparation etc will change detection limits.

Limit of Quantification (LOQ) is the lowest concentration of analyte in a sample that may be measured in a sample matrix such as impurities in bulk drug substances and degradation products in finished pharmaceuticals.

The solution was prepared which gave S/N ratio about 3 based on 25% of Linearity solution injection. To prepare this, 5 mL of lowest concentration solution 0.5 µg/mL was taken and diluted to 20 mL with diluent which resulted in solution of 0.125 µg/mL concentration. This solution was injected into the HPLC system and response was observed for the same. The signal to noise ratio was determined for this solution. Limit of detection was determined by identifying the concentration which gave a signal to noise ratio about 3.

In similar manner, limit of quantification was determined by identifying the concentration which gave a signal to noise ratio about 10.

7. Robustness

Robustness is the measurement of capability of analytical method to remain unaffected by small but deliberate variations in the method. Parameter provides an indication of its reliability during normal range (5).

Robustness testing is normally restricted to methods that are to be used repetitively in the same laboratory. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

In the case of liquid chromatography, examples of typical variations are:

- Influence of variations of pH in a mobile phase;
- Influence of variations in mobile phase composition;
- Different columns (different lots and/or suppliers);
- Temperature;

- Flow rate.

7.1 Effect of variation of pH in mobile phase:

The effect of pH variation was studied by preparing two mobile phases with the deviation of ± 0.2 in method pH. The system suitability solution (Standard preparation) as per the test method was injected into the HPLC system using both mobile phases. The system suitability values were determined by the test method for both the mobile phases.

7.2 Effect of variation in flow rate :

The system suitability solution of standard preparation as per the test method was injected into the HPLC system with ± 0.2 mL of method flow. The system suitability values were evaluated by the test method for both the flow rates.

2. Result and Discussion

Method Development

Optimization of Chromatographic conditions

Initially while developing, HPLC method when injection of standard solution of 50µL solution was injected and peak was observed at two different wavelengths, maximum area was obtained at 215 nm. Hence 215 nm was selected for further optimization and validation. Different ratios of Mobile phase were used at flow rate 0.5 to 1.0 mL/min.

Sr. No.	*Mobile phase composition*	*Flow rate mL/min*	*Approximate Retention time (min)*	*Peak observation*
1	Water: ACN (50:50)	1.0	3	Sharp
2	Water: ACN (50:50)	0.5	6	Broad with tailing
3	Water: ACN (70:30)	1.0	12	Sharp but late retention
4	Water: ACN (40:60)	1.0	5	Sharp but early retention
5	Water: ACN (45:55)	0.5	10	Sharp with slight Tailing
6	**Water: ACN (45:55)**	**1.0**	**8.06**	**Sharp and good**

Table 4.1.8 Effect of variation in mobile phase composition and flow rate

The above table shows that variation in mobile phase composition and flow rate showed significant changes in peak shape. The aim was to achieve good peak shape with low tailing at optimum retention time (R_t) and mobile phase composition of 45:55 (water: ACN) produced sharp peak at 8.06 min (R_t) without any tailing. Therefore this composition and flow rate was finalized for further validation. The final chromatographic conditions were mentioned below:

Column	: Hypersil BDS, C_{18} (250 X 4.6mm), 5µ
Mobile phase	: Water: Acetonitrile (45:55) with 0.2% TEA at pH 3.0
Injection volume	: 20 µl
Flow rate	: 1.0 mL/min
Needle wash	: Double Distilled Water
Detector	: UV Detector-215 nm
Temperature	: Ambient

Method Validation

1. System Suitability

The values of system suitability parameters are mentioned in Table 4.1.9.

Parameter (Mean ± SD)	Values
R_t (min)	8.06 ± 0.03
Tailing factor	1.11 ± 0.09
Theoretical plates	8507 ± 26.17
% RSD of area	1.03

Table 4.1.9 System Suitability parameters for valsartan

2. Linearity

Linearity was checked by using WRS solutions from 0.500 µg/mL to 50.0 µg/mL and mean area obtained for 3 injections of each concentration are presented in Table 4.1.10.

Concentration (µg/mL)	Mean Area
0.5	1.0
1	2.1
2.5	4.9
5	10.7
10	18.7
25	48.8
50	92.4

Table 4.1.10 Mean area of Valsartan solutions of different concentration

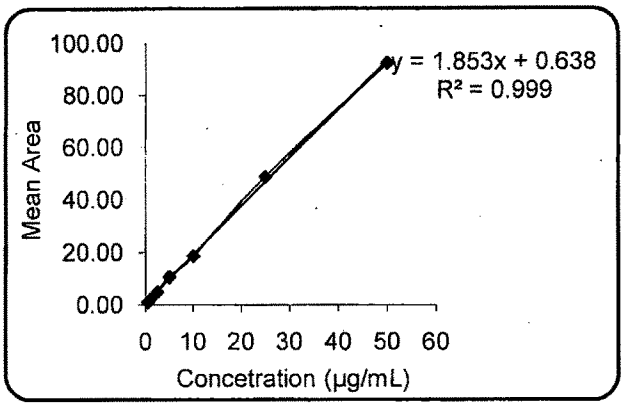


Fig 4.1.4 Linear Calibration Plot for HPLC method of Valsartan

Developed HPLC method was found to be linear over the calibration range of 0.5-50 µg/mL. The correlation of coefficient was 0.9990, Y-intercept (constant of regression) was 0.638 and slope of line was 1.853.

3. Precision

Intra day and inter day precision were determined by preparing three different concentration (Low, Medium and High) on the same day (intra day) and on next three consecutive day (inter day). The results are shown in Table no 4.1.11 and 4.1.12.

Parameters	Valsartan Concentration		
	Low QC, 0.5 µg/mL	Medium QC, 10 µg/mL	High QC, 50 µg/mL
Mean	0.497	10.04	50.35
SEM	0.003	0.040	0.220
Precision as % RSD	1.44	1.08	1.05

Table 4.1.11 Intra day Precision for Valsartan determination

As per ICH guideline, precision can be recorded in terms of % relative standard deviation (% RSD) and it should be less than 2%. Tables 4.1.11 and 4.1.12 show that for both the cases, % RSD was less than 2% which complies with specified limit.

Parameters	Valsartan Concentration		
	Low QC, 0.5 µg/mL	Medium QC, 10 µg/mL	High QC, 50 µg/mL
Mean	0.494	10.05	50.23
SEM	0.003	0.030	0.190
Precision as % RSD	1.65	0.83	0.94

Table 4.1.12 Inter day Precision for Valsartan determination

Theses results indicate that method is precise and reproducible to give the correct results.

4. Accuracy/ % Recovery

Several methods for determining accuracy are available:

a) application of the analytical procedure to synthetic mixtures of the drug product components to which known quantities of the drug substance to be analyzed have been added;

b) in cases where it is impossible to obtain samples of all drug product components , it may be acceptable either to add known quantities of the analyte to the drug product or to compare the results obtained from a second, well characterized procedure, the accuracy of which is stated and/or defined (independent procedure, see 1.2.);

c) accuracy may be inferred once precision, linearity and specificity have been established.

In this study, aliquots of standard valsartan solution were added to preanalyzed marketed formulation was added to the solution of standard drug and total amount of analyte was estimated. The % recovery was calculated on the basis of amount of drug recovered. Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations/3 replicates each of the total analytical procedure).

The concentration of marketed formulation (Table 4.1.13) was constant to 60 µg/mL and to that standard drug solution was added to get total concentration of 80 µg/mL, 100 µg/mL and 120 µg/mL.

Concentration of Valsartan (µg/mL)			Amount found# (µg/mL)	% Recovery#
Initial	Added	Total		
60	20	80	79.26 ± 0.86	99.07
60	40	100	100.10 ± 1.23	100.1
60	60	120	120.95 ± 0.55	100.8

#Mean ± S.D. of three determinations

Table 4.1.13% Recovery for Valsartan

These resulted in accuracy of 98 to 101% which complies with ICH specification limit of 100 ± 3%.

5. Ruggedness

5.1. System to system variability, Column to Column Variability and Analyst to Analyst Variability:

The method was considered rugged for system to system, column to column and analyst to analyst variability as the % RSD of assay results was not more than 2.0%.

Ruggedness Parameters	Concentration (µg/mL)	% RSD
System to System	10	1.16
Column to column	10	0.83
Analyst to analyst	10	0.97

Table 4.1.14 Ruggedness parameters for valsartan solution

5.2. Stabiltiy

The stability of solution was determined on bench top. The results of Table 4.1.15 show that solution was stable for 24 hrs.

Bench top Stability	Found Concentration (µg/mL)	% Assay
Initial	10.05	100.5
After 12 hrs	9.98	99.8
After 24 hrs	9.99	99.9

Table 4.1.15 Bench top stability of valsartan standard solution

6. Limit of Detection and Limit of Quantification

When solution of 0.125µg/mL was injected, the value of S/N ratio was 3.2. It means, 0.125µg/mL was lowest detected concentration. Similarly the solution of 0.50µg/mL gave S/N ratio value of 12.3.

	Concentration (µg/mL)	S/N ratio
LOD	0.125	3.2
LOQ	0.5	12.3

Table 4.1.16 Limit of Detection and Limit of Quantification for valsartan solution

7. Robustness

7.1 Effect of variation of pH in mobile phase:

The method is considered robust for variation of pH, if all the system suitability values meet the acceptance criteria set forth in the test method. If any of the system suitability parameters fails, the pH range would have narrowed.

7.2 Effect of variation in flow rate :

Parameter (Mean ± SD)	Variation in pH of mobile phase	Variation in Flow rate
R_t	8.3 ± 0.03	8.1 ± 0.3
Tailing factor	1.01 ± 0.09	1.1 ± 0.1
Theoretical plates	8427 ± 24.87	8560 ± 27.22
% RSD of area	1.16 ± 0.2	0.9 ± 0.2

Table 4.1.17 Robustness parameters

The method was considered robust for variation in flow rate as all system suitability criteria were match with acceptance limit.

3. Conclusion

The developed method was validated for different parameters and it was found to be linear, accurate, precise, rugged and robust. All the parameters are summarized in Table 4.1.18 to give the overview of method.

Method validation parameters	Results
Linearity and Range	
• Linearity (µg/mL)	0.5 -50.00
• Slope	0.1853
• Intercept	0.638
• Regression co-efficient (r)	0.9999
Precision (% RSD)	
• Intraday precision	1.05 – 1.44
• Interday precision	0.83 – 1.65
Accuracy	99-100.8%
Limit of Detection (µg/mL)	0.125
Limit of Quantification (µg/mL)	0.50

Table 4.1.18 Summary of Validation Parameters

Application of HPLC for Bioavailability Study

Developed HPLC method was applied for analysis of valsartan in plasma samples.

1. Experimental

Primary and secondary stock solution

To prepare primary stock solution, 100 mg of drug was transferred into 100 mL of volumetric flask. To that 70 mL of methanol was added, sonicated for about 5 min and diluted up to volume with methanol. 10 mL of above primary stock solution was transferred into 100 mL volumetric flask and diluted to mark with methanol. Secondary stock solution was stable for 24 hrs.

Calibration Standards Solution

Aliquots of secondary stock solution of 0.4, 0.6, 0.8, 1, 1.2 and 1.4 mL were transferred in 100 mL volumetric flask and diluted with diluents (water: methanol in ratio of 1:1) to prepare calibration standard solutions of 400, 600, 800, 1000, 1200 and 1400 ng/mL of valsartan.

Plasma sample preparation

0.1 mL of calibration standard solutions of each concentration was added in 5 mL polypropylene centrifuge tube with flat caps separately containing 1.0 mL of drug-free human plasma. To each tube 0.1 mL of losartan potassium solution having concentration of 10 µg/mL was added as an internal standard and mixed for 3 minutes on vortex shaker to allow through mixing. Set of ten centrifuge tube was prepared containing calibration standard solution concentration ranging from 400 to 1400 ng/mL.

Sample extraction Procedure

Sample extraction was done by protein precipitation method. In each centrifuge tube 1 mL of acetone was added to precipitate plasma proteins. Centrifuge tubes were centrifuged at 10000 RPM for 10 min in high speed homogenizer (Sigma Hi Speed Homogenizer, Japan). Supernatant from each tube was separated using micropipettes and 20 µL solution was injected into HPLC system.

Chromatographic conditions

Chromatographic conditioned were same as mentioned in section 5.1.2. Additional guard column was attached prior to HPLC column to prevent blockage due to plasma components.

2. Results and Discussion

Concentration (ng/mL)	Area of Drug	Drug/IS
10	6.18	0.013
50	59.379	0.124
100	105.43	0.220
200	201.11	0.420
400	405.243	0.845
600	604.36	1.261
800	819.719	1.710

Table 4.1.19 Mean area for different concentration of valsartan

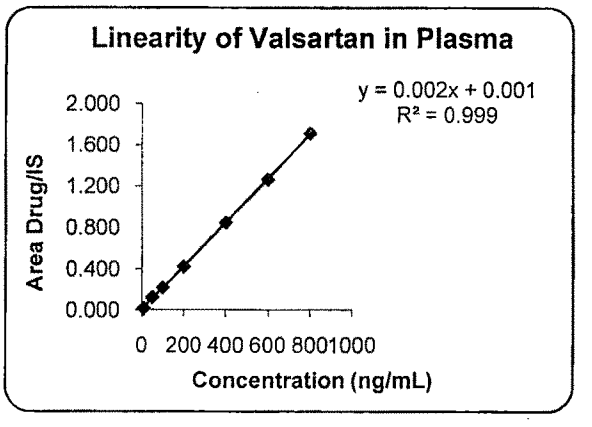


Fig. 4.1.5 Calibration Plot for Valsartan in Plasma

Calibration plot for valsartan was found to be linear within concentration range of 10 ng/mL to 800 ng/mL. Linearity was obtained by plotting graph of ratio of drug area to area of internal standard. This linearity was used as tool to determine unknown concentration of valsartan in plasma matrix.

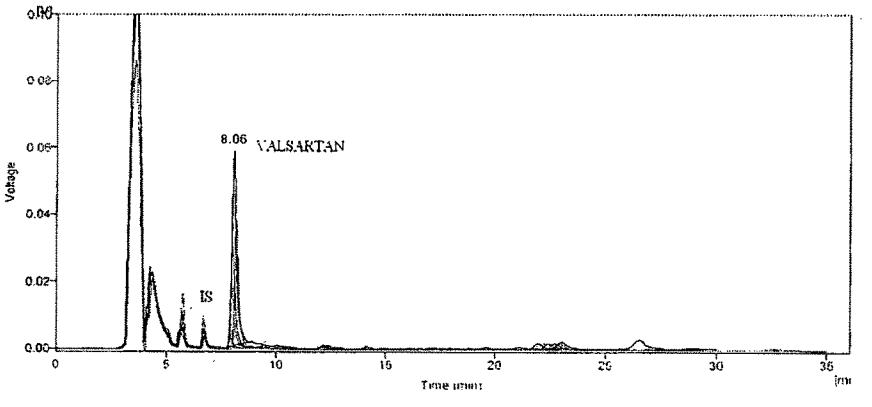


Fig 4.1.6: Chromatogram showing Linearity of Valsartan in Plasma.

The Fig. 4.1.6 shows the overlay chromatogram of different concentration of valsartan recovered from plasma. The peak for valsartan shows increase in area along with increase in concentration of drug. The peak is of internal standard shows consistency as its concentration was constant in all the samples. The results of linearity indicate that developed HPLC has application in plasma matrix.

4.2 PREPARATION OF SELF MICROEMULSIFYING DRUG DELIVERY SYSTEM (SMEDDS)

4.2.1 Experimental

4.2.1.1 Selection of Excipients:

Solubility of Valsartan was checked in different oils like vegetable oils (e.g. Peanut oil, Soyabeen oil, Cotton seed oil etc.) or medium chain triglycerides (e.g. Capmul MCM, Labrasol PG, Captex 200, Captex 355 NF) alone or in presence of surfactants (e.g. Cremophore RH 40, Tween 80, Span 20) and/or co-surfactants (e.g. Polyethyleneglycol 400, Cremophore EL, Butanol etc.). The solubility of valsartan in various components was determined by adding an excess amount of drug in 2 mL of selected oils, surfactant and co-surfactant separately in 5 mL capacity stopper vials, and mixed using a vortex mixer. The mixture vials were then kept at 25 ± 1.0 °C in an isothermal shaker (Nirmal International, Delhi, India) for 72 hr to reach equilibrium. The equilibrated samples were removed from shaker and centrifuged at 3000 rpm for 15 min. The supernatant was taken and filtered through a 0.45 μm membrane filter. The concentration of valsartan was determined in oils and water using HPLC at 215 nm as per the method described in section 4.1.3.1.

4.2.1.2 *Construction of Pseudo-ternary Phase Diagram*

The existence of microemulsion regions were determined by using pseudo-ternary phase diagram. SMEDDS were diluted under agitation condition using water titration method: the mixture of oil and surfactant/cosurfactant at certain weight ratios were diluted with water in a drop wise manner. The ratios of surfactant/cosurfactant were prepared in specific manner i.e. 1:1, 2:1, 3:1 (w/w). Each of these ratios was mixed with increasing percentage of oil i.e. 10%, 20%, 30%, 40% up to 90% of oil to get phase diagram. Then, each mixture was titrated with water and agitation was provided by magnetic stirrer. The formation of microemulsion regions were monitored visually for turbidity-transparency-turbidity. These values of oil, surfactant and cosurfactant were used to determine the boundaries of microemulsion region. After the identification of microemulsion region in the phase diagrams, the microemulsion formulations were selected at desired component ratios. To determine the effect of

drug addition in SMEDDS, phase diagrams shown in Fig. 4.2.3 were constructed in presence of drug. Black color shows self-microemulsion region and gray color indicates self-emulsion region. In order to prepare SMEDDS, selection of microemulsion region from phase diagram was based on the fact that solution remains clear even on infinite dilution.

4.2.1.3 Preparation of SMEDDS:

A series of formulations were prepared from each SMEDDS (Table 4.2.2, 4.2.3, 4.2.3) with varying ratios of oil, surfactant and cosurfactant. Formulation A, B and C were prepared using Capmul MCM as oil, Tween 80 as surfactant and PEG 400 as cosurfactant. Similarly formulations D, E and F were prepared with Capmul MCM C (10) as oil, Tween 80 as surfactant and Plurol Oleique as cosurfactant. Third system containing formulation G, H and I was prepared using combination of Captex 200 P, Cremophore EL and PEG 400 as an oil, surfactant and cosurfactant respectively. In all the formulations, the level of valsartan was kept constant (i.e. 10 mg of valsartan). Briefly, oil, surfactant and cosurfactant were accurately weighed into glass vials according to their ratios. The amount of SMEDDS should be such that it should solubilizes the drug (single dose) completely. 10 mg of drug should easily dissolve in 0.2 mg of SMEDDS. The valsartan (10 mg) was added in the mixture. Then, the components were mixed by gentle stirring and vortex mixing, and heated at 37°C in incubator, until valsartan perfectly has dissolved. The mixture was stored at room temperature until used. So, prepared SMEDDS was the concentrate of oil, surfactant, co-surfactant and drug. Water was added to SMEDDS as an external phase, diluted to infinitive dilution and optimized by in-vitro characterization. The compositions which were optically clear have been evaluated further by constructing phase diagrams. Visually clear and transparent microemulsions (ME) were considered as acceptable. Clarity was further confirmed by measuring percentage transmittance at 630 nm wavelength (Shimadzu 1700, Japan) against water as blank (Rolan I et al., 2003). ME having transmittance value greater than 99 were considered as clear. The concentrations of oil, surfactant, and cosurfactant for Valsartan SMEDDS are recorded in Table 4.2.2 (VSMS 1), Table 4.2.3 (VSMS 2) and Table 4.2.4 (VSMS 3).

4.2.2 Result and Discussion

4.2.2.1 Selection of Excepients

Solubility of valsartan in different oils, surfactant and cosurfactant is given in Table 4.2.1.

Sr. No.	Name of oil/surfactant/co-surfactant	Solubility of Valsartan (mg/mL) in oil /surfactant/co-surfactant
1	Peanut oil	0.82
2	Cotton seed oil	0.33
3	Captex 355 EP/NF	13.94
4	Captex 200 P	11.78 √ (O)
5	Labrafac PG	2.82
6	Labrafil M 2125	4.2
7	Cremophor EL	13.07 √ (S)
8	Cremophor RH 40	8.89
9	Capmul MCM	80.18 √ (O)
10	Plurol Oleique	3.71 √ (Co S)
11	PEG 400	5.67 √ (Co S)
12	Capmul MCM (C10)	20.45 √ (O)
13	Tween 80	30.62 √ (S)
14	Tween 20	0.95
15	Transcutol P	6.35±0.23

Table 4.2.1 Solubility of Valsartan in different oils, surfactant and cosurfactant

Self-microemulsion consists of oil, surfactant and co-surfactant as constructing component of the system. Drug should be completely soluble in all three components and their mixture. Therefore solubility of drug should be one of the main criteria for selection of oil, surfactant and co-surfactant. Moreover solubility of drug is also important to decide the dose of drug. Hence SMEDD should consist of such oil, surfactant and cosurfactant that accommodate dose of drug. Another factor which can be affected by solubility is partitioning effect. It drug is not soluble and stable in mixture it will be diffused towards water at the time of formation of microemulsion and as drug is water insoluble, it will precipitate out in the formulation. Considering both these facts, selection of excipients is crucial factor for successful formulation. The Table 4.2.1 shows that valsartan has good solubility in synthetic oils in comparison to vegetable oils. So, Captex 200 P, Capmul MCM and Capmul MCM (C 10) were selected as oil phase. Cremophore EL and Tween 80 which can act as

surfactant due to its high HLB values showed good solubility of valsartan. Hence they were selected as other components of SMEDDS.

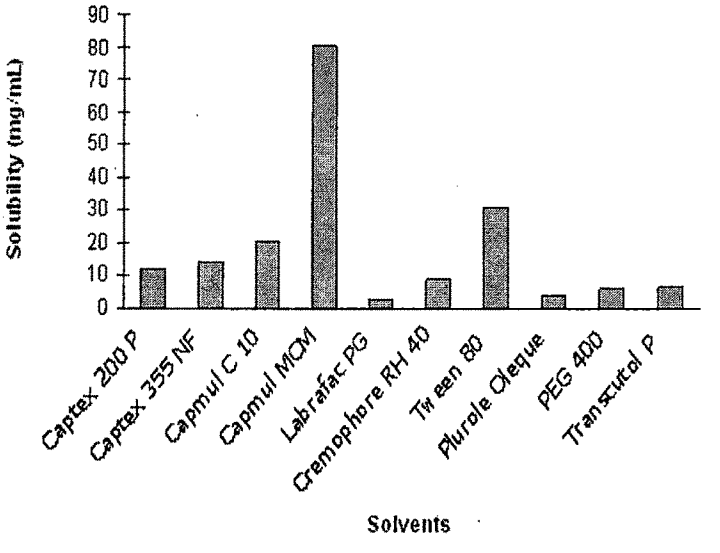
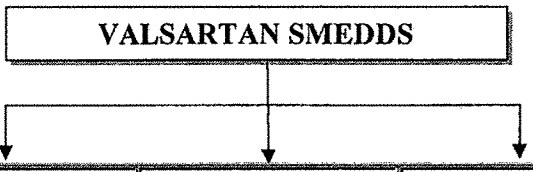


Fig 4.2.1 Graphical Presentation of Solubility of Valsartan in different solvents

The third component of SMEDDS i.e cosurfactant which is not necessarily required but may help surfactant to stabilize the system are PEG 400 and Plurol oleique. These two chemicals also showed significant solubility of valsartan.

4.2.1.2 Construction of Pseudo-ternary Phase Diagram:

Valsartan SMEDDS prepared using three different systems are summarized in figure 4.2.2.

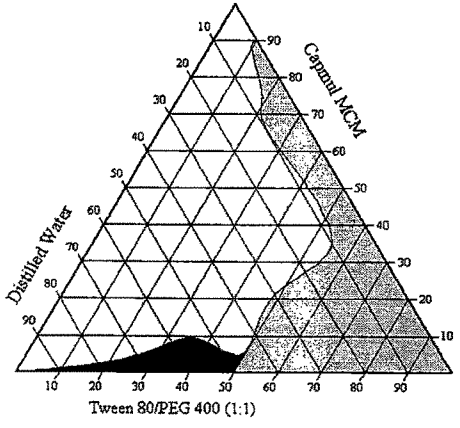


Ingredients	VSMS 1	VSMS 2	VSMS 3
Valsartan	√	√	√
Captex 200P (O)			√
Capmul MCM (O)	√		
Capmul MCM C10 (O)		√	
Cremophore EL (S)			√
Tween 80 (S)	√	√	
Plurole Oleique (CoS)		√	
PEG 400 (CoS)	√		√

√ Ingredients used □ Ingredients not used

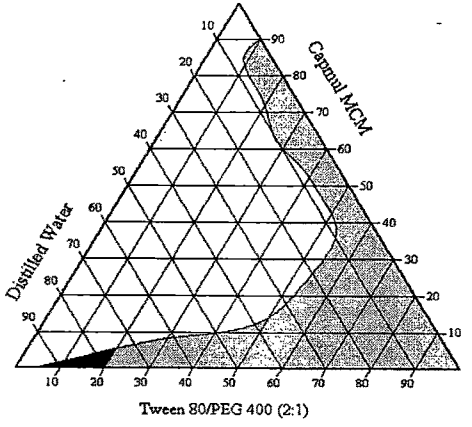
Figure 4.2.2 Excipients profiles for three different systems of Valsartan SMEDDS

VSMS 1



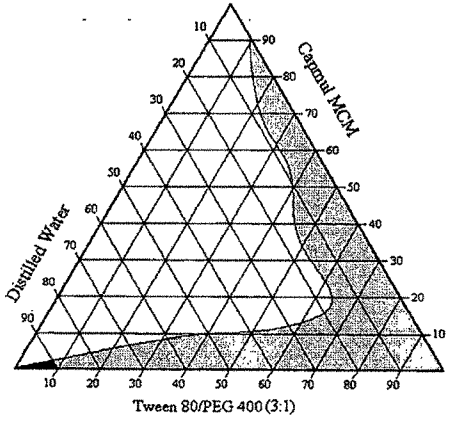
(A)

VSMS 1



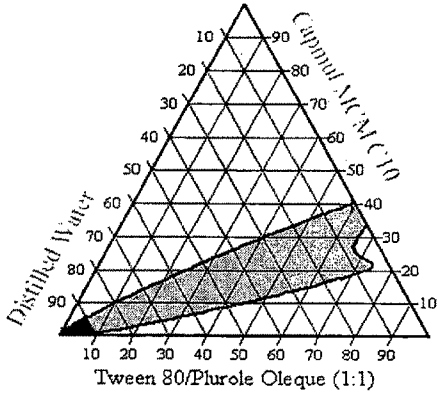
(B)

VSMS 1



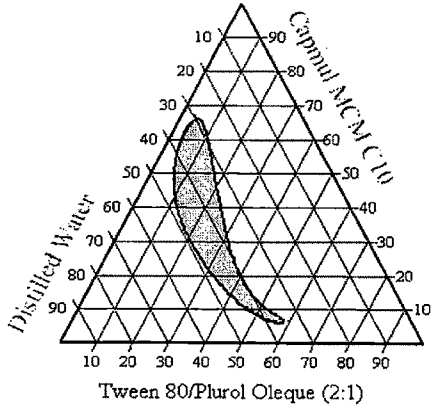
(C)

VSMS 2



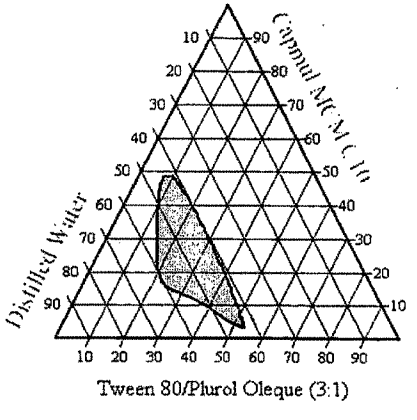
(D)

VSMS 2



(E)

VSMS 2



(F)

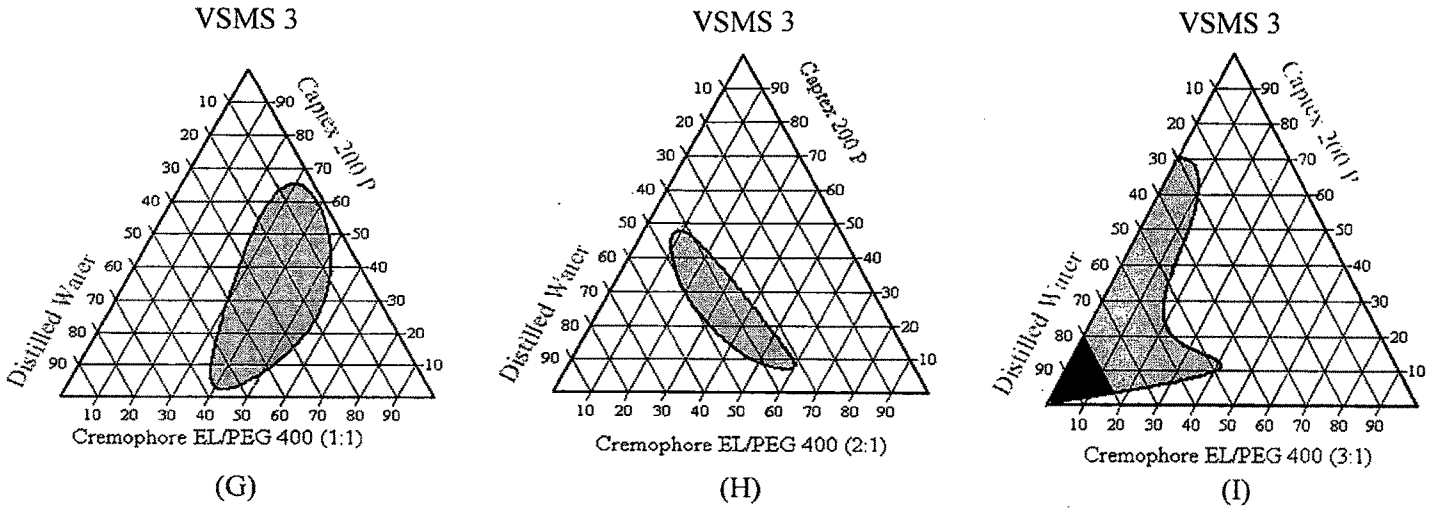


Figure 4.2.3 Pseudo-ternary phase diagrams for VSMS (System 1, 2 and 3) showing ME and emulsion regions at S: CoS ratio 1:1, 2:1 and 3:1 ◆ Self Microemulsion ◈ Microemulsion

Psudo-ternary phase diagram for each formulation shown above represents presence of microemulsion and emulsion regions. Black region represents selfmicroemulsion domain where as gray region indicates formation of coarse emulsion.

In order to form self emulsifying o/w and w/o microemulsions, an oil, a blend of two surfactants and aqueous phase were used. These four component systems can be best described by pseudo-ternary phase diagram where, a constant ratio of two of the components was used and other two were varied. To determine optimum concentration of oil, surfactant and cosurfactant, phase diagram were constructed. SMEDDS forms microemulsion when titrated with water under agitation condition. The particle size of microemulsion is less than 100 nm and as the energy required to form microemulsion is very low, it is a thermodynamically spontaneous process (6). This process is facilitated by presence of surfactant. The surfactant forms a layer around oil globule in such a way that polar head lies towards aqueous and non polar tail pull out oil and thereby reduces surface tension between oil phase and aqueous phase (7). Another factor affecting formation of microemulsion is the ratio of surfactant and cosurfactant. The lipid mixtures with different surfactant, co-surfactant and oil ratios lead to the formation of SMEDDS with different properties (8). Since surfactant and cosurfactant adsorb at interface and providing mechanical barrier to coalescence, selection of oil, surfactant, cosurfactant and mixing ratio to S/CoS, play important role in microemulsion formation (9). Six different formulations were prepared using different oils, surfactants and cosurfactants in varying ratio. VSMS 1 is prepared using Capmul MCM as oil phase, Tween 80 as surfactant and PEG 400 as cosurfactant. Formulation A was prepared with surfactant/cosurfactant (S/CoS) ratio of 1:1. As shown in Figure 4.2.3 (A), formulation A covers maximum microemulsion region as compare to other formulations. The point where amount of oil is less than 10%, the water content is 90%. At this point microemulsion can be diluted to infinite which fulfills requirement of SMEDDS and also particle size of this microemulsion is less than 100nm (described in characterization of SMEDDS). The region where oil content is more than 10% and surfactant/cosurfactant is up to 50% also forms the microemulsion but these were found to be unstable on dilution. The phase diagram shows that when S/CoS exceed to 50%, coarse emulsion forms having particle grater than 100 nm (Fig 4.2.3). Hence it can be predicted that concentration of S/CoS should be less than 50% to form self-microemulsion. Further, more amount of oil also entrap less water content and thereby results in coarse emulsion. Formulation B was

prepared using similar excipients but with S/CoS ratio of 2:1. In this system, after dilution amount of oil contained was limited up to 7% and concentration of S/CoS was also 20% (Fig. 4.2.3 (B)). Above these concentrations coarse emulsion formed. The third formulation C was prepared using S/CoS as 3:1. This involves only 2% of oil, less than 10% of S/CoS and more than 90% of water (Fig. 4.2.3 (C)) after formation of self microemulsion. Initially it formed self microemulsion but later on converted to emulsion as it moved towards higher concentration of oil. Hence putting into Nut Shell, in VSMS 1, formulation A prepared with 1:1 ratio of S/CoS forms better SMEDDS compared to other two formulations and in all the cases oil concentration should be less than 10%.

The systems VSMS 2 were prepared using Capmul MCM C10 as oil, Tween 80 as a surfactant and Plurol olieque as a cosurfactant which produced three formulations D, E and F with varying ratios of S/CoS to 1:1, 2:1 and 3:1 respectively. Formulation D (Fig. 4.2.3 (D)) creates self-microemulsion region with oil up to 7%, S/CoS 9% but at larger oil concentrations it forms emulsion region having higher particle size which were not stable for longer time. Fig. 4.2.3 (E) and (F) shows only gray region, it means formulation E and F did not generate any self-microemulsion regions. So it can be concluded that excipients used for VSMS 2 are not suitable to form a SMEDDS. Next three formulations G, H and I were prepared from third system VSMS 3 using Captex 200P as oil, Cremophore EL as surfactant and PEG 400 as cosurfactant with S/CoS ratio of 1:1, 2:1 and 3:1 respectively. The figure 4.2.3 (G), (H) and (I) clarify that first two formulation G and formulation H did not form any self-microemulsion regions where as third formulation I formed self-microemulsion with 20% of oil concentration and 20% of S/CoS but again stability of this microemulsion was poor.

4.2.2.3 Preparation of SMEDDS:

Valsartan SMEDDS 1 (VSMS 1) was prepared by using Capmul MCM as an oil phase, Tween 80 as a surfactant and PEG 400 as cosurfactant (Table 4.2.2). Second system was prepared with Capmul MCM C10 as oil, Tween 80 as a surfactant and Plurol oleique as a cosurfactant which is named as SMEDDS 2 (VSMS 2). The composition of VSMS 2 is shown in Table 4.2.3. In case of SMEDDS 3 (VSMS 3), Captex 200P acted as an oil component, Cremophore EL acted as surfactant and PEG 400 played a role of

cosurfactant (Table 4.2.4). In each system three formulations were prepared by varying ratio of surfactant and cosurfactant. Formulation A, B and C were prepared with S/CoS ratio of 1:1, 2:1 and 3:1 respectively from VSMS 1. Similarly formulations D, E, F and G, H, I were prepared from VSMS 2 and VSMS 3 respectively. In each formulation concentration of valsartan was kept constant to 10 mg/mL.

Vehicle (% w/w)	**A (1:1)**	**B (2:1)**	**C (3:1)**
Valsartan (mg)	10	10	10
Capmul MCM	10	10	10
Tween 80	45	60	67.5
PEG 400	45	30	22.5

Oil- Capmul MCM, Surfactant- Tween 80, Co-surfactant- PEG 400

Table 4.2.2 Composition of System 1 (VSMS 1)

Vehicle (% w/w)	**D (1:1)**	**E (2:1)**	**F (3:1)**
Valsartan (mg)	10	10	10
Capmul MCM C10	10	10	10
Tween 80	45	60	67.5
Plurol Olieque	45	30	22.5

Oil- Capmul MCM C10, Surfactant- Tween 80, Co-surfactant- Plurol Olieque

Table 4.2.3 Composition of System 2 (VSMS 2)

Vehicle (% w/w)	**G (1:1)**	**H (2:1)**	**I (3:1)**
Valsartan (mg)	10	10	10
Captex 200 P	10	10	10
Cremophore EL	45	60	67.5
PEG 400	45	30	22.5

Oil- Captex 200P, Surfactant- Cremophore EL, Co-surfactant- PEG 400

Table 4.2.4 Composition of System 3 (VSMS 3)

4.3 CHARACTERIZATION AND SELECTION OF SMEDDS

4.3.1 Experimental

25 µL of SMEDDS were diluted with 25 mL of distilled water. These microemulsion solutions were considered for further assessment of various *in vitro* parameters.

4.3.1.1 Appearance:

Appearances of VSMS A, VSMS 2 and VSMS 3 were tested against white and black background and turbidity were checked. The test was carried out as described in the United States Pharmacopoeia (10).

4.3.1.2 Particle size analysis:

The particle size of resultant solutions were measured by laser diffraction (Malvern Instruments, Malvern, UK) at obscuration of 10 % (n =5). Particle size distributions were characterized by d 50 % & VMD (volume mean diameter).

4.3.1.3 Zeta potential measurement:

It gives an idea about particle distribution in the solution and formation of stable microemulsion. The Nano ZS Zetasizer (Malvern Instrument, UK) was used to measure the zeta potential by electrophoresis and electrical conductivity of the formed ME (3). The electrophoretic mobility (µm/s) was converted to zeta potential by in-built software using Helmholtz- Smoluchowski equation. Measurements were performed using small volume disposable zeta cell. Average of three measurements of each sample was used to derive average zeta potential. Latex dispersion having zeta potential -50 mV ± 2.5 mV was used as a standard. The standard was evaluated after every 60 min during measurement of test samples in order to validate the results of test formulation.

4.3.1.4 Viscosity and Refractive Index:

Viscosities were measured to determine rheological properties of formulation. Brookfield LVDV 111 + CP viscometer (Stoughton, MA) at 30°C with a CPE 42 spindle at 5 rpm was used to serve this purpose. Experiment was performed in triplicate for each sample, and results were presented as average ± standard deviation.

Refractive index measures clarity of prepared system. Abby's refractometer was used to determine R.I. Refractive index of water was considered as standard to compare the values obtained samples.

4.3.1.5 Conductance:

Type of microemulsion (o/w or w/o) can be determined by measure of conductance. It was measured by conductivity meter. The electroconductivity of the resultant system was measured by an electroconductometer (CM 180 conductivity meter, Elico, Mumbai, India). For the conductivity measurements, the tested microemulsions were prepared with a 0.01N aqueous solution of sodium chloride instead of distilled water.

4.3.1.6 % Transmittance Measurement:

The percent transmittance of VSMS 1, VSMS 2 and VSMS 3 were measured at 650 nm using UV spectrophotometer (UV 1601, Shimadzu, Japan) keeping distilled water as a blank.

4.3.1.7 Assay:

VSMS 1, VSMS 2 and VSMS 3 were analyzed to determine the content of valsartan in SMEDDS. Systems were diluted as per method and amount of drug was determined by validated HPLC method.

4.3.2 Result and Discussion

4.3.2.1 Appearance

SMEDDS were checked for transparency to turbidity. SMEDDS remains clear on dilution but appeared as transparent blue colored solution due to presence of synthetic oils. In some cases, initially upon dilution, SMEDDS formed clear microemulsion but while studying bench top stability it got converted to gel form. Such SMEDDS can not be considered as stable SMEDDS. The stability of SMEDDS was determined by studying different *in vitro* characters, results of which are listed in Table 4.3.1, 4.3.2 and 4.3.3 for VSMS 1, VSMS 2 and VSMS 3 respectively.

4.3.2.2 Particle Size

The particle size of microemulsion is important criteria for evaluation and it should be less than 100 nm. It is clear from observed values that only VSMS A, B and C formed SMEDDS having particle size less than 100 nm. Constantinides reviewed about SEDDS and SMEDDS (6). As per his review there is no such specific boundary between SEDDS and SMEDDS. Self-emulsifying system is similar to SMEDDS, and only difference is in particle size.

Vehicle (% w/w)	**A (1:1)**	**B (2:1)**	**C (3:1)**
Mean Particle size (nm)	**11.8**	**12.7**	**13.6**
Zeta Potential (mV)	**-12.1**	**-8.746**	**-6.65**
Poly Dispersivity Index (PDI)	**0.203**	**0.246**	**0.281**
% Transmittance	**99.8**	**99.6**	**98.6**
pH	**5.24**	**5.18**	**5.39**
Viscosity (cP)	**0.992**	**0.945**	**0.967**
Conductivity (μS)	**98.69**	**96.28**	**97.66**

Table 4.3.1 Characterization of System 1 (VSMS 1)

Vehicle (% w/w)	**D (1:1)**	**E (2:1)**	**F (3:1)**
Mean Particle size (nm)	120.7	132.2	141.1
Zeta Potential (mV)	0.036	-0.617	-0.53
Poly Dispersivity Index (PDI)	0.196	0.234	0.326
% Transmittance	97.53	97.84	97.99
pH	5.33	5.41	5.29
Viscosity (cP)	0.987	0.965	0.973
Conductivity (μS)	94.68	95.27	95.83

Table 4.3.2 Characterization of System 2 (VSMS 2)

Vehicle (% w/w)	**G (1:1)**	**H (2:1)**	**I (3:1)**
Mean Particle size (nm)	111.9	135.4	157.5
Zeta Potential (mV)	-0.249	-0.381	-0.563
Poly Dispersivity Index (PDI)	0.341	0.442	0.386
% Transmittance	98.16	97.85	97.53
pH	5.21	5.17	5.08
Viscosity (cP)	0.963	0.948	0.932
Conductivity (μS)	93.97	94.88	94.49

Table 4.3.3 Characterization of System 3 (VSMS 3)

An efficient SEDDS should be able to form self-emulsion having particle size less than 5μm. Other formulations which have higher particle size may get converted to SEDDS upon dilution or during stability period. In our study, VSMS A, B and C having particle size 11.8, 12.7 and 13.6 nm (Fig. 4.3.1, 4.3.2, 4.3.3) respectively were considered as SMEDDS and taken for further studies.

			Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm):	11.8	Peak 1:	12.2	97.0	3.69
PdI:	0.203	Peak 2:	4880	3.0	683
Intercept:	0.950	Peak 3:	0.00	0.0	0.00

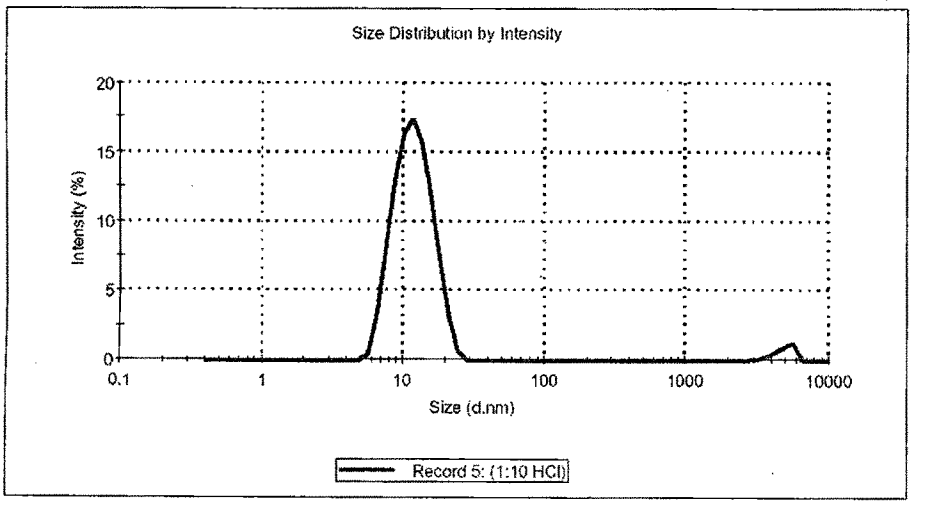


Figure 4.3.1: Particle size and Polydispersivity index (PDI) of Formulation A

			Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm):	12.7	Peak 1:	12.9	90.1	4.34
PdI:	0.246	Peak 2:	1450	9.9	741
Intercept:	0.921	Peak 3:	0.00	0.0	0.00

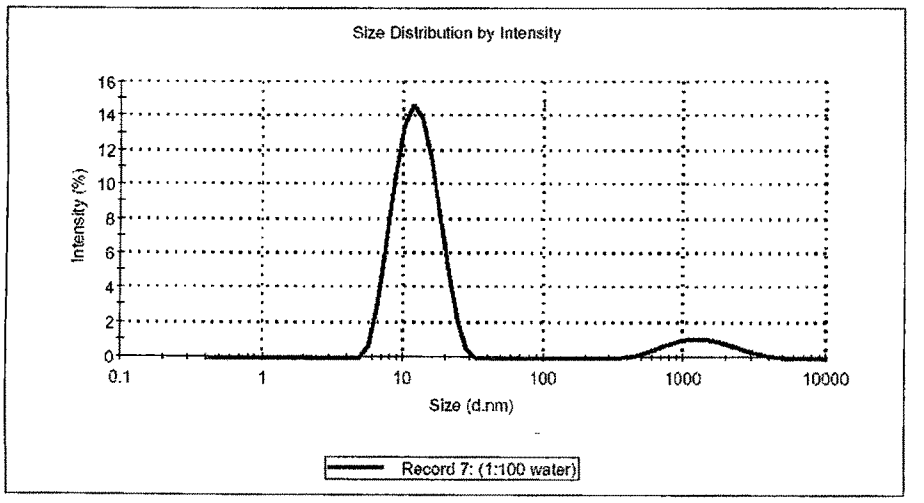


Figure 4.3.2: Particle size and Polydispersivity index (PDI) of Formulation B

			Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm):	13.6	Peak 1:	13.4	88.7	4.74
PdI:	0.281	Peak 2:	426	8.5	175
Intercept:	0.936	Peak 3:	4440	2.8	911

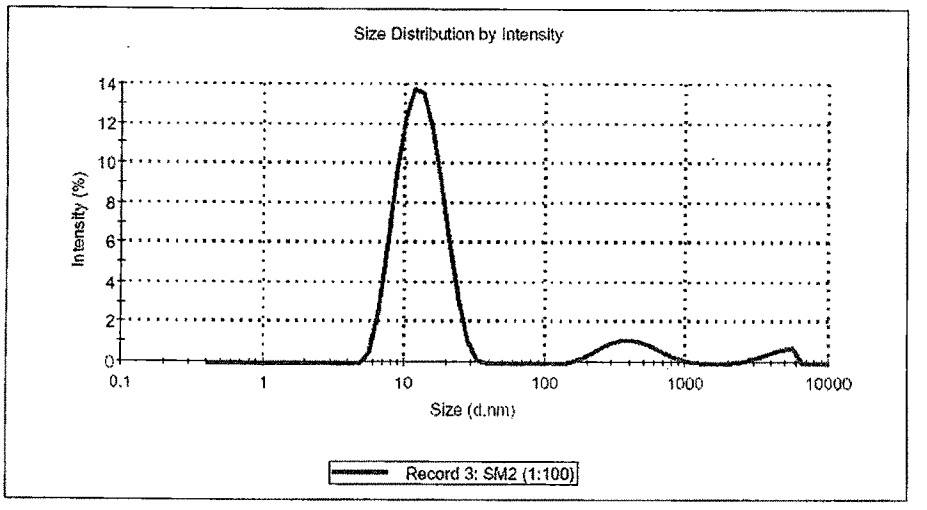


Figure 4.3.3: Particle size and Polydispersivity index (PDI) of Formulation C

4.3.2.3 Zeta Potential

Other important factor is zeta potential and it is related to surface charge of microemulsion droplet. It is highly dependent on surfactant used. The theory states that system remains stable due to deflocculation of microemulsion particles and for identical system zeta potential charge should be between ranges of -10 to -30 mV (11). In our study except VSMS A, zeta potential values for systems other than formulation A were found near to zero or less than -5 mV. This indicates that systems were not stable for long time. Formulation A has a zeta potential value -12.1 which lies in ideal limit mentioned above. Due to this electrostatic charge, particles remain flocculated and hence system shows stability.

4.3.2.4 Polydispersivity Index (PDI)

Polydispersivity which determines size range of particles in the system is measured by

$$\frac{No.\ of\ particles\ having\ size\ grater\ than\ 100nm}{No.\ of\ particles\ having\ size\ less\ than\ 100nm}$$

It is expressed in terms of polydispersivity index (PDI). An ideal SMEDDS should be widely distributed with particles less than 100 nm and so PDI should be less than 0.3 or in other words particles having size more than 100 nm should be maximum up to 23%. The data (Table 4.3.1, 4.3.2 and 4.3.3) shows that system VSMS A, B, C, D and E have PDI less than 0.3 while in opposite to that VSMS F, G, H and I have PDI greater than 0.3.

4.3.2.5 % Transmission

The clarity of microemulsions was checked by transparency, measured in terms of % Transmission (%T). SMEDDS forms o/w microemulsion since water is in external phase. VSMS A and B have %Transmission value grater than 99%. These results indicate the high clarity of microemulsions. In case of other systems %T values were about 97% suggesting less clarity of microemulsions. This may be due to greater particle size and zeta potential of the formulation. Due to higher particle size, oil globules may reduce the transparency of microemulsion and thereby values of % T. Another factor zeta potential as we discussed earlier keeps system flocculated. It can be observed from Tables 4.3.2 and 4.3.3 that VSMS D to VSMS I have zeta potential values near to zero. Moreover these systems have particle size more than 100 nm and also % T values are about 97%. In

contrast to that VSMS B and C have smaller particle size, zeta values significantly near to specified range and transparency near to 99%. Formulation A showed 99% clarity of microemulsion with small particles and significant zeta value.

4.3.2.6 pH

Another important parameter measured was pH. The excipients used in formulation decide the pH of the final preparation. Literatures (11) suggest that change in pH may change zeta potential of formulation which in turn can affect the stability of preparation. So pH is also responsible for stability of microemulsion. All the formulations prepared showed similar pH values in the range of 5.0 to 5.5. So here pH is not affecting stability. This also confirms that drug is not diffusing to the external phase as literature revels that pH of valsartan is 3.3 in water but pH values of these systems are not matching with that. So it can be assumed that drug remains in oil phase only and since water is in external phase entire system showed pH of water.

4.3.2.7 Viscosity

Viscosity of microemulsion systems can be monitored by standard rheological techniques. It depends on oils and surfactant used. In developed systems mostly medium chain triglycerides are used as oil phase. Viscosity of these chemicals is reported to be 56.7 cP (6), whereas SMEDDS forms o/w microemulsion, water remains as external phase and so viscosity of SMEDDS is near to that of water. The observed values listed in Tables 4.3.1, 4.3.2 and 4.3.3 support this statement. The viscosity of SMEDDS is 0.992 which is similar to that of water i.e. 1.0. This reveals that formulation A is very clear, transparent and low viscous liquid.

4.3.2.8 Conductivity

Presence of o/w microemulsion formation was confirmed by measuring conductivity. The initial conductivity of the studied samples was low as water fraction was less than 10% wt/wt water but a drastic increase in conductivity was observed at 30% wt/wt fraction of water. The increase in conductivity at water is most likely caused by a transition from an oil-continuous microemulsion system to a water-continuous microemulsion system (12). As all SMEDDS are water-continuous microemulsions, viscosities were found to be higher. Some decrease in conductance was due to presence of oil droplets. Systems which

have higher droplet size shows resistance to conductance and so decrease in conductivity were observed. VSMS A showed highest conductivity 98.67 as compared to all other systems.

On the basis of all the data, three formulations A, B and C were found suitable as SMEDDS. The two main criteria, particle size and zeta potential of these systems fulfills the requirement of SMEDDS. The other characteristics also support the formation of o/w microemulsion. Therefore stability studies were performed for VSMS A, B and C.

4.4 STABILITY STUDIES OF VALSARTAN SMEDDS

4.4.1 Experimental

4.4.1.1 *Robustness to dilution*

Robustness of SMEDDS to dilution was studied as per Date et al., method with slight modification (13). SMEDDS were diluted to 10, 100 and 1000 times with various dissolution media viz. water, pH 1.2 buffer, pH 4.5 buffer and pH 6.8 buffer. The diluted microemulsions were stored for 12 hours and observed for any signs of phase separation or drug precipitation.

4.4.1.2 Physical Stability

Formulation A, Formulation B and Formulation C were subjected to physical stability tests (14). The prepared microemulsions have been subjected to accelerated centrifugation for the assessment of physical phase separation, if any between the oil and aqueous phase.

Criteria for selection of batches (15)

1. Microemulsions having mean globule size below 100 nm; and
2. Zeta potential at least -5 mV.

Microemulsions having least globule size are expected to have larger surface area and therefore, may get absorbed or may transverse rapidly across the gastric mucosa. Moreover, literature citation revealed that microemulsion which are negatively charged and having zeta potential -30 mV or less exhibits moderate to excellent physical stability (16). Therefore, both the selection criteria were used as a filter prior to assessment of accelerated physical stability.

Method

Approximately 5 mL of the microemulsion was charged to the centrifugation tube and the top of the tube was tightly closed using screw-on cap. Phase separation study of the globule size- and zeta potential-fractionated microemulsions were performed using accelerated centrifugation at 3000 rpm for 10 minutes (17). Sample from top, middle and bottom were collected using 24" needle fitted on 1 mL syringe and globule size determination was performed using photon correlation spectroscopy (as mentioned under characterization in chapter, section 3.3). The results of globule size following accelerated centrifugation for VSMS A, VSMS B, and VSMS C are recorded in Table 4.4.1.

4.4.1.3 Chemical Stability

SMEDDS for valsartan were subjected to accelerated temperature and stress conditions (18) and were analyzed for physical and chemical stability. Approximately 10 mL of the formulation was filled in USP type III glass vials and sealed using VP6 crimp. The stress stability was conducted at 60°C ± 2°C in an incubator. The accelerated stability was performed at 30°C ± 2°C / 65% ± 5% relative humidity (R.H.) and 40°C ± 2°C / 75% ± 5% R.H. The duration of stability study was 6 months and samples were withdrawn at predetermined time intervals after 1 month, 2 months, 3 months and 6 months (18). The parameters such as physical separation at accelerated gravitational force, active ingredient content, globule size determination, zeta potential measurement, appearance were determined. The results for these studies are recorded in Table 4.4.2, Table 4.4.3 and Table 4.4.4.

4.4.2 Result and Discussion

4.4.2.1 Robustness to Dilution

Diluted SMEDDS did not show any precipitation or phase separation on storage in various dilution media. This suggests that all media were robust to dilution.

4.4.2.2 Physical Stability

Conventional emulsions are inherently unstable from physical standpoint. Poor physical stability is ultimately exhibited by phase separation, which can be visually monitored. Certain properties of emulsions will start to change long before this phase separation is visually apparent. An increase in particle size is particularly indicative of physical

instability, since this monitors the coalescence or flocculation that is part of the process involved in ultimate phase separation. Increases in viscosity (due to flocculation) and changes in zeta potential (arising from a decrease in droplet surface area) are both indicative of poor physical stability. The presence of drug and/or cosolvents can potentially hasten the phase separation.

The key factor in determining the stability of an emulsion is believed to be the interfacial tension between two phases, and lower interfacial tension (primarily governed by surfactant) will increase the stability. Very low interfacial tension is thought to be the primary factor leading to the stability of microemulsions. One of the most useful approaches is the so-called DLVO theory (19). According to this theory, the stability of emulsion depends on a balance of electrostatic repulsive forces and London type Vanderwaals attractive forces. Thus, higher surface charge (characterized by high absolute values of the zeta potential) will generally tends to stabilize emulsion due to higher repulsion of the droplets from one another. An overabundance of electrolytes will generally tend to destabilize emulsion due to lowering of surface charge, leading to so-called *salting out effect*, wherein the emulsion will undergo flocculation and phase separation at sufficiently high electrolyte concentration. Calcium and other divalent ions are particularly destabilizing. A parameter known as the *critical flocculation concentration* (CFC), can be derived which is the electrolyte concentration at which flocculation begins.

On the basis of results of particle size, zeta potential and viscosity of formulation A, formulation B and formulation C were determined to predict the stability of prepared system. Stability data is shown in Table 4.4.1.

System	Globule size (nm)		
	Top layer	Middle layer	Bottom layer
Formulation A	11.39 ± 0.65	13.12 ± 0.37	15.35 ± 0.67
Formulation B	12.76 ± 0.52	21.34 ± 0.35	29.67 ± 0.92
Formulation C	13.74 ± 1.45	19.26 ± 1.24	25.50 ± 1.68

Table 4.4.1 Physical Stability of SMEDDS for valsartan

The values clearly prove that after stability study, formulation A showed all the results similar to initial formulation while significant changes were observed in values of formulation B and C. In case of particle size formulation A showed least particle size 17.8 and same zeta potential -11.5 but incase of formulation B and formulation C it was observed that though particle size of VSMS B is 24.4, two types of peaks with different size were observed and hence PDI was increased to 0.334. Similarly particles size of formulation C was increased to 82.2 form original value of 13.6. This also shows presence of two peaks and PDI is 0.675. These results indicate that in stability period, chemical reaction between excipients of formulation B and C had taken place so that on dilution it formed microemulsion with increased particle size and polydispersivity. While in case of VSMS A, excipients are compatible and hence form microemulsion with same particle size. Since zeta potential governs stability of microemulsion, it is important to measure its value for stability samples. The high value of zeta potential indicates electrostatic repulsion between two droplets. DLVO theory (19) states that electric double layer repulsion will stabilize microemulsion where electrolyte concentration in the continuous phase is less than a certain value. When two droplets approach each other, the counter ions forming the diffuse part to the electric double layer begin to overlap. This overlap means that the electric potential between droplets is increased which, in turn, means that more energy must be added with reduced distance between the droplets. At the same time, there is always an attractive force between emulsion droplets, and this force becomes stronger with reduced distance between them. A negative force means a negative potential between the droplets. The interaction between droplets is decided by the total potential. In our study, zeta potential were found to be decreased for system B and C which indicates reduced repulsive forces between two particles. Due to presence of Vanderwaals attractive forces two particles come closer and hence flocculation of system occurred resulting in increased droplet size or microemulsion. As droplet size increased, the viscosities of system were also increased. Moreover presence of oil droplets reduced the transmittance of UV light and thereby %T. In case of formulation A, all these values were found to be same as in the previous study. It can be concluded that significant changes occurred in formulation B and C during stability which destabilized the

formulations but formulation A was able to form stable self-microemulsifying system. Hence formulation A was selected for further *in vitro* and *in vivo* bioavailability study.

4.4.2.3 Chemical Stability

Certain emulsion components, specially those derived from unsaturated lipids, can give rise to undesirable degradation products on storage in turn affecting the stability of SMEDDS. These can be oxidative products e.g., lipid hydroperoxides and aldehydes, or hydrolytic products e.g., free fatty acids, mono and diglycerides, and lyso-phospholipids. These degradation products can alter the surface property and zeta potential of the emulsion and distribute in the aqueous phase. Fatty acid formation can also decrease the pH and increase the conductivity of the emulsion. Hence, these two parameters should be carefully monitored to aid stability evaluation. The emulsion excipients (viz., oils, surfactants and cosolvents) should be monitored by periodic assays to ensure no loss of excipients concentration.

Formulation A	Ratio of S:CoS	Period (month)	40°C/75% RH				30°C/65% RH			
			Globule size (nm) ± SEM	Zeta potential (mV) ± SEM	Trans-mittance (%) ± SEM	Drug content (%)	Globule size (nm) ± SEM	Zeta potential (mV) ± SEM	Trans-mittance (%) ± SEM	Drug content (%) ± SEM
	1:1	0	11.8 ± 11.25	-11.5 ± 2.00	99.8 ± 0.1	99.45	11.80 ± 11.47	-11.5 ± 2.00	99.8 ± 0.1	99.45 ± 0.22
		1	20.24 ± 8.90	-10.25 ± 3.56	99.3 ± 0.2	98.18	20.15 ± 8.54	-11.89 ± 1.82	99.8 ± 0.2	98.48 ± 0.65
		2	17.66 ± 10.39	-13.67 ± 1.55	99.7 ± 0.5	96.23	17.00± 5.22	-13.37 ± 2.01	99.2 ± 0.4	97.19 ± 0.59
		3	19.85 ± 4.97	-12.43 ± 3.34	99.1 ±0.1	97.88	18.65 ± 10.28	-14.76 ± 2.78	99.6 ± 0.3	98.56 ± 0.24
		6	18.65 ± 3.34	-11.68 ± 4.97	99.4± 0.3	99.12	19.23 ±5.89	12.29 ± 1.75	99.1 ± 0.2	± 0.25

Table 4.4.2 Stability data of Formulation A

Formulation B	Ratio of S:CoS	Period (month)	40°C/75% RH				30°C/65% RH			
			Globule size (nm) ± SEM	Zeta potential (mV) ± SEM	Trans-mittance (%) ± SEM	Drug content (%)	Globule size (nm) ± SEM	Zeta potential (mV) ± SEM	Trans-mittance (%) ± SEM	Drug content (%) ± SEM
	2:1	0	24.4 ± 8.57	-8.75 ± 1.05	99.6 ± 0.2	99.18 ± 0.28	24.4 ± 5.39	-34.62 ± 0.78	99.9 ± 0.4	98.35 ± 1.20
		1	35.55 ± 6.53	-6.27 ± 3.52	95.8 ± 0.5	97.54 ± 0.96	26.86 ± 6.23	-35.00 ± 2.06	99.4 ± 0.6	98.28 ± 0.17
		2	63.25 ± 11.56	-3.66 ± 1.26	94.1 ± 0.2	96.27 ± 0.36	25.87 ± 4.59	-32.19 ± 1.85	98.4 ± 0.7	97.05 ± 1.02
		3	94.42 ± 9.25	-0.48 ± 3.10	94.7 ± 0.3	93.98 ± 0.54	23.45 ± 5.28	-34.27 ± 1.77	97.1 ± 0.5	96.21 ± 0.26
		6	122.56 ± 8.16	-0.59 ± 2.34	93.6 ± 0.1	88.3± 0.75	20± 24.63	-40.12± 0.98	96.4± 0.3	93.7± 1.49

Table 4.4.3 Stability data of Formulation B

Formulation C	Ratio of S:CoS	Period (month)	40°C/75% RH				30°C/65% RH			
			Globule size (nm) ± SEM	Zeta potential (mV) ± SEM	Trans-mittance (%) ± SEM	Drug content (%) ± SEM	Globule size (nm) ± SEM	Zeta potential (mV) ± SEM	Trans-mittance (%) ± SEM	Drug content (%) ± SEM
	3:1	0	18.23 ± 5.67	-34.12 ± 2.71	99.4 ± 0.1	99.45 ± 0.96	18.23 ± 5.28	-34.12 ± 0.78	99.4 ± 0.1	99.45 ± 0.24
		1	22.76 ± 7.93	-36.10 ± 3.34	99.3 ± 0.2	98.18 ± 0.21	19.75 ± 8.54	-30.87 ± 2.01	99.8 ± 0.3	98.48 ± 0.59
		2	18.45 ± 9.39	-31.89 ± 1.69	99.7 ± 0.5	96.23 ± 0.30	20.15 ± 4.53	-35.46 ± 1.82	99.2 ± 0.4	97.19 ± 0.65
		3	20.64 ± 8.41	-35.17 ± 1.22	99.1 ±0.1	97.88 ± 0.76	18.65 ± 9.56	-33.07 ± 2.00	99.6 ± 0.2	98.56 ± 0.29
		6	17.00 ± 12.96	-30.96 ± 2.00	99.6 ± 0.4	99.00 ± 0.22	13.86 ± 1.22	-35.00 ± 1.77	99.4 ± 0.5	98.28 ± 0.22

Table 4.4.4 Stability data of Formulation C

As the stability study was performed as per ICH guideline, conditions can be decided based on climatic condition of that particular zone. As per guideline, stability should be carried out under three different conditions. They are:

(a)	25°C/60% RH	12 months	Long term stability
(b)	30°C/65% RH	6 months	Intermediate stability
(c)	40°C/75% RH	6 months	Accelerated study

Based on the stability of the samples under these three conditions shelf life of the samples can be decided. The present study was carried out under two conditions i.e., intermediate condition at 30°C/65% RH and accelerated condition at 40°C/75% RH for 6 months. It is obvious that if samples are stable on these two conditions for at least 6 months, it will remain stable through out its shelf line. The data of stability studies is shown in Table 4.4.2, Table 4.4.3 and Table 4.4.4. The data indicate that formulation A is the most stable system. Stability of microemulsion were observed at different time intervals i.e., 0 (initial), 1, 2, 3 and 6 months. All the characteristics of formulation A were found stable even after 6 months period. In case of other two formulations, data from table 4.4.3 and 4.4.4 shows that values for particle size are continuously increased over the period of months and zeta potential was found to be decreased for formulation B and formulation C. These are indicatives of unstable systems. Other characteristics like viscosity, %Transmission, pH and conductivity support these conclusions for all three formulations. As mentioned above, there may be formation of fatty acid during stability study which may leads to degradation of drug, so assay content were also determined using developed HPLC method. The results showed reduction in actual drug content of the system. The present drug content of the system was determined by estimating assay value (Table 4.4.2). The assay for formulation A was found constant i.e., around 99% throughout 6 months period. The assay values of formulation B and formulation C were found to be decreased from 99% to 90% for formulation B and 99% to 80% for formulation C over the period of time. These data confirms the instability of formulation B and C.

On the basis of results of physical and chemical stability study it can be concluded that formulation A is most stable SMEDDS giving a microemulsion with low particle size and good zeta potential values. Formulation A was selected as final formulation as it has low

particle size and significant potential to keep system suspended. Low PDI values shows uniformity of globule size, %T indicates the clarity of microemulsion, viscosity and conductivity confirms water-continuous microemulsion system and constant pH represents solubility and stability of drug in oil droplet. Even in pseudoternary phase diagram VSMS A showed maximum microemulsion region. Therefore VSMS A was selected for further *in vitro* and *in vivo* studies.

4.5 *IN VITRO* DIFFUSION STUDY

In Vitro drug diffusion study was carried out by using two different methods.

1. Dialysis bag study.
2. Intestinal permeability study.

In this investigation, all the test formulations were assessed for *in vitro* diffusion across the dialysis technique and *in vitro* permeation across Male Sprague Dawley rat's duodenum in triplicate and the % drug release was calculated.

4.5.1 Experimental

4.5.1.1 Dialysis Bag Method

In vitro release of VSMS A was tested by using dialysis bag method (20). Dialysis bag was soaked over night into a phosphate buffer pH 6.8 for saturation purpose and then it was used for further experimental procedure. One end of the prepared dialysis bag was tied with thread, and then 0.5 mL of valsartan microemulsion was instilled into the dialysis bag (MWCO 12000, Hi-Media Industries Inc., USA). The other end of the dialysis bag was also secured with thread and was placed in 1000 mL, phosphate buffer pH 6.8 as the dissolution medium at 37°C. The revolution speed of the paddle was maintained at a rate of 50 rpm (21). Samples (5 mL) were drawn out at predetermined time intervals (0.08, 0.17, 0.25, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 8, 12 hr), and the same volume of fresh dissolution medium was replenished. The release of valsartan from SMEDDS was compared with that of from marketed capsule formulation and standard drug solution. Samples were analyzed quantitatively for valsartan dialyzed across the membrane at given time by using UV method (section 4.1.1.1). The experiments were run in triplicate and data are presented in terms of % cumulative release of drug along with SD. Same study was performed in pH 1.2 buffer and pH 4.5 buffer. Based on results

of these study release from SMEDDS of formulation was compared with that of pure valsartan suspension and marketed formulation (Valent-80®). The results are shown in tabular form in Table 4.5.1 and in Figure 4.5.1.

4.5.1.2 Intestinal Permeability Study (22, 23)

Male Sprague Dawley rats (250 – 300 gm) were killed by over dose with pentobarbitone administrated by intravenous injection. The basic aim of study was to check the intestinal permeability of the drug, orally administered as microemulsion dosage form. To check the intraduodenal permeability, the duodenal part of the small intestine was isolated and used for *in vitro* intestinal study. Separated duodenal part was washed with cold Ringer's solution to remove mucous and lumen contents and one end of the duodenum was tied with thread. SMEDDS of valsartan was diluted with pH 6.8 phosphate buffer to get 1000 times dilution. Diluted SMEDDS was filled in duodenum and study was carried out. Similarly a suspension of marketed formulation (Valent-80) was formed by using 1 mL of phosphate buffer pH 6.8. The resultant solution (10mg/mL) was injected into the lumen of the duodenum using a syringe and another side of the lumen was tightly closed with the thread. The tissue was placed in a chamber of organ bath with continuous aeration and a constant temperature of 37°C. The receiver compartment was filled with 30 mL of Phosphate buffer pH 6.8. Aliquots of 1 mL were withdrawn at different time intervals and volume of aliquots replaced with fresh dialysis medium each time. The samples were analyzed quantitatively for Valsartan dialyzed across the membrane at corresponding time by using UV-Visible spectrometric method as mentioned in section 4.1.1.1. The experiments were run in triplicate and the mean cumulative % drug diffused along with SD for pure valsartan, marketed formulation (Valent-80) and prepared SMEDDS are shown in Table 4.5.2 and graphically represented in Figure 4.5.2.

4.5.2 Result and Discussion

In vitro diffusion of formulations is a valuable tool to predict behavior of a particular formulation with respect to drug transport across the membrane. According to Gemmell and Morrison (24), *in vitro* model may have limitations in terms of prediction of drug transport across the mucosal membrane nevertheless; under the testing conditions *in vitro* studies can be helpful to assess the relative drug transport behavior across the mucosa.

Various physicochemical parameters pertaining to formulations such as flux, partition coefficient, diffusion coefficient can be derived using *in vitro* evaluation techniques. One of the disadvantages of *in vitro* evaluation techniques is that method does not mimic the behavior of living tissues/organs, for example, degradation of drug compound in presence of enzymes, capricious blood supply or metabolism etc. In practice, it virtually becomes difficult task to perform the biological studies using animals or on humans for the assessment of different formulations from the perspective of economy and time requirement. At the same time, *in vitro* models can serve as second line option which will be indicative kind of tool prior to proceeding for animal or human studies.

4.5.2.1 Dialysis Bag Method

Time (Hrs)	% Cumulative Drug Release				
	Formulation A in pH 1.2	Formulation A in pH 4.5	Formulation A in pH 6.8 Buffer	Marketed Formulation in pH 6.8	Standard Drug Solution in pH 6.8
0.08	6.73	8.55	19.01	16.87	13.90
0.17	7.95	9.68	24.93	19.52	14.64
0.25	9.24	10.83	33.89	21.64	15.07
0.33	10.08	12.05	42.93	23.97	16.44
0.5	11.53	13.54	57.79	33.29	17.50
0.75	12.99	14.85	74.23	41.98	18.25
1	14.32	16.04	84.89	51.31	19.31
1.5	15.11	16.99	90.52	58.30	19.41
2	15.48	17.59	94.64	57.66	20.79
3	14.96	18.42	95.35	58.62	21.64
4	15.06	18.26	95.97	60.58	22.48
8	15.28	18.55	95.28	60.99	22.87
12	15.15	18.75	95.68	60.53	22.66

Table 4.5.1 % Cumulative Drug Release In-vitro Release Study (Dialysis Bag Method)

When *in vitro* release of valsartan was checked for formulation A in three different medias i.e. pH 1.2, pH 4.5 and pH 6.8, formulation A showed highest released in pH 6.8 buffer (Table 4.5.1). This study indicates that release of valsartan is pH dependent. The pH 1.2 buffer and pH 4.5 buffer showed very less release up to 15% and 19% respectively. Form these data it is confirmed that valsartan has pH dependent solubility. Based on results of these data, further release of valsartan was compared with conventional formulation (Valent-80®) and standard drug suspension in pH 6.8 buffer.

The graphical release pattern (Fig 4.5.1.) shows that drug release form formulation A is faster than conventional formulation and standard drug solution.

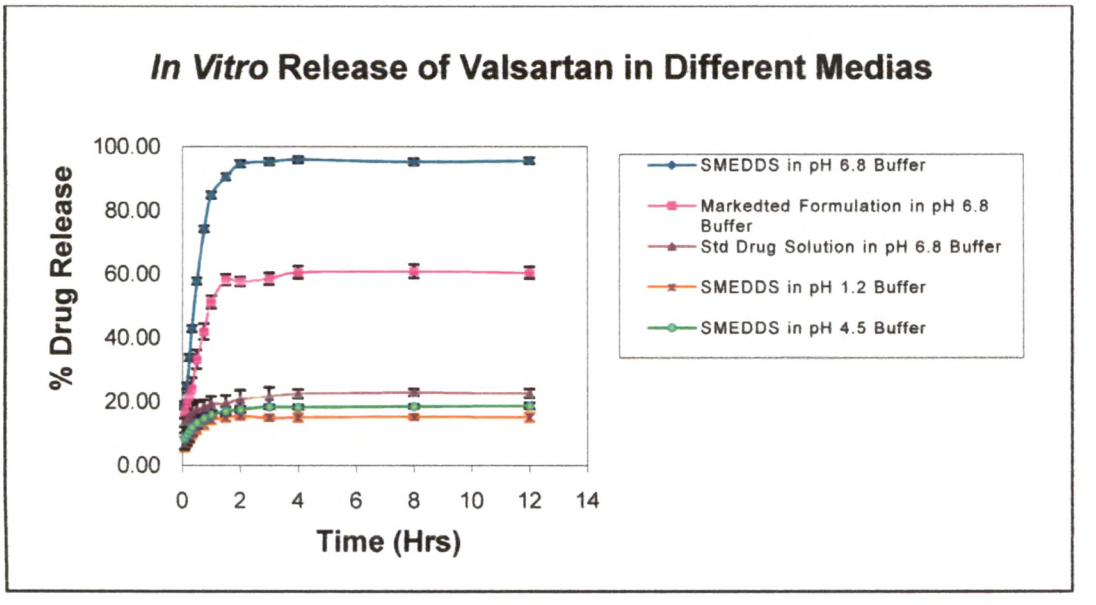


Fig 4.5.1 Comparative *In vitro* Release Study (Dialysis Bag Method)

Moreover, formulation A releases more than 85% drug within one hour while release rate is very slow in case of conventional capsule formulation i.e. only up to 50% and same for drug suspension is only up to 19% within first hour. Initially undiluted SMEDDS was filled in dialysis bag, but for the formation of self-microemulsion SMEDDS must come in contact with aqueous phase. Due to high viscosity of oil blend, it can not diffuse directly through membrane and could not form microemulsion and thereby no drug was released. So it was important to dilute SMEDDS with water before being instilled into dialysis bag. In this case, the drug was present in form of micro globules of microemulsion and water was as a aqueous phase. Due to low size of microemulsion particles, they easily diffuse through dialysis membrane. The results indicated that valsartan SMEDDS might be diluted previously with aqueous solutions before performing *in vitro* release test in a dialysis bag. Otherwise, SMEDDS will stick to the dialysis bag and circumscribe the inflow of release medium. After prior dilution with release medium, SMEDDS can be easily dispersed, and dissolved molecules can permeate out of the dialysis bag easily. The possible factors may be affecting drug release

are (a) SMEDDS with reduced particle size provides more surface area to release drug from solvents and thereby increases drug release rate (b) oil phase of SMEDDS may act as carrier molecules which itself does not diffuse through the barrier but allow drug molecules to get diffused form membrane of dialysis bag. The later statement was concluded during UV estimation of release study. UV method could not be developed for formulation due to interference of oil at same wavelength that of drug, but this interference was not observed after release of drug across dialysis bag. This proves that oil globules do not diffuse through membrane and only drug diffusion was permitted. Another possible reason for diffusion of drug may be a concentration gradient. The compartment consisting SMEDDS has high concentration of drug while opposite compartment having diffusion media was lacking of that. So based on concentration gradient the drug started to diffuse from dialysis bag to media. Although, exact mechanism is not known but it can be confirmed that any of these factors may have effect on bioavailability of drug. This *in vitro* study finally concludes that release of valsartan was greatly enhanced by SMEDDS.

4.5.2.2 Intestinal Permeability Study

Time (Hrs)	% Cumulative Drug Release		
	Formulation A	Market formulation	Standard Drug Solution
0.08	10.65	7.41	2.50
0.17	16.98	12.42	4.13
0.25	28.14	18.69	7.46
0.33	35.72	21.36	9.31
0.5	46.88	27.32	11.25
0.75	58.64	34.62	14.33
1	67.83	43.87	15.47
1.5	72.39	49.48	16.85
2	76.18	52.83	17.01
3	75.48	53.29	16.82
4	75.99	52.96	16.97
8	76.04	53.11	16.83
12	76.28	52.86	17.09

Table 4.5.2 % Cumulative Drug Release in Intestinal Permeability Study

Another approach of intestinal permeability study was also adopted to correlate drug diffusion form SMEDDS. In this study results found were different than that of dialysis bag method. Formulation A showed higher release than conventional formulation and

pure drug suspension, but amount of drug released was less compared to dialysis bag method for all there formulations. Formulation A released up to 60% while conventional capsule released only up to 53% and pure drug suspension showed least release up to 17%. Though formulation A showed highest drug diffusion, it was approximately 20% less than that by dialysis bag method. There may be many assumptions behind this reduced diffusion of drug but exact phenomenon is not known. The thickness of intestinal layer may be one of the factors. As it has been discussed earlier that oil globule act as only carrier and allow drug to diffuse through the cell, some amount of drug may get entrapped in thickness of intestinal cell along with oil globule.

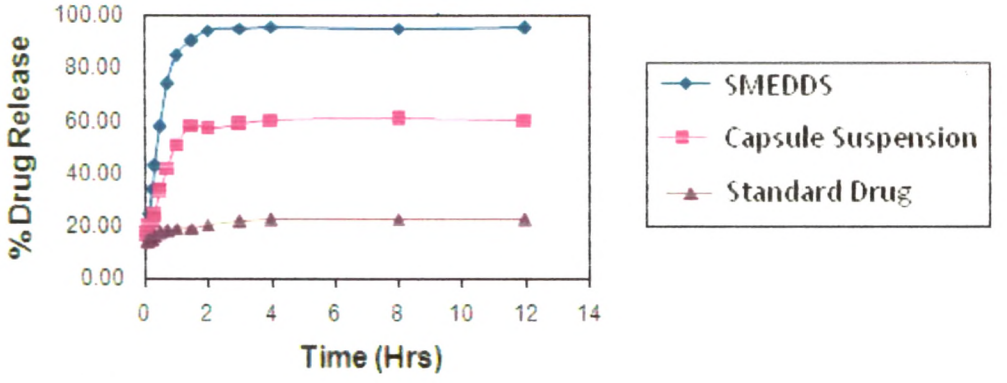


Fig. 4.5.2 Comparative *In vitro* Release Study (Intestinal Permeability Method)

This might lead to reduced drug diffusion. Another factor affecting release may be the physiology of intestinal cell membrane. Surfactants are known to increase the permeability by disturbing the cell membrane (25). The main rate-limiting barrier for the drug absorption/diffusion is the single layer of intestinal epithelial cells that covers the luminal surface of the intestinal wall. In addition, the pre-epithelial, unstirred, aqueous layer presents a barrier to hinder the poorly soluble drugs from reaching the absorption site (26). For the majority of drugs, absorption occurs via passive transcellular transport and the paracellular transport is limited due to the tight junctions between the cells (27). The data of permeability study supports the above statement and hence reduction in diffusion might be observed as compared to dialysis bag method.

In spite of variable release data from two diffusion studies, one fact is evident from both methods that valsartan SMEDDS showed best release as compared to other two formulations.

4.6 BIOAVAILABILITY STUDY

4.6.1 Experimental

Animal study was performed as per the approval of animal ethics committee, M. S. University of Baroda in accordance with Institutional Animal Ethics Committee (IAEC). Bioavailability of valsartan SMEDDS was compared with suspension of marketed valsartan capsule (Valent-80, Lupin Ltd., Deharadun, India). Valsartan suspension was prepared by milling powder from capsule with a small amount of 2.5% (v/v) hydroxymethylcellulose (15 cP, S.D. Fine Chemicals, Mumbai, India) solution and diluted to a definite volume using the same vehicle afterwards. Six rabbits were allocated at random to two treatment groups and administered SMEDDS and valsartan suspension solution in a crossover design. The washout period between the two treatments was 7 days. Male rabbits (weighing approximately 1.7 ± 0.3) kg was fasted for 12 hrs prior to the experiment and water was available ad lib. After oral administration of valsartan dose (5.6 mg/1.5 kg b.w.), about 2 mL of blood sample were collected through retro-orbital plexus into heparinized tubes at 0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 12, and 24 hr. Blood samples were centrifuged at 5000 rpm for 10 min using a high speed centrifuging machine and plasma samples were withdrawn and stored at -18°C.

4.6.1 Result and Discussion

The linear trapezoidal rule was used to calculate the area under curve ($AUC_{0\rightarrow t}$). Relative bioavailability was calculated using following formulae:

$$Relative\ BA(\%) = \frac{A \cup C_{test}}{A \cup C_{reference}} \times \frac{Dose_{reference}}{Dose_{test}}$$

Plasma concentration C_{max} and $AUC_{0\rightarrow t}$ are significantly increased for formulation A than those for the capsule suspension. T_{max} was found to be decreased for formulation A. T_{max} value for formulation A was found to be 1 which was 1.6 hr for capsule formulation. Relative bioavailability was also found to be increased 1.78 fold.

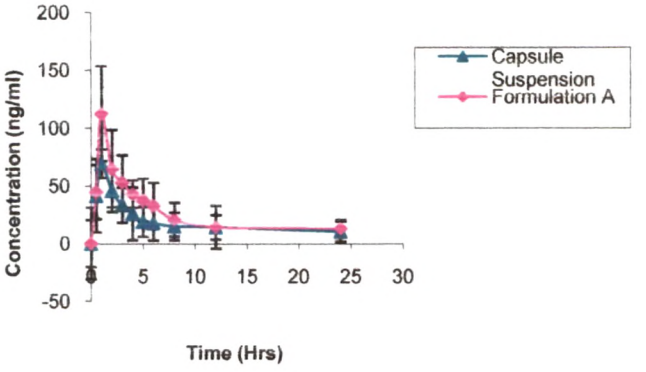


Fig 4.6.1 Plasma concentration profile of valsartan after oral administration of Formulation A (◆) and conventional capsule suspension (▲) in rabbit (n=6)

The non compartment model was used to evaluate pharmacokinetic parameters of valsartan absorption which are summarized in Table 4.6.1.

Parameters	**Formulation A**	**Capsule suspension**
t_{max} a(h)	1	1.36
C_{max} b(ng/ml)	112.61±9.13	69.24±3.98
$AUC_{0\rightarrow t}$ c(ng h/mL)	607.93	445.36
$AUC_{0\rightarrow \infty}$ d(ng h/mL)	1124.57	893.72
$AUMC_{0\rightarrow t}$ e(ng h/mL)	4752.96	3848.13
$AUMC_{0\rightarrow \infty}$ f(ng h/mL)	37933.75	33804.48
$MRT_{0\rightarrow \infty}$ g(h)	33.73	37.82
Relative bioavailability h(%)	178.70	-

a Time of peak concentration.

b Peak of maximum concentration.

c Area under the concentration time profile curve until last observation.

d Area under the concentration time profile curve extrapolated to infinity.

e Area under moment curve computed to the last observation.

f Area under moment curve extrapolated to infinity.

g Mean residence time.

h Relative bioavailability.

Table 4.6.1 Relative Bioavailability and pharmacokinetic parameter of valsartan after oral administration of VSMS A and conventional marketed formulation

The consistency in the intrinsic properties of drug may be contributing factor. Increased bioavailability of SMEDDS may be due to its lymphatic transport through transcellular pathway (28). It is also reported that the long-chain oils promote lipoprotein synthesis and subsequent lymphatic absorption (29). The main rate-limiting barrier for drug absorption/diffusion is the single layer of intestinal epithelial cell. High content of surfactants in SMEDDS could increase the permeability by disturbing the cell membrane (30). It should be noted that the surfactant with best enhancement ability requires both hydrophilic and lipophilic domains reaching a balance with intermediate values of HLB such as Tween 80 used in our study which consist of a polyoxyethylene and intermediate hydrocarbon chain. Its structural characteristics impart both lipophilic and hydrophilic properties to the surfactant, allowing it to partition between lipid and protein domains. Surfactant also demonstrated a reversible effect on the opening of tight junction of intestinal cell membrane; it may interact with the polar head groups of the lipid bilayers, modifying hydrogen bonding and ionic forces between these groups. It may also insert itself between the lipophilic tails of the bilayers, resulting in a disruption of the lipid-packing arrangement (31). In addition, *it vitro* studies indicated that Capmul MCM affected electrical and permeability properties of rabbit intestinal epithelium with the distal colon being more sensitive than ileum (20). On the basis of *in vitro* and *in vivo* correlation it can be noted that increase in release profile of valsartan from SMEDDS can lead to increase of bioavailability of valsartan. The study suggest that because of several factors like low particle size, optimized concentration and ratio of surfactant and cosurfactant, SMEDDS offers a promising approach to increase solubility and bioavailability of valsartan.

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