Part-2
Chapter 1

Development of a validated UPLC method for estimation of all-trans retinoic acid and cholecalciferol (vitamin D₃).
1.0 INTRODUCTION

Administration of vitamins especially all-trans retinoic acid (ATRA) and cholecalciferol (vitamin D₃) has been considered beneficial for the treatment of tuberculosis (Martineau et al., 2011). ATRA increases the resistance of cultured human macrophages to infection by virulent strains of *M. tuberculosis* (Crowle and Ross, 1989) while tuberculosis patients are reported to be deficient in vitamin D₃ (Sasidharan et al., 2002; Zittermann, 2003).

ATRA acts synergistically with vitamin D₃ to inhibit *Mycobacterium* entry as well as survival within macrophages, possibly through the rescue of phagosome maturation arrest (Anand et al., 2008). As *Mycobacterium* is highly resistant and can also shift strains, treatment with antitubercular drugs may fail at times, whilst the host mediated macrophage directed pathway observed with ATRA and vitamin D₃ can be a gunshot treatment for tuberculosis.

Development of a suitable analytical method for precise determination of ATRA and vitamin D₃ in plasma is a challenge due to their high affinity with proteins (Blomhoff et al., 1991). The latter complemented with their poor bioavailability results in untraceable concentrations, post administration of high and repetitive doses of both ATRA and vitamin D₃. Various methods based on the use of protein based assays or high pressure liquid chromatography have been reported for the determination of these vitamins in human fluids. Most of these methods involve tedious extraction procedures, gradient flow, long elution times and often a purification step prior to their estimation (Clements et al., 1982; Dabek et al., 1981; Gilberston and Stryd, 1977; Gueguen et al., 2002; Jones, 1978). Moreover several of these methods utilize solid phase extraction processes (Kao and Heser, 1984; Kohl and Schaefer, 1981) involving high processing cost. Furthermore there are limited reports on the simultaneous quantification of ATRA and vitamin D₃ in plasma (Qian and Sheng, 1998) demonstrated an optimized HPLC method for simultaneous determination of vitamin A, D and mixture of other vitamins in the animal feed. A rapid and quantitative determination of fat soluble vitamins (Paliakov et al., 2009) is also
reported, however the report did not include the estimation of vitamin D₃. Quantification of retinal, retinol and retinyl esters in serum and tissues using gradient elution by HPLC/UV is also reported, but with a retention time of more than 20 min, thus making the process time consuming and also expensive in terms of large volumes of solvents used (Kane et al., 2008).

Our intent was to develop a sensitive, reproducible, and accurate ultra-performance liquid chromatography (UPLC) method involving liquid-liquid extraction (LLE) for simultaneous determination of ATRA and vitamin D₃ in plasma.

2.0 MATERIALS AND METHODS

2.1 Materials

ATRA (purity: 99.80%; 1 mg=3333.3 IU) was purchased from Sigma Aldrich, India Ltd. and cholecalciferol (vitamin D₃) (purity: 99.90%; 1 mg=40,000 IU) was obtained as a generous gift from Fermenta Biotech, Kullu, Himachal Pradesh, India. HPLC grade acetonitrile and methanol, used in the preparation of mobile phase were purchased from Merck KGaA, Darmstadt, Germany. All other chemicals and reagents were of analytical grade and were used without further purification.

2.2 UPLC Analysis

Measurement of ATRA and vitamin D₃ was achieved using UPLC (Acquity, Waters Corp., U.S) with an Acquity UPLC BEH RP18 column (2.1 x 50 mm, 1.7 µm), maintained at 35°C. The mobile phase comprised of a mixture of acetonitrile: methanol: water (90:8:2 v/v/v %) at a flow rate of 0.20 ml/min. Complete separation of peaks for both the vitamins was achieved within a run time of 5 min.

The column was washed with 100% acetonitrile for 2 min at 0.50 ml/min and re-equilibrated with the mobile phase for 1 min before injecting the samples. The eluant was monitored at 351 nm and 265 nm for ATRA and vitamin D₃, respectively, using an Acquity UV detector (Waters Corp.).
2.3 Animals

Wistar male rats weighing 200–300 g were used for the study. Animals were acclimatized to laboratory conditions for a week before the start of the experiment. The protocol was duly approved by the Institutional Animal Ethics Committee (IAEC) of Panjab University, Chandigarh, India (letter No. CAH/131 dated 09.08.2010). For the validation protocol, blank plasma samples were used. Blood was withdrawn, from retro-orbital plexus of naïve rats (n= 10), transferred into heparinised vials and centrifuged at 1677 g for 10 min to obtain clear plasma which was distributed in plastic tubes, sealed tightly and stored frozen at a temperature of −20°C, till analysis.

2.4 Stock solutions

Concentrated stock solutions of ATRA and vitamin D₃ were prepared by dissolving 10 mg of each in 100 ml of chloroform : methanol (1:1) v/v (solvent I) and methanol (solvent II) to give stock solutions containing 100 μg of each vitamin per ml of respective solvent. Solvent I was used subsequently for the estimation of total drug content and entrapment efficiency of the prepared SLNs while methanol was employed for pharmacokinetic studies in rat plasma.

2.5 Preparation of calibration curve (CC) in chloroform: methanol (1:1)

A nine point CC was prepared by adding 0.45 ml of chloroform: methanol (1:1 v/v) to 25 μl each of the appropriate working dilution of ATRA and vitamin D₃, respectively, to result in the final concentrations of 1, 5, 25, 50, 100, 250, 500, 1000, and 5000 ng of each vitamin/ml. A plot for the resulting peak area for each vitamin against respective concentrations was plotted.

2.6 Preparation of the CC in plasma and quality control (QC) samples

A nine point CC was prepared as explained above by spiking 0.45 ml of blank plasma with 25 μl each of the appropriate working dilution of ATRA and vitamin D₃ in methanol.

High quality control (HQC: 4000 ng/ml), medium quality control (MQC: 2000 ng/ml) and low quality control (LQC: 75 ng/ml) samples were also prepared similarly. All
solutions were stored in the refrigerator at 5.0 ± 3.0°C. The bulk spiked CC and QC plasma samples were stored at -20°C.

2.7 Plasma sample preparation

Both the vitamins were extracted from the spiked rat plasma samples by LLE in a polypropylene tube. To the spiked plasma samples (section 2.6) and the blank rat plasma samples, 400µl of chilled methanol and 2 ml of n-hexane: ethyl acetate (1:1) was added and vortexed for 1 min. Resulting mixture was kept at -20 °C for 5 min and cold centrifuged at 1677 g for 10 min. Supernatant was decanted and another 2 ml of n-hexane: ethyl acetate (1:1) was added to the pellet, followed by vortexing (1 min) and extraction, as above, to achieve complete recovery of these vitamins. Combined supernatants were dried by purging nitrogen gas through them (Turbovap, Biotage India). Utmost care was taken to perform these steps in dim light, complemented with the use of amber colored containers or containers covered with aluminium foil to minimize light exposure. Dry samples were reconstituted with 250µl of acetonitrile, transferred to amber colored autoinjector vial and analyzed using UPLC (injected volume= 4 µl).

2.8 Method validation

2.8.1 System suitability

System suitability was performed by determining the AUC for the MQC (without spiking into plasma) sample injected into the UPLC before the start of each analytical run and its comparison with the average AUC value obtained for the MQC, upon repetitive injections (n=7).

2.8.2 Specificity

Blank plasma samples and methanol (n=6) were prepared according to the sample preparation procedure described above and screened for the presence of any interfering peaks corresponding to the retention time of either ATRA or vitamin D₃.
2.8.3 Sensitivity

The limit of quantification (LOQ) was taken as the lowest concentration in the standard curve with accuracy between 80-120% and limit of detection (LOD) was determined at a signal to noise ratio (S/N) of 3.

2.8.4 Recovery

To determine recovery, the LLE efficiency was calculated by comparing the peak areas obtained after extracting plasma standards with areas obtained for corresponding stock standards. The study was accomplished at three concentration levels (75, 2000, and 4000 ng/ml) for both ATRA and vitamin D₃ (n=6).

2.8.5 Inter-day and intra-day precision and accuracy

Inter-day and intra-day precision and accuracy were evaluated by spiking known amounts of ATRA and vitamin D₃ in plasma. The precision was expressed as % CV (coefficient of variation) and % accuracy was expressed by using the formula: measured concentration/nominal concentration x 100.

Three different concentrations (LQC, MQC and HQC) were used and samples were prepared as per the procedures described above. Inter-day precision and accuracy were assessed over a period of 3 days using replicate (n=6) determinations for the spiked plasma sample, whereas intra-day precision and accuracy were assessed on three separate occasions on the same day (n=6) for each concentration, respectively.

2.9 Stability at various storage conditions

2.9.1 Processed sample stability

In assessing the processed sample stability, the LQC and HQC samples were quantified 6 hours after they were first analyzed (0 time reading). Samples were stored at room temperature during this period.
2.9.2 Autoinjector stability
The processed replicates of LQC and HQC samples were stored in the autoinjector at 4°C after analysis and were re-injected after 24 hours. Stability of QC samples was determined in percentage by comparing the peak area values at 24 hours against the values obtained for the same samples when injected fresh (zero time).

2.9.3 Long-term stability
Long-term stability of LQC and HQC samples was determined after storing these samples for 5 days at -20°C. The QC samples were quantified against the readings of the same samples at zero time.

3.0 RESULTS
3.1 Method validation
3.1.1 System suitability
The system was found to be suitable for the determination of ATRA and vitamin D₃ under the optimized chromatographic conditions. Average peak area per injection (n=7) of MQC level was determined, and its RSD was found to be ≤3.1 %. As per USFDA bio-analytical method validation guidelines, 2001, the accuracy for the developed analytical procedure should be high and its RSD should be less than 5%.

3.1.2 Specificity
High specificity of the developed method was confirmed by the absence of any interfering peaks at the retention time of respective vitamins. Further, chromatograms obtained from spiked plasma samples were found to be specific for ATRA and vitamin D₃ as shown in Figure 1.
Figure 1: Representative chromatograms of
A) Standard ATRA (100ng/ml) in methanol (USP tailing 0.8, Resolution: 2.84)
B) ATRA spiked plasma (USP tailing: 1.08, Resolution: 2.56)
C) Blank plasma (USP tailing 0.9, Resolution: 2.40); baseline ATRA levels in rat plasma = 56.12 ng/ml
D) Cholecalciferol in methanol (USP tailing 0.8, Resolution: 2.88)
E) Cholecalciferol spiked plasma (USP tailing 0.9, Resolution: 2.67)
F) Blank Plasma (USP tailing 0.8, Resolution: 2.13); baseline vitamin D₃ levels in rat plasma = 10.83 ng/ml

λ_max for ATRA samples was 351 nm and for vitamin D₃ it was 265 nm. Retention time (RT) of various samples is indicated in the respective chromatograms.
3.1.3 Sensitivity
LOQ and LOD values for both ATRA and vitamin D3 were found to be similar and were determined as 1ng/ml and 0.5 ng/ml, respectively.

3.1.4 Recovery
Recovery (n=6) for ATRA was found to be 96.5± 1.4%, 98.1± 1.1% and 96.0± 2.4% for LQC, MQC, HQC samples, respectively. Similarly high recovery of 89.4±1.8%, 88.9± 2.3% and 87.8± 2.1%, respectively was observed for LQC, MQC and HQC samples of vitamin D3.

3.1.5 Intra and inter-day precision and accuracy
The intra-day accuracy for ATRA was found to between the 95.4-99.9% and for vitamin D3 it was in the range of 98.5-100.8% in rat plasma samples with RSD less than 2.5% and 3.1%, respectively, for the QC samples.

The inter-day accuracy of ATRA and vitamin D3 in rat plasma samples ranged from 95.3% to 101.1% and 99.3 to 101.7% respectively for the QC samples with RSD value less than 2.1% and 2.8 %, respectively (Table 1), confirming high precision of the developed method.

Table 1: Intra and inter-day precision and accuracy of ATRA and vitamin D3 in rat plasma.

<table>
<thead>
<tr>
<th>Nominal Concentration (ng/ml)</th>
<th>Observed concentrations (ng/ml)</th>
<th>% Precision</th>
<th>% Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRA-DAY (n=6)</td>
<td>ATRA</td>
<td>Vit.D3</td>
<td>ATRA</td>
</tr>
<tr>
<td>LQC (75)</td>
<td>71.6±1.8</td>
<td>73.9±2.3</td>
<td>2.5</td>
</tr>
<tr>
<td>MQC (2000)</td>
<td>1999.7±14.9</td>
<td>2012.3±16.5</td>
<td>0.7</td>
</tr>
<tr>
<td>HQC (4000)</td>
<td>3990.4±21.3</td>
<td>4035.7±25.1</td>
<td>0.5</td>
</tr>
<tr>
<td>INTER-DAY (n=6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LQC (75)</td>
<td>71.5±1.5</td>
<td>74.4±2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>MQC (2000)</td>
<td>1952.9±11.9</td>
<td>2034.2±15.7</td>
<td>0.6</td>
</tr>
<tr>
<td>HQC (4000)</td>
<td>4046.4±24.5</td>
<td>3996.2±27.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>
3.1.6 Linearity
The calibration curve for both the vitamins was linear ($r^2 =0.999$) in chloroform:methanol (1:1 v/v) and in plasma at the concentration range of 1-5000ng/ml.

3.2 Stability at various storage conditions
Methanolic solutions of ATRA and vitamin D$_3$ were found to be stable for up to 5 days at -20°C, with their mean % stability ranging between 98.6%- 99.5% and 94.5%- 90.4%, respectively. The acceptance criterion for % accuracy for stability samples is ±15%, thus the values are sufficiently within the limits. Solutions of both the agents were again found to be stable (Table 2) for up to 24 hours in the autosampler at 4°C and room temperature for 6h, as per the acceptance criteria of USFDA bio-analytical method validation guidelines, 2001 (Table 2).

Table 2: Stability of ATRA and vitamin D$_3$ under various storage conditions (n=6).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>LQC (75 ng/ml)</th>
<th>HQC (4000 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Accuracy</td>
<td>% Accuracy</td>
</tr>
<tr>
<td>Long term solution stability at -20°C for 5 days</td>
<td>ATRA</td>
<td>Vit. D$_3$</td>
</tr>
<tr>
<td></td>
<td>98.6± 1.4</td>
<td>94.5 ± 2.1</td>
</tr>
<tr>
<td>Processed sample stability for 6 h</td>
<td>103.9±2.4</td>
<td>97.6± 2.0</td>
</tr>
<tr>
<td>Autosampler stability for 24 h</td>
<td>96.5± 1.4</td>
<td>112.0±1.2</td>
</tr>
</tbody>
</table>

4.0 DISCUSSION
A UPLC method for estimation of ATRA and vitamin D$_3$ in rat plasma was developed in the present investigation and validated according to the principles of good laboratory practices. With the advent of the expected usefulness of ATRA and vitamin D$_3$ in the therapeutic regime of tuberculosis (Nonnecke et al., 2003) their quantification in biological matrix in conjunction with ascertaining the stability of samples during extraction and analysis is an important assignment. Physicochemical instability due to rapid oxidation and decomposition in the presence of light observed with both vitamins, poses a serious challenge for the
development of a suitable analytical method with minimal degradation during processing and storage of samples. Although the literature database is replete with validated methods using sophisticated analytical equipments such as HPLC, LCMS/MS, and HPLC-MS/MS method, however use of the UPLC method for the quantification of ATRA and vitamin D₃ in plasma has been investigated only to a limited extent.

Paliakov et al (2009) reported on a rapid quantitative determination of fat-soluble vitamins (vitamin A, α and β tocopherol, β- carotene, and coenzyme Q-10, vitamin K) in human serum by reverse phase UPLC (with UV detection) with a flow rate of 1.25 ml/min and a gradient flow (Paliakov et al., 2009). Mobile phase used by them, constituted of two phases, phase A (acetonitrile: de-ionized water :: 90:10) and phase B (methanol: 2 propanol :: 70:30). Kane et al (2008) also reported a HPLC/UV quantification of retinal, retinol, and retinyl esters in serum and tissues with more than 30 min of run time and a gradient elution (Kane et al., 2008). Howsoever, both the methods do not claim their suitability for the determination of vitamin D₃. Some reports on the analysis of these molecules using HPLC-MS/MS and LC/MS/MS have also been listed. Latter involves various limitations including cost of the equipment and complicated extraction procedures, apart from the need of expert staff for running the equipment, and time and labour for method development.

Presently, we have demonstrated the simultaneous estimation of ATRA and vitamin D₃ in chloroform: methanol (1:1 v/v) (for evaluating TDC and EE of the developed SLNs) and in plasma (for subsequent pharmacokinetic studies) using UPLC. The method was developed for simultaneous estimation of ATRA and vitamin D₃ in plasma, since concurrent administration of both the vitamins can help in the prophylaxis of tuberculosis (Anand et al., 2008). Method will be applied to determine stability and photostability of these molecules, in free form and when entrapped into SLNs. Suitability of the method for determining pharmacokinetic parameters in rat plasma, upon oral and subcutaneous administration of both the vitamins to rats was also established (chapter 2 and 3).
Method for simultaneous analysis of both the vitamins, was optimized using Acquity UPLC on BEH RP18 column (2.1 x 50 mm, 1.7µm) maintained at 35°C. A number of mobile phase combinations were tried to resolve the chromatographic peaks of ATRA and vitamin D₃. Various ratios of some solvent systems which were employed initially, included: methanol: water at a v/v ratio of 70:30; 50:50; and 40:60. These systems were tested at various flow rates ranging from 0.1-0.6 but invariably resulted in broad peaks in the chromatograms. In an attempt to attain a selective and a sharp chromatographic peak, a combination of acetonitrile was tried with water at various concentrations viz acetonitrile: water, 30:70, 70:30, and 90:10 v/v, again at flow rates ranging from 0.1-0.6 ml/min. However, the observed chromatograms were again unsatisfactory and non-reproducible. Thereafter mobile phase comprising acetonitrile: methanol: 50:50 and 80:20, v/v, and finally acetonitrile: methanol: water 90:5:5 v/v at flow rates of 0.15 to 0.20 ml/min were tried and the latter system resulted in a sharp chromatographic peak with significant AUC. However, after repetitive injections the peaks tended to broaden in this mobile phase, which was fine tuned to acetonitrile: methanol: water (90:8:2 v/v/v %) with a flow rate of 0.20 ml/min. This produced sharp peaks, faster elution and sufficient AUC for both the vitamins within a run time of 5 min, with reproducible results. The retention time (RT) for ATRA and vitamin D₃ in this system was found to be 0.98 and 2.58 min, respectively.

ATRA and vitamin D₃ eluants were monitored at wavelength (λ max) of 351 nm and 265 nm, respectively, with an Acquity UV detector (Waters Corp.). The method was highly specific with sharp and selective chromatograms for both the analytes in spiked plasma samples. The column was washed with 100% acetonitrile for 2 min at 0.50 ml/min before re-equilibration for 1 minute, before and after each set of determinations.

The intra-day and inter day accuracy of ATRA and vitamin D₃ in rat plasma samples was sufficiently high with a small RSD (Table 1) confirming high precision of the developed method. Stability of the ATRA and vitamin D₃ samples
were studied under various storage conditions and respective samples were found to be stable (Table 2). The validated method was linear ($r^2$ values = 0.999) over a sufficient range of concentrations both in chloroform: methanol (1:1 v/v) and in plasma. The LOD for ATRA and vitamin D$_3$ by the developed method was significantly low, at 0.5ng/ml. It may be noted that as per the US FDA guidelines of May 2001, it is stated that the recovery of the analyte need to be consistent, precise, and reproducible. The method developed and reported by us showed good recovery (> 87%) for both the vitamins, from spiked plasma samples at three concentration levels and may be considered suitable taking into account the consistency and reproducibility (n=6) of the results obtained upon repetitive evaluations.

5.0 CONCLUSIONS

A validated and highly sensitive and specific ultra-performance liquid chromatography technique for the quantization of ATRA and vitamin D$_3$ with LOD of 0.5ng/ml and linearity range from 1.0ng-5000.0ng/ml at $r^2$=0.999, was presently developed in rat plasma. The method will be used for in vitro characterization of solid lipid nanoparticles of ATRA and vitamin D$_3$ in terms of total drug content, entrapment efficiency and determination of stability including photostability of ATRA and vitamin D$_3$ loaded SLNs and their pharmacokinetic studies upon oral and subcutaneous administration, indicated for the treatment/prophylaxis of tuberculosis.
Chapter 2

Preparation, characterization and pharmacokinetic evaluation of all-trans retinoic acid loaded solid lipid nanoparticles (ATRA-SLNs).
1.0 INTRODUCTION
A variable and extremely low oral absorption of vitamin A (retinol/ all-trans retinoic acid) is reported and is attributed to its low solubility (Adamson et al., 1993; Hollander, 1980; Takitani et al., 2004). Furthermore it also shows a tendency to get readily oxidized in air and undergo photodegradation. Diverse strategies have been attempted to improve bioavailability, solubility and compromised stability of all-trans retinoic acid (ATRA), including its complexation with cyclodextrins (CDs) (Chen et al., 2011; Lin et al., 2000) or incorporation into SLNs, (Hu et al., 2004) microemulsions (Hwang et al., 2004) and micelles (Zuccari et al., 2005) and making structural modification in the parent molecule (Cui et al., 2009). The incorporation of ATRA in colloidal carriers such as liposomes (Douer et al., 2001; Ozpolat et al., 2003), for site specific drug delivery is also reported widely. Some authors also recommend administration of retinoids via intravenous route to resolve their poor aqueous solubility (el Mansouri et al., 1995). Use of ATRA loaded SLNs (ATRA-SLNs) for topical application (Jenning et al., 2000a; Jenning et al., 2000b) is also reported.

ATRA, has been demonstrated to be effective for the treatment of tuberculosis (Crowle and Ross, 1989). ATRA increases the resistance of cultured human macrophages to experimental infection with virulent *Mycobacterium tuberculosis* (Crowle and Ross, 1989). Deficiency of vitamin A in patients has been reported to increase their proneness to develop tuberculosis (Pakasi et al., 2009). Former has been attributed to a general loss of appetite, poor intestinal absorption, and increased urinary loss of vitamin A. Furthermore vitamin A deficiency lowers immunity, while its supplementation reduces TB induced morbidity and mortality (Semba, 1994). Ability of SLNs (including several reports from our group) for their ability to encapsulate both hydrophilic (Bhandari and Kaur, 2013b; Kumar et al., 2014a) and hydrophobic drug molecules (Kakkar et al., 2013b; Singh et al., 2013a) has been documented. Present chapter, explores the possibility of incorporating ATRA into SLNs to result in its improved biopharmaceutical performance after administration by subcutaneous and oral routes both in terms of improved stability and pharmacokinetics (in rats) using a validated ultra
performance liquid chromatography (UPLC) method of analysis (Kumar et al., 2014b). Such a system is proposed to be useful for the prophylaxis and treatment of tuberculosis (Kaur and Verma, 2012; 2013).

2.0 MATERIALS AND METHODS

2.1 Materials
ATRA and HPLC grade solvents as described in chapter 1. Soy lecithin (Hi Media, India), Tween 80 (S.D. Fine Chemicals Ltd., India), and Compritol® 888 ATO (Glycerylbehenate, gift sample from Gattefosse, USA) were used for preparing SLNs in the study. All other chemicals and reagents were of analytical grade and were used without further purification.

2.2 Methods

2.2.1 Preparation of ATRA loaded solid lipid nanoparticles (ATRA-SLNs) by microemulsification method
ATRA-SLNs were prepared using patented microemulsification method (Kaur and Verma, 2012; 2013). Briefly, the lipid (glyceryl behenate; 7.27%w/v) was melted at approximately 10°C above its melting temperature i.e. 82-85°C. Tween 80 (45.45% v/v), soy lecithin (0.58%w/v), and water were placed together in a beaker and heated to the lipid melt temperature. ATRA was added to the lipid phase, following which the hot aqueous emulsifier mix was dropped at once into the lipid melt, under magnetic stirring to obtain a clear microemulsion. The hot microemulsion thus formed, was transferred into an equivalent amount of cold water (~2°C) under continuous mechanical stirring for 2h to result in the formation of SLNs by crystallization of the lipid droplets present in the microemulsion. Prepared SLNs were stored in a refrigerator until further analysis.

All procedures were carried out under dimlight using amber colored containers or containers covered with aluminium foil to prevent photodegradation of ATRA.

2.2.2 UPLC analysis
As described earlier in chapter 1.

2.2.3 Physicochemical characterization of SLNs
2.2.3.1 Total drug content (TDC)
1 ml of ATRA-SLN dispersion was disrupted using an appropriate volume of chloroform: methanol (1:1) till a clear solution was obtained which was analyzed by UPLC.

2.2.3.2 Entrapment Efficiency (EE)

SLN dispersion was ultracentrifuged at 802,000 g for 2h at 4°C. Resulting pellet was dissolved using sufficient quantity of methanol:chloroform (1:1, v/v). Amount of drug in the pellet, determined using UPLC, gave a direct measure of the extent of drug entrapped; AUC value obtained for blank SLN dispersion treated in a similar manner was used as the control value to compensate for any interference of the ingredients.

Entrapment efficiency = \[
\frac{\text{Amount of drug entrapped} \times 100}{\text{Total drug incorporated as per TDC}}
\]

2.2.3.3 Particle size analysis

The mean diameter of nanolipidic particles in the dispersion (after 20 X dilution using triple distilled water) was determined by dynamic light scattering using Delsa Nano™, Beckman Coulter, USA.

2.2.3.4 TEM

Shape of the prepared ATRA-SLNs was examined by staining the samples with 2% phosphotungstic acid (PTA) in water, for 5 min, after which the excess PTA was removed. Stained samples were spread on a carbon coated copper grid and examined under TEM (H 100, Hitachi Ltd., Japan) at a voltage of 80 kv.

2.2.3.5 Differential scanning calorimetry (DSC)

DSC studies were performed for ATRA, physical mixture of Compritol® 888 ATO and ATRA, Compritol® 888 ATO and lyophilized SLN dispersion with a Perkin-Elmer (DSC 821-e, Mettler Toledo, Switzerland) differential scanning calorimeter. Samples were placed in conventional aluminum pans and heated from 10°C to 300°C at a scanning speed of 10°C/min.

2.2.3.6 Powder X-ray diffraction (PXRD)

The encapsulation of drug inside the SLNs was confirmed by X-ray diffraction measurements carried out with an X-ray diffractometer (XPRT-PRO,
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PANalytical, Netherlands). Studies were performed by exposing the samples to CuKα radiation (45 kV, 40 mA) and scanning from 5° to 50°, 2θ at a step size of 0.017° and scan step time of 25 s. Samples used for PXRD analysis were same as those of DSC analysis. The instrument measures interlayer spacing d which is calculated from the scattering angle 2θ, using Bragg’s equation 2d sin θ = nλ where λ is the wavelength of the incident X-ray beam and n is the order of the interference.

2.2.3.7 FTIR spectra analysis
The IR spectroscopy analysis of the ATRA, lipid and ATRA-SLNs was performed. The respective peaks obtained were compared for any significant changes.

2.2.3.8 In vitro release
Dialysis bag method was used to study the release of ATRA from SLNs using 0.01 M phosphate buffer (pH 7.4) + 3% tween 80, as the release medium. Tween 80 was added to increase the solubility of ATRA (reported solubility <0.01 mg/ml) in the dissolution media. The dialysis bag (12 kDa, Hi Media) was soaked in de-ionized water for 12 h before use. One milliliter (3.70 mg ATRA/ml) of SLN dispersion was placed in the dialysis bag which was tied tightly at both the ends. The bag was dipped in 100 ml of the release medium in a beaker, maintained at 37±0.5°C and stirred at a rate of 50 rpm. Release medium aliquots were withdrawn and replaced from time to time with an equal volume of fresh medium to maintain sink conditions. The samples were suitably diluted and analyzed for ATRA at 351 nm using UPLC.

2.3 Stability and photostability studies (PS) on ATRA-SLNs
ATRA-SLNs were stored in amber colored screw capped vials at 5±3°C for 1 year. Total drug content and the entrapment efficiency were determined at the end of 1 year. Photostability studies were performed using cool white fluorescent light to provide an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 watt hours/square, to the ATRA-S (free ATRA solubilised in 25 % w/v aqueous solution of tween 80) and ATRA-SLNs in clear and amber color glass vials, respectively, for 10 days at 25°C in a photostability chamber (Binder, Germany). TDC and EE (for SLNs only)
of the samples before and after exposure was measured using the developed UPLC method (Kumar et al., 2014b).

3.0 PHARMACOKINETIC STUDIES

In vivo pharmacokinetic studies were performed using male wistar rats weighing 250–300 g. The protocol was duly approved by the Institutional Animal Ethics Committee (IAEC) of Panjab University (letter No. CAH/131 dated 09.08.2010) Chandigarh, India. The animals were divided into four groups (n=5 per group). The single subcutaneous injection of 20 mg/kg body weight, of free ATRA (ATRA-S) solubilized in aqueous tween 80 (25%) and similar dose of ATRA loaded SLN dispersion (ATRA-SLNs; comprising both free/unentrapped and entrapped drug (84.6%)) respectively, was administered to two groups of rats. Similarly, single oral dose of 7.5 mg/kg body weight, of ATRA-S and similar dose of ATRA-SLNs was administered orally to another two groups of rats, respectively. Blood samples (1 ml) were withdrawn from retro-orbital plexus and, collected into heparinized microcentrifuge tubes (containing 20 ml of 1000 IU heparin/mL of blood) at 0.5h, 1h, 2h, 4h, 8h, 12h, 24h, and 48h post administration. Samples were analyzed using liquid-liquid extraction (LLE) in a polypropylene tube. To 0.50 ml of plasma samples, 400 \mu l of chilled methanol and 2 ml of n-hexane: ethyl acetate (1:1) was added and vortexed for 1 min. Resulting mixture was kept at -20 °C for 5 min and cold centrifuged at 1677 g for 10 min. Supernatant was decanted and another 2 ml of n-hexane: ethyl acetate (1:1) was added to the pellet, followed by vortexing (1 min) and extraction, as above, to achieve complete recovery of ATRA. Combined supernatants were dried by purging nitrogen gas (Turbovap, Biotage India). Utmost care was taken to perform these steps in dim light, complemented with the use of amber colored containers to minimize exposure to light. Dry samples were reconstituted with 250 \mu l of acetonitrile, transferred to amber colored autoinjector vials and analyzed using UPLC (injection volume= 4 \mu l).

3.1 Data analysis

The pharmacokinetic parameters were calculated based on a non-compartmental model. The area under the concentration–time curve from time zero to time t
(AUC_{0-t}) was calculated using the trapezoidal method. Peak concentration (C_{max}) and time of peak concentration (T_{max}) were obtained directly from the individual plasma concentration–time profiles. The area under the total plasma concentration–time curve from time zero to infinity was calculated by:

\[ \text{AUC}^{\infty}_{0-\infty} = \text{AUC}^{0-t} + \frac{C_t}{K_e}. \]

where \( C_t \) is the drug concentration observed at last time, and \( K_e \) is the apparent elimination rate constant obtained from the terminal slope of the individual plasma concentration–time curves after logarithmic transformation of the plasma concentration values and application of linear regression. The data obtained from pharmacokinetic parameters were analyzed statistically using WinNonlin software. Statistically significant differences were assumed at \( p \leq 0.05 \). All values are expressed as their mean ± S.D.

4.0 RESULTS

4.1 Physicochemical characterization of the ATRA-SLNs

The total drug content and entrapment efficiency of ATRA-SLNs were estimated to be 92.50± 2.10% and 84.60±3.20% (n=6), respectively. High values of TDC and EE indicate the efficiency of the method of preparation of SLNs and insignificant losses incurred during formulation of SLNs. Average particle size of ATRA-SLNs was found to be 131.3 ± 5.0 nm, with polydispersity index (P.I) of 0.283, and zeta potential of -17.45 mV (Figure 2 and 3) when measured using photon correlation spectroscopy. TEM studies (Figure 4) reveal rod shaped ATRA-SLNs. Most of the particles observed under TEM were of size less than 50 nm. The smaller size of nanoparticles observed under TEM (Figure. 4) in comparison to the particle size (131.3 nm) obtained using Delsa Nano™ may however be attributed to the fact, that the latter system is based on the principle of photon correlation spectroscopy (PCS), and electrophoretic light scattering which at times may not detect small particles, due to the higher brownian movement of these particles. TEM on the other hand, involves observation of only a small number of representative particles (in 2-3 fields) of a dilute dispersion such that the particle size observed under TEM may not at times match with results obtained by the PCS technology which gives population statistics.
Figure 2: Particle size analysis by Delsa Nano™
Figure 3: Zeta potential of ATRA-SLNs
Figure 4: TEM micrograph of ATRA-SLNs

4.2 DSC

DSC is a thermo analytical technique in which the difference in the amount of heat required to maintain the sample and reference at same temperature is measured as a function of temperature and time. It uses the fact that different lipid modifications possess different melting points and enthalpies.

In case of pure ATRA, the melting endotherm appeared at 187.34°C corresponding to its melting point at 180-189°C and was associated with 84.03
J/g of enthalpy, while Compritol® 888 ATO showed a sharp peak at 73.06°C and an enthalpy of 108.3 J/g. Physical mixture (Figure 5) showed distinct endothermic peaks corresponding to both Compritol® 888 ATO and ATRA. However, lesser intensity for peak corresponding to ATRA is attributed to the fact that the amount of ATRA present in the physical mixture was 10 times less than Compritol® 888 ATO. The ATRA-SLN dispersion however showed a broad endotherm (which it seems is a merger of several small peaks) starting from 63.81°C to 102.99°C with a low enthalpy of 13.43 J/g associated with the peak corresponding to the lipid at 73.06°C. Broadening of the peak indicates amorphous nature of ATRA-SLNs.

Further, the observation that enthalpy for the peak corresponding to the lipid is significantly lower than that of the pure lipid indicates the change in its polymorphic state from the crystalline β form to the amorphous (α, β') form with more imperfections in the crystal lattice. Latter will comfortably incorporate large amounts of drug within the lipid matrix as indicated by a high EE of 84.60% achieved presently.

![DSC thermograms](image)

**Figure 5:** DSC thermograms of ATRA (A), physical mixture of Compritol® 888 ATO and ATRA (AP), ATRA-SLNs (ASLN), and Compritol® 888 ATO (Compritol).
4.3 PXRD

PXRD patterns of ATRA, Compritol® 888 ATO, lyophilized blank SLN (B-SLN), and ATRA-SLN are shown in Figure 6A. PXRD pattern of ATRA exhibited sharp peaks at 2θ scatter angles 10.49, 14.64, 18.79, 23.43 and 29.51 which indicate its crystalline nature. PXRD pattern of Compritol® 888 ATO also showed sharp peaks at 2θ scatter angles of 21.16, 23.37, 23.52 and 35.76; again establishing its crystalline state. However, no characteristic peaks in lyophilized B-SLNs indicate the amorphous nature of lipid after transformation into SLNs. Typical pattern of peaks corresponding to those of free ATRA, were also found missing in the PXRD of lyophilized ATRA-SLNs, re-confirming the loss of crystallinity and a shift towards the amorphous state. Latter may also be taken to indicate that ATRA incorporated within the SLNs is now present in a soluble and hence a more bioavailable form (Figure 6B).

Figure 6A: P-XRD of a) ATRA, b) Compritol® 888 ATO, c) blank- SLNs, and d) ATRA-SLNs.
Figure 6 B  
a) Free drug dispersion of ATRA (ATRA-S) in water (solubility <10 μg/ml)  
b) ATRA presented as a fine disperse form (comparable to soluble form) containing 3.7 mg ATRA/ml (ATRA-SLNs)

4.4 FTIR spectroscopy
The IR peaks obtained with the developed formulation of ATRA-SLNs reveal an inter-molecular stretching of the –OH groups (3400-3200 cm⁻¹) (Figure 7), which is not apparent in the spectra for the ATRA and the lipid. Furthermore, the intensity of peak at 3445.5 cm⁻¹ in ATRA-SLNs is of a significantly high intensity than the similar peak observed for Compritol® 888 ATO at 3448.9 cm⁻¹. This may be regarded as an indication confirming formation of ATRA-SLNs.

Figure 7: FTIR spectrum of a) ATRA, b) Compritol® 888 ATO, and c) ATRA-SLNs.
4.5 In vitro release

In vitro release study of ATRA-SLNs using 0.01M phosphate buffer pH 7.4 + 3% tween 80 is shown in Figure 8. Tween 80 was added as the dissolution enhancer for ATRA. The free drug was released into the dissolution media within 2h while only 19.20% ATRA was released from ATRA-SLNs at 2h. ATRA-SLNs showed 61% release of ATRA at 8h and 100% release was observed at the end of 24 h. Of the various release models, Korsmeyer peppas model was best fitted to the data with n value of 0.66 (Table 3)

Values of n higher than 0.45 (0.45 < n < 0.89) indicate that the mechanism of mass transfer follow a non-fickian model (anomalous transport), where release is controlled by a combination of diffusion and lipid dissolution.

Figure 8: Invitro release of ATRA-S and ATRA-SLNs in 0.01M phosphate buffer (pH 7.4 )+ 3% tween 80.
Table 3: In vitro release kinetics of ATRA-SLN in 0.01M phosphate buffer (pH 7.4) + 3% tween 80.

<table>
<thead>
<tr>
<th>Time points (h)</th>
<th>Value of n</th>
<th>Mechanism of drug release</th>
<th>R²</th>
<th>Korsmeyer Peppas</th>
<th>Higuchi</th>
<th>First order</th>
<th>Zero order</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>n=0.66</td>
<td>Non fickian diffusion and erosion controlled</td>
<td>0.9503</td>
<td>0.9045</td>
<td>0.7852</td>
<td>0.9176</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td></td>
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4.6 Stability studies and photostability studies (PS)

After storage at 5±3°C for 1 year the ATRA-SLN dispersion showed an insignificant (p<0.05) change of <3.0% both in the values of TDC and EE. Storage of samples under cool white fluorescence light for 10 days (photostability studies) in amber colored and clear glass containers reduced the total drug content to 48.5% and 29.6% respectively for ATRA-S; while >97% and 85% ATRA remained when stored as ATRA-SLNs (Table 4).
Table 4: TDC and EE of ATRA-SLNs when stored in amber container at 4°C for 1 year and when exposed to UV light of 1.2 million lux h in amber and white containers, respectively.

<table>
<thead>
<tr>
<th>Stability studies</th>
<th>TDC</th>
<th>EE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATRA-SLNs (Amber) 0 month</td>
<td>92.5± 2.1%</td>
<td>84.6±3.2%</td>
</tr>
<tr>
<td>12 months</td>
<td>89.7±1.5%*</td>
<td>82.4±2.3%*</td>
</tr>
<tr>
<td>Photo stability studies</td>
<td>TDC</td>
<td>EE</td>
</tr>
<tr>
<td>ATRA-S (Amber)</td>
<td>48.5±2.1%</td>
<td>-</td>
</tr>
<tr>
<td>ATRA-S (Clear)</td>
<td>29.6±1.5%</td>
<td>-</td>
</tr>
<tr>
<td>ATRA-SLNs (Amber)</td>
<td>90.5±2.1%*</td>
<td>82.7±3.2%*</td>
</tr>
<tr>
<td>ATRA-SLNs (Clear)</td>
<td>79.3±3.6%*</td>
<td>81.2±2.2%*</td>
</tr>
</tbody>
</table>

*The values are insignificantly different (p<0.05) from the initial values of TDC and EE respectively, for the ATRA-SLNs at zero time.

4.7 Pharmacokinetic studies

The plasma ATRA concentration versus time plots after oral and subcutaneous administration of ATRA-SLNS is shown in Figure 9. At each time interval, the ATRA levels in plasma, after oral and subcutaneous administration of the ATRA-SLNs were significantly higher than those obtained for ATRA-S. Furthermore, the plasma concentration of ATRA in the ATRA-S group dropped to less than 20 ng/ml level at 8 h after dosing (by both the routes), while > 60 ng/ml ATRA was still detectable until 48 h in the SLN groups. Concentration-time curves in both the cases were however described by a non-compartmental pharmacokinetic model. The single dose subcutaneous pharmacokinetic parameters of the two formulations are shown in Table 5. The Cmax, AUC0-t, and AUC0-∞ of the ATRA-SLNs reached 3.19µg/ml, 14.98µg*h/ml and 31.23 µg*h/ml, respectively, while it was only 1.39µg/ml, 2.82 µg*h/ml and 5.77µg*h/ml, respectively, for the free ATRA. As being analyzed with sigmastat statistical software, all three parameters for the ATRA-SLNs were significantly higher (p<0.05) than that for the free ATRA. A significant reduction in Kel (10.6 times) and increase in half life (14.4 times).
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times) was observed for ATRA-SLNs as compared to ATRA-S in case of subcutaneous administration. Consequently, the subcutaneous bioavailability of ATRA-SLNs was 5.40 times higher than that achieved with ATRA-S, as calculated from AUC(0-$\infty$) values.

![Figure 9: The mean plasma concentration-time curves for ATRA-SLNs (A & C) and ATRA-S (B & D) upon oral (7.5 mg/kg body weight; C & D) and subcutaneous (20 mg/kg body weight; A & B) administration.](image)

In case of oral administration the $C_{\text{max}}$, $AUC_{0-2}$, and $AUC_{0-\infty}$ of the ATRA-SLNs reached $1.04 \mu g/ml$, $10.19 \mu g*\mu h/ml$ and $24.99 \mu g*\mu h/ml$ respectively, while it was significantly less ($p \leq 0.05$) at $0.151 \mu g/ml$, $0.356 \mu g*\mu h/ml$ and $0.713 \mu g*\mu h/ml$, respectively, for the free ATRA (Table 6). The oral bioavailability of ATRA-SLNs was 35.03 times higher than ATRA-S. As a BCS II drug, the solubility of ATRA is a significant limitation to its oral bioavailability. Latter was suitably attended to by the formulation of SLN dispersion (Figure 6B (b) which can present the drug in a more accessible form for uptake as is done by solubilisation. A significant reduction in $K_{el}$ (8.5 times) in case of orally administered ATRA-SLNs versus ATRA-S, points towards a prolonged circulation time of ATRA-SLNs. Another,
parameter depicting the longer plasma availability is the half life ($t_{1/2}$), which was increased by 10.4 times after incorporation into SLNs in case of orally administered ATRA-SLNs. It may however be noted that rather than administering free ATRA as a dispersion it was administered after dissolving in tween 80, used at a concentration present in final SLN formulation. Latter may indicate the bioavailability of ATRA-S to be higher than when actually administered orally as a solid dosage form.

Table 5: Pharmacokinetic parameters of single dose 20 mg/kg body weight subcutaneous injection of ATRA as free drug (ATRA-S) and upon loading into SLNs (ATRA-SLNs) to rats (n=5).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>$C_{max}$ (ng/ml)</th>
<th>$T_{max}$ (h)</th>
<th>AUC (0-t) hr*ng/ml</th>
<th>AUC (0-04) hr*ng/ml</th>
<th>$K_e$ (h$^{-1}$)</th>
<th>$T_{1/2}$ (h)</th>
<th>MRT (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATRA-SLNs</td>
<td>3190.54</td>
<td>1.0</td>
<td>14984.04</td>
<td>31238.04</td>
<td>0.07</td>
<td>13.71</td>
<td>6.234</td>
</tr>
<tr>
<td>ATRA-S</td>
<td>1396.98</td>
<td>0.50</td>
<td>2824.50</td>
<td>5770.05</td>
<td>0.74</td>
<td>0.95</td>
<td>1.26</td>
</tr>
</tbody>
</table>

Table 6: Pharmacokinetic parameters of a single oral dose of 7.5 mg/kg body weight of ATRA as free drug (ATRA-S) and upon loading into SLNs (ATRA-SLNs) administered to rats (n=5).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>$C_{max}$ (ng/ml)</th>
<th>$T_{max}$ (h)</th>
<th>AUC (0-t) hr*ng/ml</th>
<th>AUC (0-04) hr*ng/ml</th>
<th>$K_e$ (h$^{-1}$)</th>
<th>$T_{1/2}$ (h)</th>
<th>MRT (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATRA-SLNs</td>
<td>1045.72</td>
<td>2.0</td>
<td>10199.05</td>
<td>24996.72</td>
<td>0.02</td>
<td>50.29</td>
<td>16.73</td>
</tr>
<tr>
<td>ATRA-S</td>
<td>151.27</td>
<td>1.0</td>
<td>356.55</td>
<td>713.51</td>
<td>0.17</td>
<td>4.84</td>
<td>1.48</td>
</tr>
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</table>
Table 7: Plasma concentration-time values obtained after oral and subcutaneous administration of ATRA-S and ATRA-SLNs.

<table>
<thead>
<tr>
<th>Conc. (ng/ml)</th>
<th>ATRA-S</th>
<th>ATRA-SLNs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oral route</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>102.86±11.21</td>
<td>498.76±22.3</td>
</tr>
<tr>
<td>1</td>
<td>151.27±9.93</td>
<td>779.36±18.3</td>
</tr>
<tr>
<td>2</td>
<td>96.79±7.56</td>
<td>1045.72±27.25</td>
</tr>
<tr>
<td>4</td>
<td>36.41±4.25</td>
<td>287.46±12.41</td>
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<td>8</td>
<td>18.72±3.26</td>
<td>128.35±11.3</td>
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<tr>
<td>16</td>
<td>16.25±1.60</td>
<td>114.12±7.21</td>
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<tr>
<td>24</td>
<td>10.36±2.4</td>
<td>96.36±5.46</td>
</tr>
<tr>
<td>48</td>
<td>8.70±1.18</td>
<td>61.2±2.8</td>
</tr>
<tr>
<td><strong>Subcutaneous route</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1396.98±12.0</td>
<td>2798.95±98.0</td>
</tr>
<tr>
<td>1</td>
<td>816.73±21.0</td>
<td>3190.54±112.0</td>
</tr>
<tr>
<td>2</td>
<td>508.27±12.6</td>
<td>1862.65±56.25</td>
</tr>
<tr>
<td>4</td>
<td>30.58±2.56</td>
<td>445.50±24.0</td>
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<td>8</td>
<td>18.25±3.11</td>
<td>94.04±14.56</td>
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<td>16</td>
<td>15.60±2.67</td>
<td>84.7±10.92</td>
</tr>
<tr>
<td>24</td>
<td>18.45±2.14</td>
<td>79.5±16.35</td>
</tr>
<tr>
<td>48</td>
<td>14.23±1.05</td>
<td>75.6±13.5</td>
</tr>
</tbody>
</table>

5.0 DISCUSSION

Numerous drugs have been developed for controlling TB, curing the patient and preventing further transmission of the disease. Unfortunately, first-line treatment with antitubercular drugs elicits serious side effects leading to poor compliance, and subsequent emergence of multidrug resistant (MDR) strains of *M. tuberculosis*. Nevertheless, tailoring of the existing antitubercular drugs for better effectiveness and reduced side effects, so as to improve patient compliance are the priority for the scientists, (Bhandari and Kaur, 2013a; Kumar et al., 2014b;
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Singh et al., 2013a); nonetheless, scope of alternative prophylactic or curative interventions like supplementation of prone or affected population with vitamin A, cannot be ruled out. Historically, administration of vitamin A is considered beneficial for the treatment of tuberculosis.

Low aqueous solubility and dissolution rate of poorly water soluble drugs in the aqueous gastrointestinal fluids often lead to poor bioavailability and can thus be enhanced by increasing the solubility of these agents in the gastrointestinal fluids (Kumar et al., 2011; Sharma et al., 2009; Yellela, 2010). SLNs with their established potential as drug delivery carrier for hydrophobic drugs were thus considered as the system of choice for incorporating ATRA to improve its BA by enhancing its solubility and permeability across biological membranes including gut walls (for oral administration) and blood vessels (for subcutaneous administration) in addition to providing it with a protective cover with the lipid matrix against oxidation and photodegradation. The SLNs are also expected to protect ATRA against the fast metabolic degradation in the liver followed by its elimination (Hu et al., 2004).

Microemulsification method used for the preparation of ATRA-SLNs resulted in rod shaped particles with reproducible values of TDC (92.50± 2.10%) and EE (84.60±3.20%), for batches prepared on six different occasions. A small particle size of 131.30 nm is reported for the presently developed ATRA-SLNs. Earlier studies report preparation of ATRA loaded SLNs for topical application with a particle size varying from 246±38 to 350 nm (Lim et al., 2004; Pople and Singh, 2006) and 154.9 nm (Lim and Kim, 2002) and 300 nm (Jenning et al., 2000b). High entrapment of ATRA within SLNs is attributed to lipophilic oily nature of ATRA. Latter may have resulted in formation of NLCs. Though the percentage of oil:lipid in such cases is usually high we used only 10% w/w of vitamin with respect to the Compritol® 888 ATO used as the solid lipid. The stability studies indicated that the prepared ATRA-SLNs were stable after one year of storage at 5±3°C (Kumar et al., 2014b). PS studies indicate that incorporation into SLNs could protect ATRA against light degradation. It may be noted that considerable
degradation (51.5%) occurred for the free drug solution even in amber colored containers which may be attributed to the induction of a combination of oxidative mechanisms and photodegradation.

The EE of ATRA-SLNs did not undergo any significant change (p≤0.05) even upon storage under refrigerated conditions. Latter is attributable to the possibility that free ATRA (not removed from the dispersion; 0.5 mg/ml) is present in equilibrium and probably above its saturation solubility with the small volume of water (as only 1:1 dilution of microemulsion is done presently versus a usual 1:10 to 1:50 dilution) in SLN dispersion so that the leakiness of ATRA from its SLNs is minimal. It may be noted that ATRA is a poorly soluble drug with a solubility of < 10μg/ml which may partly increase in the presence of tween 80 and the formulated SLNs. Improvement in solubility of poorly soluble drugs in the presence of SLNs (Singh et al., 2013a) and niosomes (Aggarwal et al., 2004) has been reported by us.

In vitro release of ATRA from ATRA-SLNs in 3% tween 80 in 0.01 M phosphate buffer pH 7.4 by diffusion/dissolution of lipid was prolonged up to 24 h. It is reported, that for highly lipophilic molecules like Coenzyme Q 10 (Ankola et al., 2007) an aqueous solution containing a surfactant or solvent in which the drug is soluble may be used to enhance the solubility of the drug and provide sink conditions in addition to maintaining the stability of the drug. A non-fickian diffusion and lipid dissolution controlled release was observed for ATRA-SLNs. DSC, PXRD and FTIR studies confirmed the formation of SLNs and their amorphous nature. Latter indicates that drug/lipid is present in a soluble form.

Pharmacokinetic studies with the developed SLNs revealed 35.03 and 5.4 times enhanced bioavailability post administration of ATRA-SLNs as compared to ATRA-S, by oral and subcutaneous routes, respectively, in rats. Further, mean residence time for SLNs administered by oral and subcutaneous route was increased by 11.3 and 5.0 times as compared to free ATRA administered at the same dose, confirming their prolonged plasma circulation.
Drugs administered by subcutaneous (s.c) route are absorbed at a slower rate compared to other parenteral routes, providing a sustained effect. Both the aqueous solutions or oily fluids and depots of oily materials for slow absorption, solid pellets, suitably sized osmotic minipumps or other implants can be administered by s.c route. Advantage of subcutaneous route is that out of all the parenteral routes, s.c administration does not require trained personnel, or a paramedical staff and the absorption of applied medicament, even though slow, is usually complete. The exact mechanism of absorption though unknown, is expected to be due to direct uptake of molecules within the subcutis by small capillaries underlying the skin, with minimal lymphatic absorption (Khan et al., 2013) However, for lipid soluble molecules like ATRA lymphatic uptake cannot be ruled out.

It is probably due to the above stated facts that free ATRA (ATRA-S) underwent significant absorption from the s.c route both in comparison to the free drug administered by oral route ($C_{\text{max}}$ of 151.27ng/ml for 7.5mg/kg dose versus 1396.98 ng/ml for 20mg/kg dose by s.c route) and also in comparison ATRA-SLNs administered subcutaneously.

A faster onset of action achieved upon subcutaneous administration (1.0 h) especially with ATRA-SLNs versus oral administration (2 h) indicates faster uptake from the s.c route. Since we could not achieve quantifiable levels of ATRA in plasma upon administration of 7.5 mg/kg subcutaneous dose hence, we enhanced the subcutaneous dose to 20 mg/kg body weight. The improvement in BA shown by ATRA-SLNs upon s.c. administration though significant (5.4 times; $p<0.05$) did not compare equally to the oral route (35.03 times). This may be attributed to a poor oral BA of free drug per se from the oral route due to a multitude of factors like poor solubility, enzymatic degradation in gut and blood and high first pass metabolism (Yamamoto et al., 2000). Howsoever, the substantially improved s.c BA observed with ATRA-SLNs can be attributed to their accumulation at the site of injection. It is reported that particles greater than 100nm ($D_{50}$ of ATRA-SLNs is 131.3 nm) are trapped at the s.c injection site for a
significant period of time (Khan et al., 2013b; Oussoren et al., 1997). ATRA released slowly from these SLNs is expected to be taken up through pores in the walls of the capillaries and may directly enter blood circulation. On the other hand ATRA-SLNs with size at or below 100 nm (Oussoren et al., 1997) may undergo a preferential uptake via lymphatics. The particle size distribution (Figure. 2) of ATRA-SLNs indicates 27.5% particles ≤100nm. Particles greater than 100 nm are also reported to undergo lymphatic uptake, but at a much slower rate. Furthermore, drug encapsulation in the protective lipid matrix of SLNs, may reduce the exposure of ATRA to the enzymatic milieu below the skin. Further to this negatively charged particles (zeta potential of ATRA-SLNs= -17.45) have been reported to show higher lymphatic uptake than neutral or positively charged surfaces. This is attributed to the fact that the interstitial matrix has a net negative charge such that the anionic carrier particles in the interstitium encounter an electrostatic repulsion and move more quickly into the lymphatics (Hawley et al., 1995; Porter, 1997). Furthermore, it is suggested that phagocytosis by macrophages is one of the major mechanism of uptake of colloidal particles in lymph nodes (Hagan et al., 1992). This can be of special significance in antitubercular therapy and for immune-modulatory effects.

Significant enhancement (35.03 times) in the oral BA of ATRA upon incorporation into SLNs may be attributed firstly to the small particle size (<200 nm) of ATRA-SLNs which are predicted to bypass the reticuloendothelial system (Kaur et al., 2008; Kumar et al., 2014a), thus enhancing systemic drug levels and helping to achieve prolonged circulation times. Secondly, it is hypothesized that the use of polysorbate 80, an efficient cytochrome P-450 inhibitor (Ren et al., 2008) prevents the metabolism of ATRA, by CYP enzymes located in liver and intestinal microsomes leading to its increased BA (Christiansen et al., 2011). Additionally paracellular and transcellular (transcytosis) uptake of SLNs by enterocytes (which represent 90–95% of the epithelial cells of the intestine) and M (microfold) cells (Des Rieux et al., 2006), also contribute to the achievement of high plasma levels of the drug on oral administration. Bioavailability
enhancement of vitamin A from a self nanoemulsified drug delivery system in rats at a dose of 7.5 mg/kg body weight by oral administration reports $C_{\text{max}}$ of 799.5 ±48.6 ng/ml, and AUC (0–$\infty$) of 3080.7±190.2 ng/ml (Taha et al., 2007) whereas we could achieve $C_{\text{max}}$ of 3190.54 ng/ml and AUC (0–$\infty$) of 31238.04 ng/ml i.e., 3.99 and 10.14 times higher $C_{\text{max}}$ and AUC (0–$\infty$) values, respectively than that reported at the same oral dose. Ozpolat et al (2003) determined single and multiple dose pharmacokinetics of liposomal-all-trans-retinoic acid (L-ATRA) following administration by the intravenous route in comparison to free ATRA by oral route administered to healthy volunteers (Ozpolat et al., 2003). However, valid comparisons cannot be drawn as they did not evaluate free ATRA by intravenous route and L-ATRA by oral route. Howsoever, their results indicated 13 fold greater AUC values with i.v L-ATRA in comparison to oral free ATRA after single dose administration. In our study however approx. 5 and 35 times higher BA by subcutaneous and oral routes, respectively after packaging ATRA into solid lipid nanoparticles in comparison to free drug administration by the respective route is of great significance.

Several studies also report on the enhancement in BA of ATRA using inclusion complexes. A report revealed a 2.89-fold increase in AUC for ATRA/HP/βCD inclusion complex solution compared with free ATRA suspension, in rats, with oral dose of 10 mg/kg (Chen et al., 2011) Lin et al., reported pharmacokinetic study of ATRA/HP/βCD inclusion complex pellets, in dogs, with oral dose of 10 mg/kg (Lin et al., 2000). Significant concentration of ATRA was maintained only upto12 h, in their study whereas we could achieve significant concentrations (more than 60 ng/ml) even after 48 h post administration of ATRA-SLNs both by the oral and subcutaneous routes in rats, proving a prolonged delivery. Another study (Francois et al., 2000) revealed non- significant levels of ATRA in plasma (30 ng/ml) after 5 h of drug administration by intra-peritoneal route, however we could achieve 75.6 ng/ml and 61.2 ng/ml (2.52 and 2.04 times higher) plasma concentrations even after 48 h post administration of ATRA-SLNs via subcutaneous and oral routes respectively reestablishing their prolonged and
sustained effect Table 7. Providing a protective layer to ATRA, by preparing SLNs, thus not only enhances its solubility but also its overall biopharmaceutical performance which was clearly depicted by the plasma concentration time values obtained after oral and subcutaneous administration of ATRA-S and ATRA-SLNs pharmacokinetic profile of ATRA-SLNs.

It is noteworthy to report here that the only FDA approved product of ATRA is available under the trade name of Vesanoid® (tretinoin), in the form of capsules and is marketed by Roche. The pharmacokinetics of Vesanoid® in humans reveals an attainment of peak concentration of 347ng/ml post single oral dose of 80 mg (2,66,667 IU) to patients suffering from acute promyelocytic leukaemia (CDER, Application No: NDA 20-38/S-001). We could however achieve 1045ng/ml of ATRA after administering 7.5 mg/kg by oral route. Latter comes to \[ \frac{7.5 \times 70 \text{ (adult weight) }}{6} \] (correction factor for higher metabolism in rats) almost the same dose of 87 mg. Results clearly highlight the potential of SLNs as a viable carrier system for effective oral administration of vitamin A. No oral or s.c SLN formulation of ATRA is reported till date.

6.0 CONCLUSIONS

A major limiting factor to the systemic use of particulate delivery systems is the rapid clearance of the carrier from the blood circulation by reticuloendothelial system (RES). Various techniques such as suppression of RES (reducing particle size; 120-200 nm) (Lu and Chen, 2004) and modification of surface characteristics of drug carriers by coating with hydrophilic agents/block copolymers have been attempted to reduce the RES uptake. The second approach has been shown to be highly effective in altering the biodistribution pattern of colloidal drug carriers. ATRA is light and air sensitive and water insoluble hence is not readily absorbed from gastrointestinal tract; we incorporated ATRA into SLNs comprising hydrophilic tween 80, and evaluated its pharmacokinetic profile post administration by oral and subcutaneous routes. Intent was to achieve a controlled and sustained release so as to maintain its therapeutic concentration in plasma. Low serum level of ATRA is observed in
tuberculosis patients. In vitro studies indicate that maintaining 1 µM (0.3 µg/ml) concentration of ATRA daily for a month can help overwhelm macrophage maturation arrest resulting from *M. tuberculosis* infection and subsequent death of the pathogenic bacteria. In our study we could achieve and maintain significant plasma concentrations of ATRA in plasma for 48 h post administration of single dose of the oral and subcutaneous routes of administration hence the developed formulation can be successfully explored clinically for the treatment /prophylaxis of tuberculosis as well as for majority of other disorders like cancers, acne and age related macular degeneration (AMD).
Chapter 3

Development, characterization and pharmacokinetic evaluation of vitamin D₃ (Cholecalciferol) loaded solid lipid nanoparticles.
Compromised physicochemical properties like hydrophobicity, limited water solubility, and instability in presence of light and air, result in inadequate levels of the vitamin D₃ in plasma inspite of the administration of a dose as high as 1 lac I.U (Ilahi et al., 2008; Martineau et al., 2011). There are reports on clinical failures of the vitamin D₃ therapy when administered at even higher doses (Ilahi et al., 2008).

Vitamin D deficiency is a global problem prevalent in all age groups. Estimates suggest that 1 billion people around the world have vitamin D deficiency or insufficiency i.e level ≤20 ng/mL (Holick, 2007). In humans, the main source of vitamin D is from cutaneous synthesis through a process initiated by incidental natural sun exposure/ultraviolet B irradiation. However, prolonged sun exposure are presently discouraged in humans, because of the increased risk of skin cancers, and a threshold of sun exposure sufficient to maintain a healthy vitamin D status without measurable cancer risk is difficult to define. The ability to synthesize vitamin D in the skin varies considerably by skin type, season, and geography. At some latitudes, cutaneous synthesis of vitamin D is precluded during the winter months (Terushkin et al., 2010). The use of sunscreens commonly advocated now a days, also almost completely block cutaneous vitamin D synthesis (Matsuoka et al., 1990). Only small amounts of vitamin D can be derived from dietary sources like oily fish, eggs, and from vitamin fortified foods such as dairy products and breakfast cereals. As vitamin D is fat soluble, manufacturing challenges are encountered when attempting to fortify food products and beverages. Thus, only small amounts of vitamin D can feasibly be provided through such dietary supplements.

Vitamin D₃ is available as oral sachet (60000 IU; 1.5mg), oral solution as 8000 IU/ml (200 µg/ml), capsule 50,000 IU (1.25mg), and tablets of 400 IU (10µg), and 2000 IU (50µg). Vitamin D supplementation is indicated for a variety of clinical situations viz. osteoporosis, rickets, hypoparathyroidism, and familial hypophosphatemia. However, no parenteral formulation of vitamin D₃ is available in the market (Martineau et al., 2007).
Vitamin D₃ is known to regulate calcium and phosphorus homeostasis, intestinal transport, bone metabolism and renal calcium reabsorption, as well as blood pressure and insulin secretion (Gonnet et al., 2010; Lind et al., 1995; Schedl et al., 1984). Additionally it regulates immune system and controls cell differentiation (Cantorna et al., 2000). Vitamin D₃ deficiency (VDD) is also reported to be one of the aetiological factors for tuberculosis (TB) which significantly distresses the cell-mediated immune response of the body (Gibney et al., 2008; Nnoaham and Clarke, 2008; Sita-Lumsden et al., 2007; Srinivasan et al., 2013; Ustianowski et al., 2005; Wilkinson et al., 2000). Immunomodulatory functions of vitamin D₃ provide biological mechanisms for this supposition and these observations call for the clinical evaluation of vitamin D as an adjunctive therapy in the treatment of active tuberculosis (Martineau et al., 2007). Diverse forms of vitamin D have been proposed for the treatment of TB of scrofula, lymph nodes, abdomen and even pulmonary TB (Ellman and Anderson, 1948; Ruiter and Groen, 1949; Trautwein and Stein, 1952).

The survival of pathogenic mycobacteria, within the host, is linked to their successful establishment in an intracellular niche. Macrophages have a unique paradoxical role in tuberculosis infection, serving both as a first line of defense and also creating the primary site for mycobacterial replication and dissemination (Cosma et al., 2003; Ferrari et al., 1999; Pieters, 2001). Mycobacterial survival within macrophage is achieved primarily by the convolution of mycobacterial phagosome with the endogenous macrophage tryptophan-aspartate-containing coat protein (TACO), also known as coronin-1 that specifically restricts phagosomes containing pathogenic mycobacteria from entering the late endosomal/lysosomal pathway (Ferrari et al., 1999; Pieters, 2001). Vitamin D₃ inhibits Mycobacterium entry as well as survival within macrophages, possibly through rescue of phagosome maturation arrest. It thus provides a host protective mechanism rather than the Mycobacterium directed mechanism shown by commonly used antitubercular drugs (ATDs); since mycobacteria is highly resistant and can also shift strains, treatment with ATDs is less reliable while a host mediated pathway can be a gunshot treatment even for MDR and XDR forms of tuberculosis (Srinivasan et al., 2013).
Endogenously synthesised vitamin D₃ is transported in the plasma via plasma vitamin D binding protein (DBP) and is very slowly delivered to liver (Haddad et al., 1993) ensuring a more sustained increase in plasma 25-hydroxy cholecalciferol (OH)D levels. In contrast exogenously administered oral vitamin D₃ is associated with chylomicrons and lipoproteins leading to a receptor-mediated, rapid hepatic delivery of vitamin D₃ and a reportedly rapid but a less sustained increase in plasma 25(OH)D levels. Thus, it may be considered relevant to deliver vitamin D₃ entrapped within a lipicid coat which will not only protect the latter against photolytic and oxidative degradation but will also enhance its permeability across the gut, limiting the association with lipoproteins and subsequent liver uptake. Delivery of intact vitamin D₃ to the plasma and its presence in plasma over prolonged periods will ensure sustained beneficial effects.

Solid lipid nanoparticles (SLNs) are a potential colloidal carrier system alternative to polymeric nanoparticles, and identical to oil in water emulsion for parenteral nutrition, with the liquid lipid being replaced by a solid lipid. They have advantages such as good biocompatibility and low toxicity in addition to their superior suitability for delivery of lipophilic drug. Solid state of these lipidic nanoparticles slows down their digestion by pancreatic lipases (Harde et al., 2011).

Present chapter describes preparation and characterization of vitamin D₃ loaded SLNs and, explores the enhanced pharmaceutical performance of the same vitamin D₃-SLNs, after administration by oral and subcutaneous routes both in terms of improved stability including photostability and pharmacokinetics (in rats) using a validated ultra performance liquid chromatography (UPLC) method of analysis (Kumar et al., 2014b).

2.0 MATERIALS AND METHODS

2.1 Materials

Cholecalciferol (vitamin D₃) and HPLC solvents as described in chapter 1. Soy lecithin, Tween 80, and Compritol® 888 ATO as described in chapter 2. All other
chemicals and reagents were of analytical grade and were used without further purification.

2.2 Methods

Note: Method of preparation, and physicochemical characterization including in vitro release and stability of vitamin D₃ loaded SLNs (vitamin D₃-SLNs) is the same as described for ATRA-SLNs in chapter 2.

3.0 IN VIVO STUDIES

3.1 Animals

Wistar male rats weighing 200–300 g were used for the study. Animals were acclimatized to laboratory conditions for a week before the start of the experiment. The protocol was duly approved by the Institutional Animal Ethics Committee (IAEC) of Panjab University, Chandigarh, India (letter No. CAH/131 dated 09.08.2010).

3.2 Pharmacokinetic studies

Pharmacokinetic studies were conducted after subcutaneous and oral administration at respective doses of 0.54 mg/kg and 0.21 mg/kg b/w of free vitamin D₃ (dissolved in 25% w/v aqueous solution of teen 80) and vitamin D₃ - SLNs, respectively, to rats (n=5/group). The oral and subcutaneous dose to be administered was determined on the basis of reported (Martineau et al., 2011) human oral dose of 2.5mg which becomes 2.5 X 6 (factor for conversion to rat dose as per USFDA)/70 kg (taken as the weight of healthy adult) =0.21 mg/kg by oral route, and 2.5X2.5 (higher dose selected for subcutaneous route) X6/70 =0.54 mg/kg by subcutaneous injection. The blood samples (1 ml) were withdrawn from retro-orbital plexus and collected into microcentrifuge tubes (containing 20 µl of 1000 IU heparin/mL of blood) at 0.5h, 1h, 2h, 4h, 8h, 12h, 24h and 48h post administration of free drug or drug loaded SLNs. Plasma was separated by centrifuging the blood samples at 1677 g for 10 min at 4°C. After centrifugation, the obtained plasma samples were stored at -20°C until analysis.
3.3 Procedure for drug extraction from plasma samples

Vitamin-D₃ was extracted from rat plasma samples by liquid liquid extraction (LLE), in a polypropylene tube. To 500 µl of plasma samples, 400 µl of chilled methanol and 2 ml of n-hexane: ethyl acetate (1:1) was added and vortexed for 1 min. Resulting mixture was kept at -20 °C for 5 min and cold centrifuged at 1677 g for 10 min. Supernatant was decanted and another 2 ml of n-hexane: ethyl acetate (1:1) was added to the pellet, followed by vortexing (1 min) and extraction, as above, to achieve complete recovery. Combined supernatants were dried by purging nitrogen gas through them (Turbovap, Biotage India). Utmost care was taken to perform these steps in dim light, complemented with the use of amber colored containers to minimize light exposure. Dry samples were reconstituted with 250µl of acetonitrile, transferred to amber colored auto-injector vials and analyzed using UPLC (injected volume= 4 µl).

3.4 Data analysis

The pharmacokinetic parameters were calculated based on a non-compartmental model. The area under the concentration–time curve from time zero to time t (AUC₀₋ₜ) was calculated using the trapezoidal method. Peak concentration (Cₘₐₓ) and time of peak concentration (Tₘₐₓ) were obtained directly from the plasma concentration–time profile. The area under the total plasma concentration–time curve from time zero to infinity was calculated by:

\[ AUC₀₋∞ = AUC₀₋ₜ + Cₜ/K_e \]

where Ct is the drug concentrations observed at the last time t, and Ke is the apparent elimination rate constant obtained from the terminal slope of the log plasma concentration–time curve upon linear regression. The data obtained from pharmacokinetic parameters were analyzed statistically using WinNonlin software.

4.0 RESULTS

4.1 Preparation and physicochemical characterization of vitamin-D₃ SLNs

Microemulsification technique for preparation of SLNs was highly reproducible in terms of the particle size, TDC and EE. Being a spontaneous method, it requires simple lab equipment, is easy to scale up (Kakkar and Kaur, 2012) and results
in a significantly small particle size (Bhandari and Kaur, 2013a; Bhandari and Kaur, 2013b).

TDC and EE of vitamin D₃-SLNs were estimated to be 88.2%±1.2 and 87.45%±2.70 respectively (n=6). High values of TDC and EE indicate the efficiency of the method and insignificant losses during formulation. Average particle size (D50) of vitamin D₃-SLNs was 196.5±3.0 nm, with polydispersity index (P.I) of 0.142. (Figure 10) and zeta potential of -14.72 mV (Figure 11) when measured using the dynamic light scattering technique (DelsaNano™).

Figure 10: Particle size of vitamin D₃-SLNs by DelsaNano™
Zeta (Potential (mV))
Version 3.73 / 2.30
Mobility Distribution
Measurement Results
Zeta Potential: -14.72 (mV)
Mobility: -1.148e-004 (cm²/Vs)
Conductivity: -0.0119 (mS/cm)
Doppler shift: 9.11 (Hz)
Base Frequency: 120.7 (Hz)
Conversion Equation: Smoluchowski

Zeta Potential of Cell
Upper Surface: -16.95 (mV)
Lower Surface: -19.28 (mV)

Cell Condition
Cell Type: Flow Cell
Avg. Electric Field: -16.43 (V/cm)
Avg. Current: 0.01 (mA)

Diluent Properties
Diluent Name: WATER
Temperature: 25.0 (°C)
Refractive Index: 1.3328
Viscosity: 0.8878 (cP)
Dielectric Constant: 78.3

Peak Data Table of Distribution Graph
<table>
<thead>
<tr>
<th>Peak Frequency (Hz)</th>
<th>Intensity</th>
<th>Half Width (Hz)</th>
<th>Zeta Potential (mV)</th>
<th>Mobility (cm²/Vs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.11</td>
<td>14.76</td>
<td>5.87</td>
<td>-14.72</td>
</tr>
<tr>
<td>2</td>
<td>1.148e-004</td>
<td>5.87</td>
<td>-14.72</td>
<td>-1.148e-004</td>
</tr>
</tbody>
</table>

Figure 11: Zeta potential of vitamin D₃-SLNs
TEM studies (Figure 12) revealed the morphology of vitamin D3-SLNs to be rod shaped. The size of particles observed under TEM was invariably in the range of 66±5.0 nm (n=50 particles).

Figure 12: TEM micrograph of vitamin D3-SLNs

4.2 DSC

DSC is a thermoanalytical technique in which the difference in the amount of heat required to maintain the sample and reference at same temperature is measured as a function of temperature and time. The breakdown or fusion of the crystal lattice by heating or cooling the sample yields information about the internal polymorphism, crystal order, and glass transition processes (Uner, 2006). The basic principle underlying the technique is that, when the sample
undergoes a physical transformation (such as melting, desolvation), some amount of heat is required to flow to it, depending on whether the process is exothermic or endothermic, to maintain both reference and sample at the same temperature. DSC measures this heat flow into or from the sample and thus both qualitative and quantitative information about the involved physicochemical changes (i.e. endothermic, exothermic processes or changes in heat capacity) can be drawn from the observations. It uses the fact that different lipid modifications possess different melting points and enthalpies.

In pure vitamin D$_3$, a melting endotherm appeared at 90.74°C corresponding to its melting point at 85-95 °C (Koshy and Beyer, 1984) and was associated with 41.69 J/g of enthalpy. While Compritol$^\circledR$ 888 ATO showed a sharp peak at 73.06°C and an enthalpy of 108.3 J/g. Physical mixture (Figure 13c), showed distinct endothermic peaks corresponding to both Compritol$^\circledR$ 888 ATO and vitamin D$_3$. Peak for the latter was however of a lower intensity, because amount of vitamin D$_3$ present in the physical mixture was 10 times less than Compritol$^\circledR$ 888 ATO. The vitamin D$_3$-SLN dispersion showed a broad endotherm merging several peaks, including one at 81.77 °C and the other at 112.81°C. Broad peak indicates amorphous nature of vitamin D$_3$-SLNs. Further, enthalpy for the peak corresponding to the lipid (observed at 81.77° C) is 85.58 J/g (Figure 13 d), which is significantly lower than that of the pure lipid (108.3 J/g) (Figure 13 b) indicating a probable change in polymorphic state of the Compritol$^\circledR$ 888 ATO from the crystalline $\beta$ form to the amorphous ($\alpha$, $\beta'$) form with more imperfections in the crystal lattice. Latter will comfortably incorporate vitamin D$_3$ within the lipid molecules.
4.3 PXRD

PXRD patterns of vitamin D₃, Compritol® 888 ATO, lyophilized blank SLN (BSLN), and vitamin D₃-SLN are shown in Figure 14. PXRD pattern of vitamin D₃ exhibited sharp peaks at 2θ scatter angles from 10-30°, which indicate its crystalline nature. Compritol® 888 ATO also showed sharp peaks at 2θ scattered angles 21.16, 23.37, 23.52 and 35.76°; again establishing its crystalline state (Figure 14b).

However, no characteristic peaks in lyophilized BSLN (Figure 14c) indicate the amorphous nature of lipid after transformation into SLNs. Typical pattern of peaks corresponding to those of free vitamin D₃ (Figure 14a), were also found missing in the PXRD of lyophilized vitamin D₃-SLN sample (Figure 14d), reconfirming the loss of crystallinity and a shift towards the amorphous state.
4.4 In vitro release

The release of the drug from vitamin D₃-SLNs was delayed to 24 h while almost 100%, free drug was released in less than 2h in 0.01 M phosphate buffer (pH 7.4) + 3% tween 80 (Figure 15). The release of vitamin D₃ from corresponding SLNs was observed to be triphasic with only 18% of vitamin D₃ being released in the initial 2 h, more than 35 % being released up to 8 h, and almost 91 % at 24h. Alginate ester (OAE) based nanoparticles of vitamin D₃ have been developed and reported to show an initial burst release in simulated intestinal fluid, with almost 60 % release in 8 h (Li et al., 2011), while presently prepared SLNs showed 60% release in almost double the time i.e in 16 h; eventhough the former studies did not use tween 80 in the SIF. The initial release (upto 2 h) of the drug may be due to presence of adsorbed drug on the surface of nanoparticles, while the subsequent steady and slow release is probably due to the release of vitamin D₃ dispersed uniformly within the lipid core of SLNs (Figure 15). Application of various models to release kinetics, indicated a higher linearity ($r^2=0.9986$) for the zero order model in comparison to Higuchi ($r^2=0.9651$), the Korsmeyer-Peppas ($r^2= 0.9805$) and first order ($r^2= 0.9818$) release models, (Table 8) for the
developed vitamin D₃-SLNs. Application of Korsmeyer-Peppas model, indicated that the value for “n” was more than 0.5 conveying that the SLN formulation follows non-fickian diffusion and dissolution controlled release.

Figure 15: In vitro release of vitamin D₃ SLNs in 0.01 M phosphate buffer (pH 7.4)+ 3% tween 80.

Table 8: In vitro release kinetics of vitamin D₃-SLNs in 0.01 M phosphate buffer (pH 7.4)+ 3% tween 80.

<table>
<thead>
<tr>
<th>Time points (h)</th>
<th>Value of n</th>
<th>Mechanism of drug release</th>
<th>R²</th>
<th>Korsmeyer</th>
<th>Higuchi</th>
<th>First order</th>
<th>Zero order</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5</td>
<td>Non fickian diffusion and dissolution controlled</td>
<td>0.9805</td>
<td>0.9651</td>
<td>0.9818</td>
<td>0.9986</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.712</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.5 Stability and photostability studies (PS)

After storage at 5±3°C for 1 year the vitamin D₃-SLN dispersion showed an insignificant (p<0.05) change of <3.0% both in the values of TDC and EE. PS studies also revealed an insignificant change (p<0.05) in TDC and EE, for vitamin D₃-SLNs when stored in amber colored vials. However, in case of free drug solution in 25% tween 80 (vitamin D₃-S); a significant loss in concentration was observed. A 34.5% higher degradation occurred in vitamin D₃-S sample stored in
clear glass vial, in comparison to amber colored vial. It may also be noted that in comparison to an insignificant degradation observed for the vitamin loaded SLNs stored in amber colored container, the corresponding free drug solution showed a degradation of 56.2%. This confirms that the lipid enclosure of SLNs provides significant protection to vitamin D₃. SLN sample even on storage in clear glass vials showed only a minimal degradation of 9.05% (Kumar et al., 2014b) (Table 9).

Table 9: TDC and EE of vitamin D₃-SLNs when stored in amber container at 4°C for 1 year and when exposed to UV light of 1.2 million lux hours in amber and white containers, respectively.

<table>
<thead>
<tr>
<th>Stability studies</th>
<th>TDC</th>
<th>EE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D₃-SLNs (Amber)</td>
<td>88.2±1.2%, 87.4±3.2%</td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>88.2±1.2%, 87.4±3.2%</td>
<td></td>
</tr>
<tr>
<td>12 months</td>
<td>86.4±3.8%* 84.1±1.1*</td>
<td></td>
</tr>
<tr>
<td>Photostability studies</td>
<td>TDC</td>
<td>EE</td>
</tr>
<tr>
<td>Vitamin D₃-S (Amber)</td>
<td>56.2±1.2%</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin D₃-S (Clear)</td>
<td>36.8±3.8%</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin D₃-SLNs (Amber)</td>
<td>86.2±1.2% 83.5±3.2%*</td>
<td></td>
</tr>
<tr>
<td>Vitamin D₃-SLNs (Clear)</td>
<td>81.1±2.6% 80.3±4.1%*</td>
<td></td>
</tr>
</tbody>
</table>

*The values are insignificantly different (p≥0.05) from the initial values of TDC and EE respectively, for vitamin D₃-SLNs at zero time.

5.0 PHARMACOKINETIC STUDIES

The pharmacokinetic studies in rats revealed a significant improvement (p<0.05) of almost 30 times or more in bioavailability, both after subcutaneous and oral administration, with respect to free drug solution (vitamin D₃-S) administered similarly. Cₘₐₓ value for single oral vitamin D₃ loaded SLN dose, was 374.79 ng/ml whereas it was only 14.72 ng/ml for the free drug administered at the same dose (Table 10). A Cₘₐₓ not more than 75 nmol/ml (30ng/ml) in plasma is reported after oral administration of 4 repetitive doses of 2.5 mg of vitamin D₃ in humans. However, presently, significant drug levels i.e. 97 ng/ml were observable in
plasma, even after 48 h post administration of vitamin D₃ loaded SLNs. Further Kᵦᵩ of oral vitamin D₃ loaded SLNs was significantly low (2.33 times) and t½ was increased by almost 10 times, as compared to free drug (vitamin D₃-S), confirming the sustained effect of vitamin D₃-SLNs.

Table 10: Single dose (0.21 mg/kg body weight) oral pharmacokinetics of vitamin D₃ administered to rats, as free drug (vitamin D₃-S) and upon loading into SLNs (vitamin D₃-SLNs).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cmax (ng/ml)</th>
<th>Tmax (h)</th>
<th>AUC₀-7 (hr*ng/ml)</th>
<th>AUC₀-∞ (hr*ng/ml)</th>
<th>Kᵦᵩ (h⁻¹)</th>
<th>T½ (h)</th>
<th>MRT (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D₃-SLNs</td>
<td>374.79</td>
<td>8</td>
<td>9803.84</td>
<td>21120.24</td>
<td>0.03</td>
<td>29.61</td>
<td>15.67</td>
</tr>
<tr>
<td>Vitamin D₃-S</td>
<td>14.72</td>
<td>0.5</td>
<td>293.00</td>
<td>608.00</td>
<td>0.07</td>
<td>2.59</td>
<td>3.86</td>
</tr>
</tbody>
</table>

Table 11: Pharmacokinetic parameters of single dose subcutaneous injection of vitamin D₃ (0.54 mg/kg body weight), to rats (n=5), as free drug (vitamin D₃-S) and upon loading into SLNs (vitamin D₃-SLNs).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cmax (ng/ml)</th>
<th>Tmax (h)</th>
<th>AUC₀-7 (hr*ng/ml)</th>
<th>AUC₀-∞ (hr*ng/ml)</th>
<th>Kᵦᵩ (h⁻¹)</th>
<th>T½ (h)</th>
<th>MRT (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D₃-SLNs</td>
<td>322.50</td>
<td>8.0</td>
<td>10110.57</td>
<td>24579.87</td>
<td>0.02</td>
<td>45.60</td>
<td>21.10</td>
</tr>
<tr>
<td>Vitamin D₃-S</td>
<td>18.0</td>
<td>1.00</td>
<td>274.00</td>
<td>834.30</td>
<td>0.09</td>
<td>7.30</td>
<td>4.26</td>
</tr>
</tbody>
</table>
Table 12: Plasma concentration-time values obtained after oral and subcutaneous administration of vitamin D₃ and vitamin D₃-SLNs.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>vitamin D₃ -S</th>
<th>vitamin D₃-SLNs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. (ng/ml)</td>
<td></td>
</tr>
<tr>
<td>Oral route</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>13.32±4.2</td>
<td>27.09±5.3</td>
</tr>
<tr>
<td>1</td>
<td>14.72±2.6</td>
<td>92.86±12.2</td>
</tr>
<tr>
<td>2</td>
<td>14.44±3.2</td>
<td>165.03±16.5</td>
</tr>
<tr>
<td>4</td>
<td>13.55±2.8</td>
<td>245.33±20.2</td>
</tr>
<tr>
<td>8</td>
<td>13.62±2.5</td>
<td>374.79±46.2</td>
</tr>
<tr>
<td>16</td>
<td>13.85±1.8</td>
<td>211.48±21.0</td>
</tr>
<tr>
<td>24</td>
<td>13.70±1.2</td>
<td>173.41±10.1</td>
</tr>
<tr>
<td>48</td>
<td>11.77±1.16</td>
<td>97.00±3.8</td>
</tr>
<tr>
<td>Subcutaneous route</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>13.53±2.4</td>
<td>78.61±12.6</td>
</tr>
<tr>
<td>1</td>
<td>18.02±1.4</td>
<td>136.30±11.7</td>
</tr>
<tr>
<td>2</td>
<td>17.24±1.3</td>
<td>175.29±28.2</td>
</tr>
<tr>
<td>4</td>
<td>15.38±2.2</td>
<td>270.86±19.5</td>
</tr>
<tr>
<td>8</td>
<td>12.51±3.6</td>
<td>322.50±23.2</td>
</tr>
<tr>
<td>16</td>
<td>12.32±1.7</td>
<td>253.63±16.3</td>
</tr>
<tr>
<td>24</td>
<td>11.12±3.2</td>
<td>140.57±24.8</td>
</tr>
<tr>
<td>48</td>
<td>10.25±1.1</td>
<td>111.00±6.7</td>
</tr>
</tbody>
</table>

Cmax value for single subcutaneous injection of vitamin D₃ loaded SLN was 322.50 ng/ml whereas levels of vitamin D₃ post free drug administration were 18.0 ng/ml. Significant amount was detectable (111ng/ml) in plasma (Table 12), even after 48h (t½ 45.60h) post-administration of vitamin D₃-SLNs subcutaneously in the present investigation. Further, Ksp of vitamin D₃-SLNs was significantly low (4.7 times) and t½ was 6.2 times longer than vitamin D₃-S, corroborating the prolonged retention and circulation of these nanoparticles upon subcutaneous administration (Table 11).

Bioavailability enhancement achieved with vitamin D₃-SLNs by either route, is attributed to small particle size which in turn improves absorption, enhances systemic levels and prolongs circulation of vitamin D₃ in plasma. This is achieved by the ability of SLNs to i) adhere to git mucosa, ii) undergo lymphatic uptake, bypassing first pass hepatic metabolism, iii) overwhelm reticuloendothelial system pickup in plasma including removal by liver and spleen cells due to size < 200 nm and hydrophilic coating provided by tween 80 present in the SLNs, iv) protect the entrapped vitamin by reducing its exposure to the enzymatic and metabolic milieu of the liver, thus ensuring a sustained
conversion to 25(OH)D, resembling the effect produced by endogenously synthesized vitamin D₃.

6.0 DISCUSSION

Vitamin D deficiency has been defined by the Institute of Medicine (IOM) as the 25(OH) D concentrations of less than 20 ng/ml, while vitamin D insufficiency is defined as a 25(OH) D concentrations of 21-29 ng/ml (Bischoff-Ferrari et al., 2008; Hansen et al., 2008; Heaney, 2004; Heaney et al., 2003; Holick, 2007; IOM 2011; Malabanan et al., 1998). In accordance with these definitions, it has been estimated that 20-100% of U.S., Canadian, and European elderly men and women are vitamin D deficient (Chapuy M et al., 1996; Greene-Finestone et al., 2011; Holick, 2006; Holick, 2007; Holick et al., 2005; Lips et al., 2006). An association of vitamin D₃ deficiency with increased risk of more than a dozen cancers, including colon, prostate, breast, and pancreas; autoimmune diseases, including both type 1 and type 2 diabetes, rheumatoid arthritis, crohn’s disease, and multiple sclerosis; infectious diseases; and cardiovascular disease is established. Vitamin D supplementation/treatment is inexpensive and would be cost-effective, particularly in treating entities such as osteoporosis, rickets, and osteomalacia.

In the United States and Canada, milk is fortified with vitamin D, as are some bread products, orange juices, cereals, yogurts, and cheeses (Holick, 2007). However, excess fortification or intake may lead to undesired effects such as hypocalcaemia, kidney stone and hypertension. There was an outbreak of vitamin D intoxication in young children in Europe upon consumption of such products (Holick et al., 2011).

Preparation and characterization of hydrophobic alginate derivative for sustained release of vitamin D₃ has been reported (Li et al., 2011). Objective of the authors was to elaborate nanoparticles from hydrophobically modified alginate to develop a carrier for oral administration of vitamin D₃. The authors purport that the BA of vitamin D₃ can be enhanced by improving its water solubility. An entrapment efficiency of 67.6% was reported by these workers whereas we could achieve an entrapment efficiency of more than 85%. The particle size achieved by them is also high i.e. more than 500 nm while presently we could achieve a particle size of less than 200 nm, which in turn results in a higher plasma concentration and
prolonged circulation in plasma, resulting in an almost 3000% or more increase in bioavailability.

Furthermore, the most popular approach to improve the oral bioavailability of lipophilic drugs is the utilization of lipid based formulations including lipidic nanoparticles (Humberstone and Charman, 1997; Pouton, 2000). Latter are expected to enhance the dissolution and solubilization of the drugs by stimulation of biliary and pancreatic secretions, prolongation of GIT residence time, stimulation of lymphatic transport, increased intestinal wall permeability and reduced metabolism and efflux activity (Dintaman and Silverman, 1999).

In continuation to above, another group has reported on the pharmacokinetics of these oleoyl alginate ester (OAE) nanoparticles (Sun et al., 2012). In vivo pharmacokinetic studies using OAE nanoparticles showed a 1.4 times increase in the absorption of vitamin D$_3$. Its application in the treatment of rickets was assayed using a model of nutritionally induced vitamin D-deficiency rickets. The results showed that the encapsulated vitamin D$_3$ had better efficacy than that of the free drug in vivo.

Tripalmitin SLNs of ergocalciferol (vitamin D$_2$) are also reported (Patel and San Martin-Gonzalez, 2012). The SLNs were prepared using high pressure homogenization method, with up to 20% loading; however the authors do not report in vitro release and in vivo pharmacokinetics of the developed SLNs. Also they did not quantify the formulation for total drug content, entrapment efficiency, stability and photostability studies and have used UV/VIS spectrophotometer for all the analysis. Howsoever, particle size and shape of our formulation was found to be similar to the one reported by them.

Solid lipid nanoparticles of vitamin D$_3$ were formulated presently to achieve i) drug solubilisation, ii) enhanced permeability across the biological membranes including gut and across blood vessels (upon s.c administration), iii) protection against oxidation by light and air, iv) protection against direct delivery to and a fast conversion to 25(OH) D in an equally efficient elimination from liver. Incorporation into SLNs will not only carry intact vitamin D$_3$ (across gut, without any enzymatic including biliary metabolism and degradation) into the plasma, but will also limit its fast conversion to 25(OH) D as observed for exogenously administered naked/free vitamin D$_3$. Vitamin D$_3$ produced endogenously in the
skin, upon sun-exposure, lasts at least twice as long in blood as that ingested orally (Haddad et al., 1993). Vitamin D3-SLNs will thus behave in a manner similar to that exhibited by endogenously synthesised vitamin D3. This is confirmed by the fact that we presently determined and report vitamin D3 concentration in the plasma rather than the serum calcidiol (calcifediol) determinations made and reported by most workers. Presently vitamin D3-SLNs were prepared by novel method of microemulsification (Kaur and Verma, 2012; 2013). Latter is a spontaneous process, based on crystallization of oil droplets in microemulsion, post transferring in an equal volume of aqueous media under stirring at 2-8°C, to result in a highly concentrated and hence stable SLN dispersion (Kumar et al., 2014b). Compritol® 888 ATO is reported to result in high entrapment efficiencies in addition to producing stable dispersions with small particle size (Bhandari and Kaur, 2013a; Bhandari and Kaur, 2013b; Kakkar et al., 2011b; Singh et al., 2013a). Small particle size (<200nm) and encapsulation of vitamin D3 within the lipid overwhelms its RES pick up in liver, resulting in prolonged circulation times and avoidance of metabolic degradation by liver enzymes (Kaur et al., 2008). Drugs administered by subcutaneous (s.c) routes are absorbed at a slower rate compared to other parenteral routes again providing a sustained effect. The exact mechanism of absorption though unknown, is expected to be due to direct uptake of molecules within the subcutis by small capillaries underlying the skin, with minimal lymphatic absorption (Khan et al., 2013). The microemulsification technique was highly reproducible in terms of particle size, its distribution and also EE (n=6). When observed under TEM, particles appeared rod shaped. It is reported that weak circular and ellipsoidal structures appear as thin platelets in top view (Jores et al., 2004). Platelet-like particles consisting of a few molecular layers, have already been observed in dispersions of saturated monoacid triglycerides in the h-modification (Bunjes et al., 2000; Unruh et al., 1999). Some authors also report the formation of platelet like structures for dispersions of a complex glyceride mixture with comparatively low triglyceride content (Jores et al., 2004). Compritol® 888 ATO employed by us presently for the preparation of SLNs is also a mixture of approximately 15% mono, 50% di and 35% triglycerides of behenic acid (C22).
In vitro release study showed a zero order prolonged release of vitamin D₃. Latter may be attributed to an additive effect of diffusion and dissolution from the lipid. DSC and PXRD studies confirmed the formation of SLNs as indicated by disappearance of sharp peaks otherwise observed otherwise for free vitamin D₃ and Compritol® 888 ATO. Latter may indirectly indicate the possibility of an improved solubility, both of vitamin D₃ and of the absolute system per se. The stability studies indicated that the prepared vitamin D₃-SLN were stable after one year of storage at 5±3°C (Kumar et al., 2014b). PS studies indicate that incorporation into SLNs could protect vitamin D₃ against light degradation while >40.0 % and >60.0 % of the free drug degraded on exposure to white florescence light at 1.2 million lux hr for 10 days after storage in amber or clear glass vials, respectively (Kumar et al., 2014b). It may be noted that considerable degradation occurred for the free drug solution even in amber colored containers which may be attributed to the oxidative photodegradation triggered by even a mild exposure to light. The EE of vitamin D₃-SLN did not undergo any significant change (p≤0.05) even upon storage under refrigerated conditions for upto 1 year. Latter is attributable to the possibility that free vitamin D₃ (not removed from the dispersion; 0.44 mg/ml) is present in equilibrium with the small volume of water (as only 1:1 dilution of microemulsion is done presently versus a usual 1: 10 to 1: 50 dilution) in SLN dispersion so that the leakiness of vitamin D₃ from its SLNs is minimal (Kumar et al., 2014b). It may be noted that vitamin D₃ is a poorly soluble drug, with a solubility of < 0.01 mg/ml.

The few pharmacokinetic studies reported for vitamin D in humans, report very low serum and plasma concentrations, by oral route of administration owing to its poor absorption. An improved patient response to antimicrobial treatment for pulmonary tuberculosis after administration of daily dose of 625 μg to 2.5 mg of vitamin D is reported (Martineau et al., 2011). Ilahi et al. (Ilahi et al., 2008) report on pharmacokinetics of a single, large dose (100000 IU) of cholecalciferol, with a prompt rise in serum calcidiol from a mean (SD) baseline of 27.7±7.7 ng/mL to a concentration maximum of 42.0±9.1 ng/ml. However the authors measured serum calcidiol levels rather than the cholecalciferol (vitamin D₃) levels.
We presently report a 35.0 and 29.5 times enhanced bioavailability of cholecalciferol post administration of vitamin D₃-SLN as compared to vitamin D₃-S, after oral and subcutaneous routes, respectively in rats. The reported values are corrected for the baseline plasma levels of vitamin D₃ in rats (10.83 ±1.5 ng/ml]). Significantly higher C_{max}, AUC_{0-t}, AUC_{0-∞} complemented with longer t_{1/2} and reduced Kel values (Table 10 & 11) also confirmed improved biopharmaceutical performance of vitamin D₃ upon incorporation into SLNs. Further, mean residence time for SLNs administered by oral and subcutaneous route was 4 times and 5 times, respectively, that obtained for vitamin D₃-S administered at the same dose and by the same route, confirming prolonged plasma circulation of vitamin D₃-SLN.

Administration of encapsulated vitamin D₃ by subcutaneous route is especially predicted to simulate the endogenous synthesis of vitamin D₃ upon exposure to UV light. It is reported that vitamin D₃ produced in the skin lasts twice as long in the blood, compared to ingested vitamin D. It may be noted that an unprotected sun exposure is the major source of vitamin D for both children and adults. However, concerns about melanoma and other types of skin cancer in addition to various allergies and skin diseases, necessitate avoidance of excessive exposure to midday sun. These observations strengthen the arguments for vitamin D₃ supplementation, especially for people living above 33° latitude (Grant et al., 2009). However as discussed and reported above latter can be achieved effectively only by incorporation of vitamin D₃ into a suitable carrier and success achieved by administration of free vitamin D is highly speculative and unreliable.

Srinivasan et al (Srinivasan et al., 2013) reported, that patients with TB show a deficiency of cholecalciferol and 13-cis-retinoic acid (13-cis-RA) in comparison to healthy volunteers, indicating a need to conduct a trial on supplementation of available formulations of vitamin A and D (cholecalciferol and 13-cis-RA) as the novel anti-tubercular drug therapy. Administration of nanoformulations of vitamin D₃ in such patients can thus be a revolutionary step in the treatment/prophylaxis of tuberculosis.
7.0 CONCLUSIONS

We ascertain here that incorporating vitamin D₃ into SLNs protected it against degradative effects of light and air with significantly enhanced bioavailability post administration both by the subcutaneous and oral route. Potential of vitamin D₃-SLNs is also revealed from the maintenance of significant concentrations of vitamin D₃ in plasma even after 48 h post administration of single dose by either route. Vitamin D₃-SLNs are proposed herein as an intervention for tubercular patients, once the pharmacodynamic proof of this concept for the treatment of tuberculosis is established. It can further be extended to the dosage regimens for more deadly diseases like cancers, in addition to their use for vitamin D related nutritional and therapeutic supplementation in rickets, osteomalacia, osteoporosis and calcium absorption.