CHAPTER-7

Analytical Method Development of Naringenin

7.1 EXPERIMENTAL METHODS

7.1.1 Pre-formulation studies

The details of Pre-formulation studies were same as mentioned in ***Section 5.1.1***. The following studies were performed:

- Organoleptic characteristics and melting point of naringenin
- Analytical method development and validation of naringenin by UV-visible spectrophotometer
- Analytical method development and validation of naringenin by high performance liquid chromatography (HPLC)
- Solubility studies of naringenin

7.1.1.1 Organoleptic characteristics and melting point of naringenin (NAR)

The organoleptic characteristics and melting point of NAR was determined by the same method as described in ***Sub-section 5.1.1.1***. Briefly, small amount of NAR was incorporated in capillary tube and placed into the melting point apparatus and melting temperature of NAR was recorded from 247-255°C.

7.1.1.2 Analytical Method Development and Validation of NAR by UV-Visible Spectrophotometer

7.1.1.2.1 Selection of media

The selection of media was done on the basis of NAR solubility. Phosphate buffer solution (PBS) of pH 7.4 was selected for preparation of calibration curve.

7.1.1.2.2 Scanning for λ_{max}

10 mg of NAR was dissolved in little volume of PBS, pH 7.4 and finally diluted to 10 ml in volumetric flask to get a concentration of 100 µg/ml. This was treated as stock solution. Various aliquots of stock solution were diluted further to get different concentrations. Resultant solutions were scanned for λ_{max} in the ultra-violet range of 200-400 nm using UV-visible spectrophotometer.

7.1.1.2.3 Sample Preparation

Sample preparation of NAR was same as mentioned in ***Sub-section 5.1.1.2.3***. The absorbance of the resultant solutions was measured at 290 nm. Freshly prepared solutions were made for the calibration curve on three consecutive days.

7.1.1.2.4 Method Validation

The validation of an analytical procedure is same as described in ***Sub-section 5.1.1.2.4***.

7.1.1.2.4.1 Specificity

The specificity of an analytical procedure is same as mentioned in ***Sub-section 5.1.1.2.4.1***.

7.1.1.2.4.2 Linearity

The linearity of analytical procedure is same as mentioned in ***Sub-section 5.1.1.2.4.2***.

7.1.1.2.4.3 Range

The specified range is normally derived from the linearity studies. It was same as described in ***Sub-section 5.1.1.2.4.3***.

7.1.1.2.4.4 Accuracy and Precision

The accuracy and precision of the analytical procedure was same described in ***Sub-section 5.1.1.2.4.4***.

7.1.1.2.4.5 Limits of detection

The limit of detection (LOD) is the lowest amount or concentration of the analyte in a sample that is detectable, but not necessarily quantifiable. The LOD determination was same as described in ***Sub-section 5.1.1.2.4.5***.

7.1.1.2.4.6 Limits of quantitation

The limit of quantitation (LOQ) is the lowest amount or concentration of the analyte in a sample that can be determined. The precision expressed as the relative standard deviation of samples containing an analyte at the LOQ is usually 10%. The LOQ determination was same as described in ***Sub-section 5.1.1.2.4.6***.

7.1.1.3 Analytical method development and validation of NAR by High performance liquid chromatography (HPLC) in biological samples

7.1.1.3.1 Chromatographic systems and conditions

The HPLC system (Shimadzu Corporation, koyoto, Japan), equipped with UV-Visible detector (SPD-20A), a pump (LC-20AD), a degasser (DGU-$20A_3$) and a rheodyne manual injector (SIL-20A) attached with 20 µl sample loop. A C_{18} reverse-phase column (Phenomenex, 250mm×4.6mm, Particle size 5µ, Enable) and a C_{18} guard column were utilized for drug separation and quantification. The isocratic elution was performed at a flow rate of 1 ml/min with a mobile phase consist of methanol: acetonitrile (80:20) and water (HPLC grade) containing 2% (v/v) acetic acid at the ratio of 70:30. The mobile phase was filtered through 0.45µm nylon filter (Himedia, India) and degassed in ultrasonic bath for 10 min prior to use. The detection and quantification was performed by monitoring the elute at 288 nm wavelength.

7.1.1.3.2 Stock and working standard solutions

Stock standard solutions of NAR (1 mg/ml) and Apigenin (1 mg/ml; internal standard, IS) were prepared by dissolving 10 mg of drug in 10 ml of mobile phase containing (methanol: ACN, 80:20 and 0.02% BHT), respectively.

Different working standard solutions of NAR (concentrations ranging from 5 to 1000 ng/ml) were obtained by diluting the stock solutions with mobile phase. These solutions were protected from light and stored at -20°C between experiments, and it was found to be stable for at least 3 weeks (Kanaze *et al.,* 2004). The apigenin solution was subsequently diluted with mobile phase to make a working IS solution of 10 µg/ml.

Calibration standards for plasma were prepared daily for three days by spiking 90 µL of blank plasma with 10 µL of the appropriate working standard solutions resulting in concentrations of 5 to 1000 ng/ml (Ma *et al.,* 2007).

7.1.1.3.3 Plasma sample preparation procedures

A liquid-liquid extraction method was employed for extraction of drug from the plasma (Wan *et al.,* 2011). Plasma containing NAR and internal standard (Apigenin, 10 µg/ml) was thawed. 100 µl was transferred to a 1.5 ml eppendorf tube and 900 µl methanol: ACN was added to precipitate plasma proteins. This mixture was then vortexed for 5 min and centrifuged at 5000 rpm for 10 min. Supernatant was transferred to a glass tube and evaporated in a vacuum oven at 40°C. The residue was reconstituted in 100 µl mobile phase and 20 µl of reconstituted samples was injected onto the HPLC column for drug content after filtration through 0.2 µm syringe filter.

7.1.1.3.4 Recovery

The recoveries of NAR from plasma were determined by spiking an equal amount of the drug into the corresponding blank sample and methanol: ACN (BHT 0.02%) (solvent used in the final chromatographic step). The detailed procedure was same as mentioned in ***Sub-section 5.1.1.3.4***.

7.1.1.3.5 Linearity, precision and accuracy

Calibration curves of NAR in plasma were established over the concentration range: 5-1000 ng/ml. For construction of the calibration curves, six different calibration standards were prepared and processed for the range 5-

1000 ng/ml in plasma. The detailed procedure and their limits are same as mentioned in ***Sub-section 5.1.1.3.5***.

7.1.1.3.6 LOD and LOQ

LOD and LOQ are estimated from the signal-to-noise ratios (ICH Q2B, 1996) as described in ***Sub-section 5.1.1.3.6***.

7.1.1.4 Solubility studies of NAR

Saturation solubility was determined in buffer solutions of different pH by shake flask method (Aulton, 2007). The detail procedure is same as described in ***Section 5.1.1.4***.

7.2 RESULTS AND DISCUSSION

7.2.1 Organoleptic Characteristics

The pure NAR possesses white-crystalline powder, odorless and tasteless. The physical appearance of pure NAR was found to be crystalline solid. The melting point of pure NAR was found to be 254°C. It is noninflammable and light sensitive drug.

7.2.2 Analytical Method Development and Validation of NAR by UV-Visible Spectrophotometer

UV-visible spectrum of NAR was taken to determine the λ_{max} of NAR in PBS pH 7.4. The 100 µg/ml solution of NAR in the test medium were scanned for absorption maxima in the ultra-violet range of 200-400 nm, and are illustrated in Table 7.1 and Figure 7.1, respectively and therefore chosen for the drug estimation for further studies.

Table 7.1 Scanned λ_{max} and absorbance value of NAR prepared in PBS, pH 7.4

Strength (µg/ml)	Scanned λ_{max}	Absorbance
100	290	0.371

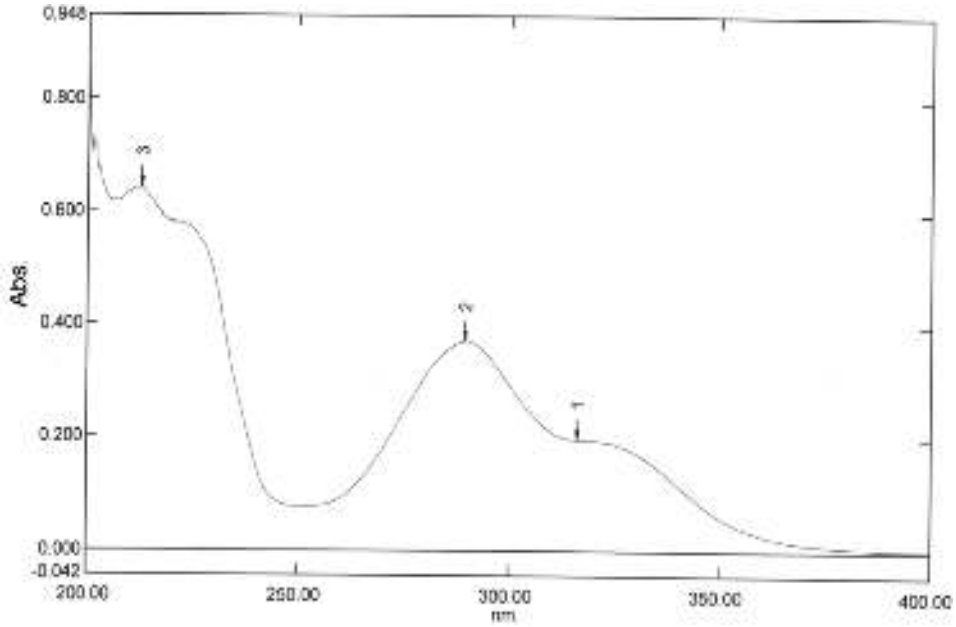

Figure 7.1 UV-Visible absorbance spectra of pure NAR in PBS, pH 7.4

Calibration curve of NAR were prepared in PBS, pH 7.4 on three consecutive days at λ_{max} 290nm. The absorbance values (mean of three determinations) with their standard deviations at different concentration in the range of 1-6 µg/ml is shown in Table 7.2 and their linear plot is presented in Figure 7.2. Drug was found to obey Beer-Lambert's law in the concentration range of 1-6 µg/ml with regression coefficient (R^2) values 0.999. The regression equation was found to be y = 0.155X + 0.001 in PBS, pH 7.4.

Table 7.2 Absorbance for plot of calibration curve of NAR in PBS, pH 7.4

S. No.	Concentration (µg/ml)	Absorbance (mean±SD; n=3)
1	0	0.000 ± 0.000
2	1	0.151 ± 0.012
3	2	0.312 ± 0.021
4	3	0.475 ± 0.032
5	4	0.625 ± 0.038
6	5	0.779 ± 0.045
7	6	0.928 ± 0.056

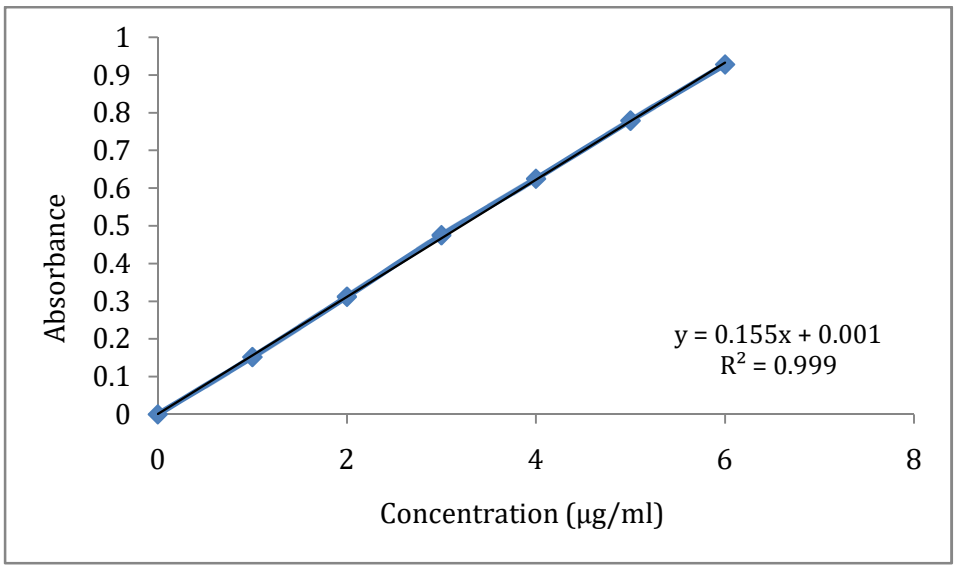

Figure 7.2 Calibration curve of NAR in PBS, pH 7.4.

Recovery of NAR from PBS, pH 7.4 was determined by spiking an equal amount of the drug into the corresponding known amounts of pre-analyzed standard solutions of NAR and subjected for UV-visible spectrophotometery at concentrations of 2, 4 and 6 µg/ml using three replicates at each concentration. The results of recovery studies are summarized in Table 7.3. The data proved the suitability of this recovery method for the analysis of PBS, pH 7.4 samples.

Table 7.3 Recovery studies of NAR from PBS, pH 7.4

S.No.	Concentration (µg/ml)	Concentration recovered (µg/ml) (mean±SD; n=3)	Recovery (%) (mean±SD; n=3)	RSD (%)	Bias (%)
1	2	2.08±0.067	104.00±4.525	4.35	3.84
2	4	4.01±0.123	100.25±6.623	6.06	0.25
3	6	6.11±0.362	101.84±5.224	5.13	1.80

For intra-day and inter-day precision study, calibration curves prepared in PBS, pH 7.4 were run in triplicate on same day for three days as well as three consecutive days, respectively and Percentage Relative Standard Deviation (%R.S.D.) calculated for all the cases. The intra-day variability of the assay

method (%R.S.D.) for NAR in PBS, pH 7.4 were found to be less than 9.15% and all data are shown in Table 7.4 whereas, corresponding inter-day variability were found to be less than 8.07%, and all data are summarized in Table 7.5. The data indicate that the assay method is reproducible within the same day and within different days; %R.S.D. are less than 15% for all sample types over the concentration ranges assayed (Shah *et al.,* 1992).

Table 7.4 The Intra-day precision of the method

Day 1	**Intra-Day**			
S. No.	**Expected Concentration (µg/ml)**	**Measured Concentration (µg/ml) (mean±SD; n=3)**	**% R.S.D.**	**% Bias**
1	0	0.000 ± 0.000	0.00	0.00
2	1	0.919 ± 0.044	4.78	-8.10
3	2	2.213 ± 0.142	6.41	10.65
4	3	3.022 ± 0.266	8.80	0.74
5	4	4.122 ± 0.377	9.15	3.05
6	5	5.088 ± 0.292	5.74	1.76
7	6	6.209 ± 0.369	5.94	3.48
Day 2				
1	0	0.000 ± 0.000	0.00	0.00
2	1	1.002 ± 0.042	4.19	0.20
3	2	1.915 ± 0.106	5.53	-4.25
4	3	2.932 ± 0.132	4.50	-2.26
5	4	4.089 ± 0.311	7.61	2.22
6	5	4.984 ± 0.345	6.92	-0.32
7	6	6.122 ± 0.411	6.71	2.04
Day 3				
1	0	0.000 ± 0.000	0.00	0.00
2	1	1.015 ± 0.056	5.51	1.50
3	2	1.965 ± 0.084	4.27	-1.75
4	3	3.045 ± 0.207	6.79	1.50
5	4	4.201 ± 0.356	8.47	5.02
6	5	5.111 ± 0.422	8.25	2.22
7	6	6.025 ± 0.388	6.44	0.42

Table 7.5 The Inter-day precision of the method

Day 1-3	Inter-Day			
S. No.	**Expected Concentration (µg/ml)**	**Measured Concentration (µg/ml) (mean±SD; n=3)**	**% R.S.D.**	**% Bias**
1	0	0.000 ± 0.000	0.00	0.00
2	1	1.022 ± 0.051	4.99	2.20
3	2	1.992 ± 0.086	4.32	-0.40
4	3	3.165 ± 0.141	4.45	5.50
5	4	4.122 ± 0.239	5.79	3.05
6	5	4.991 ± 0.403	8.07	-0.18
7	6	6.095 ± 0.422	6.92	1.58

Linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Data from the regression line is helpful to provide mathematical estimates of the degree of linearity. Table 7.6 shows the linearity and range data for calibration curves prepared in PBS, pH 7.4.

LOD and LOQ of calibration curve were calculated which was based on the standard deviation of y-intercept of regression line (SD) and the slope (S) of the calibration curve. The LOD and LOQ values are shows in the Table 7.6.

Table 7.6 Other validation parameters for calibration curve prepared in PBS, pH 7.4

Parameters	Values
Linearity correlation coefficient	0.999
y- intercept (SD)	0.019446
Slope	0.155
Range	1-6 µg/ml
LOD	0.414 µg/ml
LOQ	1.254 µg/ml

The developed method of estimation of NAR using UV spectrophotometry technique is sufficiently rapid, simple, sensitive, precise as well as accurate that complies with FDA guidelines. The linearity, accuracy, precision, limit of detection and quantification and specificity-selectivity of the method were established. In addition to analysis of drug during assay and release studies, this rapid and reproducible analytical method was successfully used for analysis of drug-excipient compatibility in a newly developed formulation in our laboratory and also for their stability studies.

7.2.3 Analytical method development and validation of NAR by HPLC in biological samples

7.2.3.1 Chromatography spectrum

The selectivity of HPLC analysis was studied by analyzing plasma samples, which did not show any interfering components. A typical chromatogram of a drug-free plasma sample spiked with NAR and IS is shown in Figure 7.3. Separation of NAR and IS was achieved successfully. The retention times of NAR and IS were approximately 4.868 and 3.292 min, respectively.

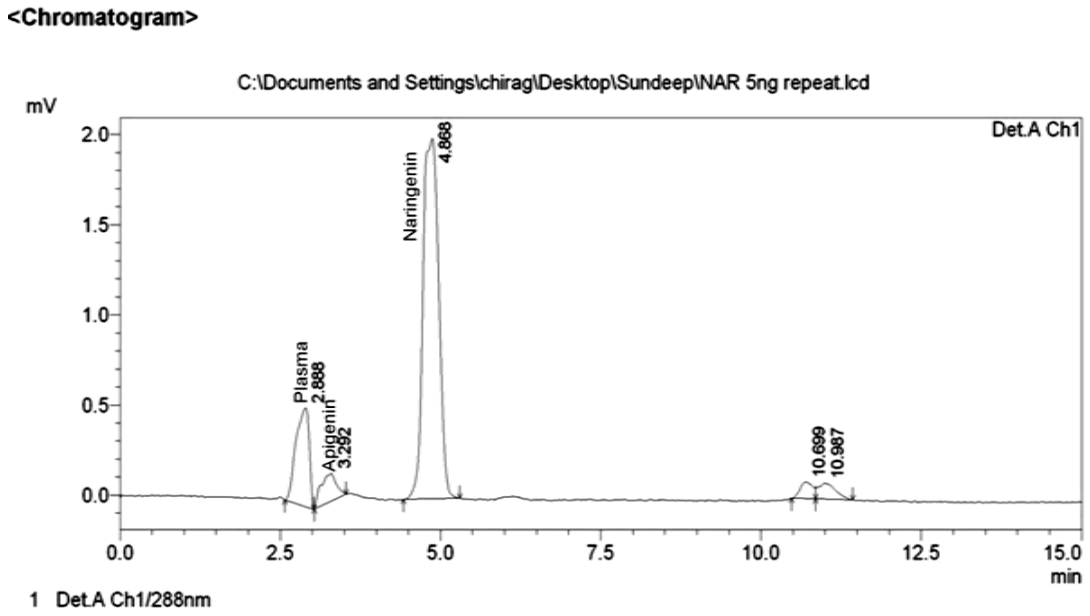

Figure 7.3 Typical HPLC chromatogram of blank plasma and blank plasma spiked with NAR and IS.

7.2.3.2 Extraction Efficiency

The absolute recovery of NAR from plasma was determined by comparing peak area obtained from extracts of spiked plasma samples with that obtained from the direct injection of known amounts of standard solutions of NAR. Assessed at concentration of 10, 200 and 1000 ng/ml using six replicates at each concentration, the recoveries were 93.87, 96.80 and 94.17%, respectively as shown in Table 7.7. The data proved the suitability of this extraction method for the analysis of plasma samples.

Table 7.7 Recovery of NAR from rat plasma

S. No.	Concentration (ng/ml)	Recovery (%) (mean±SEM; n=6)	RSD (%)
1	10	96.283±1.455	1.511
2	200	97.418±1.207	1.239
3	1000	94.502±0.639	0.676

7.2.3.3 Precision and Accuracy

Table 7.8 shows a summary of intra-day and inter-day precision and accuracy for NAR in rat plasma. The intra-day and inter-day of NAR ranged from 91.83 to 109.84% over a wide range of NAR concentrations with precision (%CV) less than 7.481 and 2.067%, respectively. Precision and accuracy studies indicated that the developed HPLC method is reproducible, accurate and therefore applicable to the pharmacokinetic studies of NAR in rats.

Table 7.8 The intra-day (n=6) and inter-day (n=6) precision of the method

	Intra-day			Inter-day		
Expected Concentration (ng/ml)	(mean±SEM; n=6) (ng/ml)	%CV	%Bias	(mean±SEM; n=6) (ng/ml)	%CV	%Bias
10	10.889±0.814	7.481	8.896	9.104±0.188	2.067	-8.966
200	203.694±1.155	0.567	1.847	201.156±0.823	0.409	0.578
1000	1006.317±1.058	0.105	0.631	996.06±2.023	0.203	-0.394

7.2.3.4 Linearity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentrations of analyte in the sample. The linearity of the assay for the NAR was evaluated with a total of six calibration standards. The linear regression analysis of NAR was constructed by plotting the peak-area ratio of NAR to IS vs the concentration of NAR in ng/ml. The calibration curve was highly linear (R^2>0.999) within the range of 5-1000 ng/ml for NAR extracted from rat plasma samples as shown in Figure 7.4. Typical values for the regression parameters a (the slope) and b (the y-intercept) were calculated to be 0.004 and 0.0638, respectively.

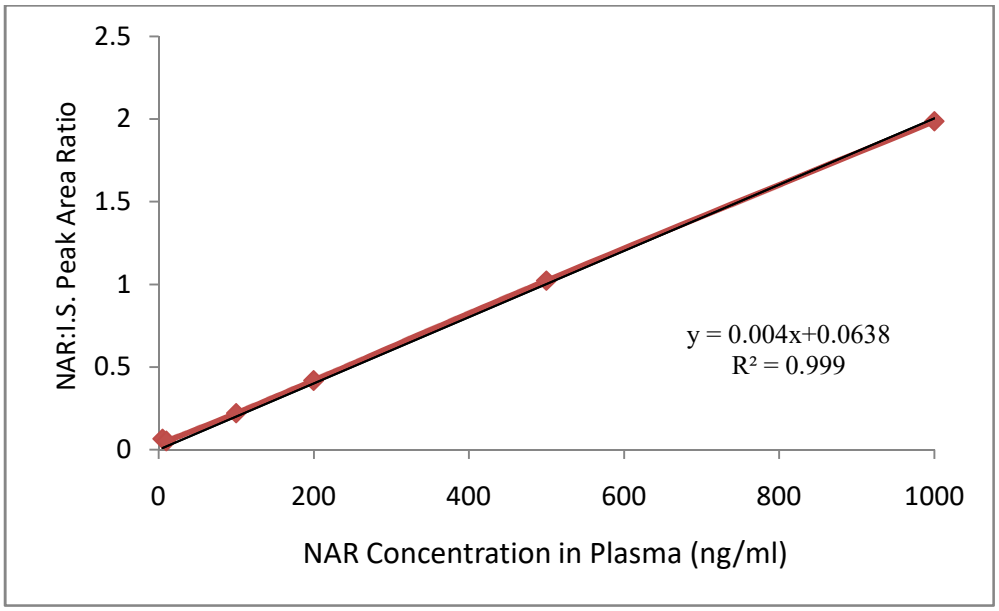

Figure 7.4 A representative standard curve for NAR in rat plasma extending from 5 to 1000 ng/ml.

7.2.3.5 LOD and LOQ

LOD and LOQ of calibration curve were calculated which was based on the standard deviation of y- intercept of regression line (SD) and the slope (S) of the calibration curve at levels approximating the LOD and LOQ, LOD = 3.3 (SD/S) and LOQ =10 (SD/S) (Chow & Shao, 2002). LOD and LOQ values of the assay method were found at 0.4629 ng/ml and 1.4027 ng/ml, respectively.

The described HPLC method is highly specific, sensitive and reproducible for the quantitative analysis of NAR in rat plasma samples. No interfering peak co-eluted with the NAR and IS were observed when rat plasma samples were analyzed. Good linearity, precision and accuracy for NAR proved the feasibility of this assay in the pharmacokinetic studies.

7.2.4 Solubility studies

The saturation solubility profile of pure NAR in different media at 25±2°C was determined. The solubility of pure NAR was found to be 0.8716±0.043, 0.9812±0.049, 1.9234±0.096 and 2.2412±0.112 µg/ml in water, acidic buffer (pH 1.2), phosphate buffer (pH 6.8) and PBS, pH 7.4, respectively as shown in Figure 7.5. The results proved that the pure NAR is able to provide a clear solution when dissolved in PBS, pH 7.4.

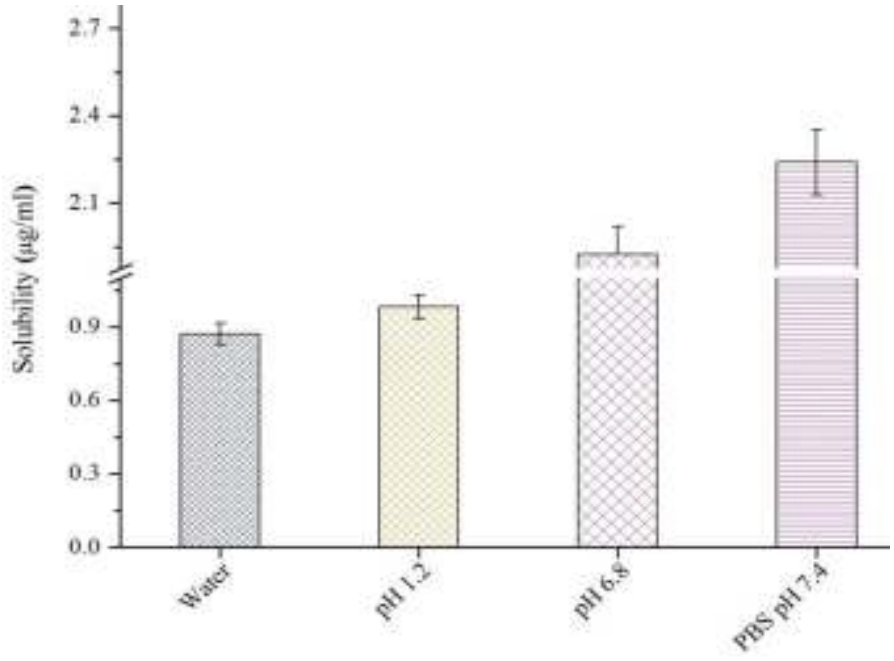

Figure 7.5 Solubility profile of pure NAR in different media at 25±2°C (Vertical bars represent ±SD; n=3).