4.1 Materials
Repaginide and metformin were provided by Ranbaxy (Gurgaon, India) and Sefsol 218 (Propylene glycol-monocaprylic ester) from Nikko Chemicals (Tokyo, Japan) as gift samples. Tween 80 (Polyoxyethylene sorbitan monooleate) and Hydrogenated castor oil (HCO), purchased from Tongliao Tonghua Castor Chemical Co, Ltd (Inner Mongolia, China) and Merck (Schuchardh, Hokenbrunn, Germany). Water was taken from Milli-Q water purification system (Millipore, Billerica, MA).

All other chemicals and reagents used were of analytical (AR) grade and procured from Merck (India) and S.D. Fine Chem. (India). All components used for the formulation of nanoemulsion were pharmaceutically acceptable for oral administration.

4.2 Preformulation and authentication of drug

4.2.1 Differential Scanning Colorimetry (DSC)
DSC thermogram was obtained in the temperature range of 40-400°C at 10°C/min rate on Pyris 6 DSC instrument and scan was obtained.

4.2.2 Ultra Violet Spectrum (UV)
An UV spectrum of drug was taken at a concentration of 10 µg/mL in methanol from 200 to 400 nm range at room temperature. 10 mg drug sample was dissolved in small amount of methanol and final volume was made up to 250 mL (stock solution) and out of this stock solution, 0.1 mL was taken in 10 mL volumetric flask and diluted to 10 mL by methanol. The spectra were taken in other solvents also like octanol, 0.1N HCl, phosphate buffer (pH 6.8) and distilled water.

4.2.3 Fourier Transform Infra Red Spectroscopy (FTIR)
An FTIR spectrum was obtained by pellet technique using potassium bromide. A small amount of drug was mixed with triple amount of potassium bromide and pellets were made.

4.2.4 pH solubility profile
The solubility of each drug in different pH was determined by equilibrium solubility method which employed a saturated solution of the material, obtained by stirring an excess of material in the solvent for a prolonged period until equilibrium was achieved. For determination of pH solubility of the API, flask shake method was used. The concentration was determined by UV spectrophotometer at 240 nm and 234 nm respectively.

4.2.5 Stability at different temperatures

To study the degradation effect of temperature (37°C and 8°C), the drug solutions in methanol (10 µg/mL) were kept in stoppered volumetric bottle for 24 hrs and analyzed by UV spectrophotometer.

4.3 Analytical methodology

4.3.1 Repaglinide

To our knowledge, no article related to the stability-indicating HPTLC determination of repaglinide in pharmaceutical dosage forms had been reported in the literature. An ideal stability-indicating method was one that quantifies the standard drug alone and also resolved its degradation products. Basic acceptance criteria for evaluation of validation experiments based on practical experience for planar chromatographical procedures was explained, which may be used at different levels either in qualitative identity testing, assays, semi-quantitative limit tests or quantitative determination of impurities (Ferenczi-Fodor et al, 2001). The parameters for robustness testing of the given procedures and quality assurance of quantitative planar chromatographical testing had been described as per International Conference on Harmonization (ICH) guidelines. The aim of the present work was to develop an accurate, specific, repeatable and stability-indicating HPTLC method for the determination of repaglinide in the presence of its degradation products.
and related impurities for assessment of purity of bulk drug and stability of its bulk dosage forms. The proposed method was validated as per ICH guidelines (ICH Guidelines Q2A, Q2B, 1994 & 1996). Acid-induced degradation kinetics were investigated by quantitation of drug by validated stability-indicating HPTLC method.

4.3.2 Metformin
An already developed simple reverse phase high-performance liquid chromatographic method for determining the concentration of metformin was used. The method employed C18 column (300 mm × 2.4 mm i.d.), ammonium acetate (0.15 M) and acetonitrile (90:10; pH-5.5; 1.0 ml/min) as mobile phase and ultraviolet detection at 234 nm. Acetonitrile was used to simultaneously deproteinize rat plasma and extract metformin. The assay was linear in the concentration range of 0.33 μg-16.6 μg/ml with co-efficient of correlation 0.994. The retention time was 4.7 min (Wanjari et al, 2008).

4.4 Formulation development
4.4.1 Repaglinide
A. Screening of components
All components used for the formulation of nanoemulsion should be pharmaceutically acceptable for oral administration. For development of nanoemulsion drug delivery system following components were taken.

i. Oils
Differents oils were selected and solubility of drug was checked in the following oils, so that highest solubility of RPG could be achieved.

- Oleic acid
- Propylene glycol monocaprylic ester (Sefsol 218)
- Isopropyl myristate (IPM)
- Glycerol triacetate (Triacetin)
ii. Surfactants
The surfactants used in the study were:
- Polyoxyethylene (20) sorbitan mono oleic acid (Tween 80)
- Tween 20
- Span 20 etc.

iii. Cosurfactants
The cosurfactants used in the study were:
- Ethyl alcohol
- Transcutol
- n-Butanol
- Isopropyl alcohol etc.

B. Phase solubility studies
Three mL of selected oils [Oleic acid, isopropyl myristate (IPM), glycerol triacetate (Triacetin), caproyl 90, propylene glycol monocaprylic ester (Sefsol 218), propylene glycol laurate (Lauroglycol), labrafac] were taken in small vials (5.0 mL capacity) and excess amount of drug was added in the oils and kept in biological shaker (Nirmal International, Delhi, India) for 72 h at a constant temperature (25 ± 1.0 °C) to reach to an equilibrium (Shafiq et al, 2007, Shakeel et al, 2007). The samples were removed from shaker and centrifuged at 3000 rpm for 15 min. The supernatant was filtered through a 0.45 μm membrane filter and the concentration of RPG was determined by taking absorbance using HPTLC at λmax of 240 nm after dilution.

C. Pseudoternary phase diagram construction
Different volume ratios (1:0, 1:1, 1:2, 1:3, 2:1, 3:1) of surfactant (Tween 80) and co-surfactant (Transcutol) mixture ($S_{mix}$) were made and stocks of 100 mL from each group were prepared (Chennamsetty et al, 2005). For each phase diagram, sixteen different combinations of oil (Sefsol 218) and $S_{mix}$ [1:9, 1:8, 1:7, 1:6, 1:5, 2:8 (1:4), 1:3.5, 1:3, 3:7 (1:2.3), 1:2, 4:6 (1:1.5), 5:5 (1:1), 6:4 (1:0.7), 7:3 (1:0.43), 8:2 (1:0.25), 9:1 (1:0.1)] were made in different volume ratios from 1:9 to 9:1 so that maximum ratios were covered for the study (Lawrence & Rees, 2000). The mixture of selected oil and $S_{mix}$ were titrated against distilled water. After every 5% addition of aqueous phase to the oil and $S_{mix}$ mixture, visual observation was made and recorded. The percentage of water, oil and $S_{mix}$ in which nanoemulsion forms were selected and plotted on ternary phase diagrams with one axis representing the aqueous phase, the other representing the oil and the third representing the $S_{mix}$. These observations were made for each $S_{mix}$ ratio in each group separately. (Table 4.1 & Table 4.2)

Different formulations were selected from each phase diagram plotted for different $S_{mix}$ ratios on the basis of following points.

- The oil concentration was such that it dissolved single dose (2 mg) of repaglinide easily.
- Oil concentration from each phase diagram was selected as a multiple of five %, i.e. 5%, 10% 15% and 20%.
- For each oil percentage selected, the concentration of surfactant was minimum for nanoemulsion preparation (Shafiq et al, 2007).
**Table 4.1.** Preparation of $S_{\text{mix}}$ ratios

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Volume of Surfactant (mL)</th>
<th>Volume of Cosurfactant (mL)</th>
<th>Ratio of $S_{\text{mix}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>50</td>
<td>1:1</td>
</tr>
<tr>
<td>2</td>
<td>33.3</td>
<td>66.7</td>
<td>1:2</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>75</td>
<td>1:3</td>
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<tr>
<td>4</td>
<td>66.7</td>
<td>33.3</td>
<td>2:1</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>25</td>
<td>3:1</td>
</tr>
</tbody>
</table>
Table 4.2. Titration chart to find out nanoemulsion region

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Oil µl</th>
<th>Surfactant (S&lt;sub&gt;mix&lt;/sub&gt;) µl</th>
<th>Water µl</th>
<th>Water added µl</th>
<th>Total µl</th>
<th>Oil %</th>
<th>Surfactant (S&lt;sub&gt;mix&lt;/sub&gt;) %</th>
<th>Water %</th>
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</thead>
<tbody>
<tr>
<td>1:9</td>
<td>10</td>
<td>90</td>
<td>10</td>
<td>10</td>
<td>110</td>
<td>9.09</td>
<td>81.82</td>
<td>9.09</td>
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<td>90</td>
<td>300</td>
<td>65</td>
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<td>23</td>
<td>75</td>
</tr>
<tr>
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<td>10</td>
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<td>400</td>
<td>100</td>
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<td>18.00</td>
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<td>10</td>
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<td>2000</td>
<td>1100</td>
<td>2100</td>
<td>0.48</td>
<td>4.29</td>
<td>95.24</td>
</tr>
</tbody>
</table>

D. Thermodynamic stability
Selected formulations were subjected to thermodynamic stability stress tests as heating cooling cycle, centrifugation and freeze–thaw cycle: Heating–cooling cycles between 45 °C temperature and room
temperature (25 ± 2 °C) with storage time of 24 h at each temperature (six cycles each) followed by centrifugation (5000 rpm for 30 min) and then Freeze–thaw cycles were done after storing the samples below −20 °C in a deep freezer (Vest frost, Hyderabad, India) and thawed at room temperature (25 ± 2 °C) for 24 h were carried out six times (six cycles each).

**E. Dispersibility tests**

The efficiency of self emulsification of oral nanoemulsion was assessed using a standard USP XXII dissolution apparatus. One mL of nanoemulsion was mixed with 500 mL of media (distilled water and 0.1N HCl, separately) maintained at 37 ± 0.5 °C. The dissolution paddle rotated at a speed of 50 rpm to provide gentle mixing. The *in vitro* performance of the formulations was visually assessed on the basis of following grading system (Table 4.1) (Ping et al, 2005).

**Table 4.3** Grading system for dispersibility test.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Grade</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>Rapidly forming (within 1 min) nanoemulsion, having a clear or bluish appearance.</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>Rapidly forming, slightly less clear nanoemulsion, having a bluish white appearance.</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>Fine milky emulsion that formed within 2 min.</td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td>Dull grayish white emulsion having slightly oily appearance that is slow to emulsify (longer than 2 min).</td>
</tr>
<tr>
<td>5</td>
<td>E</td>
<td>Formulation exhibiting either poor or minimal emulsification with large oil globules present on the surface.</td>
</tr>
</tbody>
</table>
F. Formulation of drug containing nanoemulsion

Drug containing nanoemulsion formulations were prepared by dissolving 2 mg/kg body weight of drug in 5%, 10%, 15% and 20% of oil and respective $S_{\text{mix}}$ ratios on vortex mixer and added required quantity of aqueous phase. The resulting mixture gave nanoemulsion.

G. Characterization of repaglinide nanoemulsion

Nanoemulsions can be characterized by using different types of techniques.

i) Visual observation

Visual observation was done to differentiate between nanoemulsion and macroemulsion.

ii) Surface morphology

Surface morphology of nanoemulsion was studied by Transmission Electron Microscopy (TEM) TOPCON 002B (Topcon, USA) (Shafiq et al, 2007, Shakeel et al, 2007). A drop of nanoemulsion was diluted with distilled water (1:100), filtered (0.22 μm) and applied on carbon coated grid with 2% phosphotungestic acid and kept it for 30 s. The dried coated grid was taken on a slide and covered with a cover slip. The slide was observed under the light microscope operating at 200 KV.

iii) Droplet size analysis (particle size distribution)

Polydispersity is the ratio of standard deviation to the mean droplet size and denotes the uniformity of droplet size within the formulation. The lower the polydispersity value, higher is the uniformity of the droplet size in the formulation. Droplet size measurement is the important parameter to optimize the nanoemulsion formulation as well as to distinguish between the nanoemulsion from microemulsion.

iv) Viscosity determination

Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) with spindle # CPE40
at 25 ± 0.5 °C was used for the determination of viscosity of the formulations. The optimized parameters used were: Sample size/wt: 0.5 g, Speed: 30 rpm, Data interval: 1.0, Loop start: 1, Wait time: 30 min, Temperature: 25 ± 0.3 °C, Shear rate: 60 s⁻¹.

v) **Refractive index**
Refractive index of formulation was determined using an Abbes type of refractrometer (Precision Standard Testing Equipment Corporation, India), which was calibrated using castor oil prior to use.

vi) **Electrical conductivity**
The conductivity (σ) of nanoemulsion was determined by using conductometer, CDM 230 (Radiometer, Copenhagen, Denmark). The reading was taken at the frequency of 94 Hz, having a cell constant of 0.11 cm⁻¹. The measurements were performed at 25 ± 1 °C.

4.4.2. **Metformin**

A. **Nanoemulsion component selection**
All components used for the formulation of nanoemulsion should be pharmaceutically acceptable for oral administration. For development of nanoemulsion drug delivery system following components were taken.

i. **Oils**
Differents oils were selected and solubility of drug was checked in the following oils, so that highest solubility of MET could be achieved.

- Oleic acid
- Hydrogenated castor oil
- Isopropyl myristate (IPM)
- Glycerol triacetate (Triacetin)
- Caproyl 90
- Propylene glycol laurate (Lauroglycol)
- Labrafac etc.
The surfactants used in the study were:

- Polyoxyethylene (20) sorbitan mono oleic acid (Tween 80)
- Tween 20
- Span 20 etc.

iii. Cosurfactants

The cosurfactants used in the study were:

- Transcutol
- $n$-Butanol
- Isopropyl alcohol etc.

B. Formulation development and optimization

Three mL of selected oils [Oleic acid, isopropyl myristate (IPM), glycerol triacetate (Triacetin), caproyl 90, Hydrogenated castor oil (HCO), propylene glycol laurate (Lauroglycol), labrafac] were taken in small vials (5.0 mL capacity) and excess amount of drug was added in the oils and kept in biological shaker (Nirmal International, Delhi, India) for 72 h at a constant temperature (25 ± 1.0 °C) to reach to an equilibrium (Shafiq et al., 2007 & Shakeel et al., 2007). The samples were removed from shaker and centrifuged at 3000 rpm for 15 min. The supernatant was filtered through a 0.45 μm membrane filter and the concentration of drug was determined by taking absorbance using HPLC method at $\lambda_{\text{max}}$ of 234 nm after dilution.

Different formulations were selected from each phase diagram plotted for different $S_{\text{mix}}$ ratios on the basis of following points.

- The oil concentration was such that it dissolved single dose of (125 mg) of metformin easily.
- Oil concentration from each phase diagram was selected as a multiple of five %, i.e. 5%, 10% 15% and 20%.
- For each oil percentage selected, the concentration of surfactant was minimum for nanoemulsion preparation. (Shafiq et al., 2007).
Different volume ratios (1:0, 1:1, 1:2, 1:3, 2; 1, 3:1) of surfactant (Tween 80) and co-surfactant (Transcutol) \textbf{(Chennamsetty et al, 2005)}. Mixture ($S_{\text{mix}}$) was made and stocks of 100 mL from each group were prepared. For each phase diagram, sixteen different combinations of hydrogenated castor oil (HCO) and $S_{\text{mix}}$ [1:9, 1:8, 1:7, 1:6, 1:5, 2:8 (1:4), 1:3.5, 1:3, 3:7 (1:2.3), 1:2, 4:6 (1:1.5), 5:5 (1:1), 6:4 (1:0.7), 7:3 (1:0.43), 8:2 (1:0.25), 9:1 (1:0.1)] were made in different volume ratios from 1:9 to 9:1 so that maximum ratios were covered for the study. \textbf{(Lawrence & Rees, 2000)}. The mixture of selected oil and $S_{\text{mix}}$ were titrated against distilled water. After every 5% addition of aqueous phase to the oil and $S_{\text{mix}}$ mixture, visual observation was made and recorded. The percentage of water, oil and $S_{\text{mix}}$ in which nanoemulsion forms were selected and plotted on ternary phase diagrams with one axis represents the aqueous phase, the other representing the oil and the third representing the $S_{\text{mix}}$. These observations were made for each $S_{\text{mix}}$ ratio in each group separately.

C. Phase diagram construction

Pseudoternary phase diagrams were developed using the aqueous titration method. Slow titration with the aqueous phase was performed for each combination of oil and $S_{\text{mix}}$, separately. The amount of aqueous phase added was varied to produce a water concentration in the range of 5% to 95% of total volume at around 5% intervals. The phase behavior of nanoemulsion system comprising oil, water and $S_{\text{mix}}$ ratio can be studied with the aid of ternary phase diagram in which each corner of the diagram represents 100% of that particular component. Special care was taken to ensure that observations are not made on metastable systems.

D. Thermodynamic stability studies

Selected formulations were subjected to thermodynamic stability stress tests as heating cooling cycle, centrifugation and freeze–thaw cycle:
Heating–cooling cycles between 45 °C temperature and room temperature (25 ± 2 °C) with storage time of 24 h at each temperature (six cycles each) followed by centrifugation (5000 rpm for 30 min) and then Freeze–thaw cycles were done after storing the samples below −20 °C in a deep freezer (Vest frost, Hyderabad, India) and thawed at room temperature (25 ± 2 °C) for 24 h were carried out six times (six cycles each).

E. Dispersibility tests
The efficiency of self emulsification of oral nanoemulsion was assessed using a standard USP XXII dissolution apparatus. One mL of nanoemulsion was mixed with 500 mL of media (distilled water and 0.1N HCl, separately) maintained at 37 ± 0.5°C. The dissolution paddle rotated at a speed of 50 rpm to provide gentle mixing. (Ping et al, 2005)

F. Formulation of drug containing nanoemulsion
Drug containing nanoemulsion formulations were prepared by dissolving 125 mg/kg body weight of drug in 5%, 10%, 15% and 20% of oil and respective \( S_{mix} \) ratios on vortex mixer and added required quantity of aqueous phase. The resulting mixture gave nanoemulsion.

G. Characterization of metformin nanoemulsion
i. Visual observation
Visual observation was done to differentiate between nanoemulsion and macroemulsion.

ii. Surface morphology
Surface morphology of nanoemulsion was studied by Transmission Electron Microscopy (TEM) TOPCON 002B (Topcon, USA). A drop of nanoemulsion was diluted with distilled water (1:100), filtered (0.22 μm) and applied on carbon coated grid with 2% phosphotungestic acid and kept it for 30 s. The dried coated grid was taken on a slide and covered
with a cover slip. The slide was observed under the light microscope operating at 200 KV.

iii. Droplet size analysis
Droplet size of the nanoemulsion was determined by photon correlation spectroscopy using Zetasizer 1000 HS (Malvern Instruments, Worcestershire, UK). (Shafiq et al, 2007 & Shakeel et al, 2007) The formulation was diluted with distilled water and filtered through 0.22 μm membrane filter in order to eliminate multiscattering phenomena and experimental errors. Light scattering was monitored at 25 °C at a scattering angle of 90°.

iv. Viscosity determination
Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) with spindle # CPE40 at 25 ± 0.5 °C was used for the determination of viscosity of the formulations. The optimized parameters used were: Sample size/wt: 100 mg. Speed: 30 rpm, Data interval: 1.0, Loop start: 1, Wait time: 30 min, Temperature: 25 ± 0.3 °C, Share rate: 60 s⁻¹.

v. Refractive index
Refractive index of formulation was determined using an Abbes type of refractrometer (Precision Standard Testing Equipment Corporation, India), which was calibrated using castor oil prior to use.

vi. Electrical conductivity
The conductivity (σ) of nanoemulsion was determined by using conductometer, CDM 230 (Radiometer, Copenhagen, Denmark). The reading was taken at the frequency of 94 Hz, having a cell constant of 0.11 cm⁻¹. The measurements were performed at 25 ± 1 °C.

4.5 In vitro drug release
In vitro release test was performed for selected repaglinide nanoemulsions in 500 mL of distilled water and simulated gastric fluid
using dissolution apparatus # 2, at 50 rpm and 37 ± 0.5 °C (Hanson Research SR8 plus, California, United States). One millilitre of nanoemulsion formulation was placed in treated dialysis bag (MWCO 1200 g/mole, Sigma Aldrich, USA). One millilitres samples were withdrawn at regular time intervals (0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 24 h) and aliquot amount of distilled water/simulated gastric fluid was replaced. The samples were analysed for the drug content using validated HPTLC method at 240 nm.

*In vitro* release test was also performed for selected metformin nanoemulsions in 500 mL of distilled water and simulated gastric fluid using dissolution apparatus # 2, at 50 rpm and 37± 0.5 °C (Hanson Research SR8 plus, California, United States). One millilitre of nanoemulsion formulation was placed in treated dialysis bag (MWCO 1200 g/mole, Sigma Aldrich, USA). One millilitres samples were withdrawn at regular time intervals (0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 24 h) and aliquot amount of distilled water/simulated gastric fluid was replaced. The samples were analysed for the drug content using HPLC method at 234 nm.

4.6 Biochemical evaluation

Approval to carry out *in vivo* study was obtained from Integral University, Institutional Animal Ethics Committee, Lucknow, Uttar Pradesh (Registration No. IU/Pharm/Ph.D./CPCSEA/10/17) and CPCSEA guidelines were adhered for the complete study. The animals used for *in vivo* experiments were diabetic Sprague dawley (SD) rats. The *in vivo* study was performed to carry out oral administration of repaglinide formulations in diabetic rats. Diabetes was induced by single Streptozotocin (STZ) injection (100 microgram/ gram i.p.) in rat pups (30-40 g) under extremely aseptic condition. The general health conditions of rats were observed daily. When rats became adult (125-
150g) they were checked whether diabetic, having blood glucose more than 300 mg/dl. (Arulmozhi et al, 2004, Jain et al, 2005 & Weiss, 1982) The blood glucose level was estimated with glucometer One Touch Basic Plus® (Life scan Inc. California, USA) using the strips (glucose oxidase method).  

**Statistical analysis**

The pharmacokinetic data among different formulations were compared for statistical significance by the one-way ANOVA followed by Turkey-Kramer multiple comparisons test using Graph Pad Instat software (Graphpad Software Inc., CA, USA).

**4.7 Stability studies**

The optimized nanoemulsion formulations were put into an air tight glass vials and subjected to stability studies at 8 ± 2°C (in refrigerator) and at different temperatures and ambient humidity conditions 25°C/60% and 40°C/75% RH. Samples were charged to stability chambers (Thermolab, Mumbai, India) in glass bottles with humidity and temperature control. They were withdrawn at specified time intervals of 0, 30, 60 and 90 days and drug content, viscosity, particle size, refractive index and electrical conductivity were determined. The drug content analysis was carried out using HPTLC method at 240 nm for RPG and by HPLC method at 234 nm respectively over a period of 3 months under accelerated conditions.