Chapter – III

Visible spectrophotometric and HPLC methods for the estimation of Niguldipine
Niguldipine (NGD) is a calcium channel blocker and A1-adrenergic receptor antagonist. It is also showed potent calcium channel blocking activity and has been investigated as an antihypertensive. Niguldipine has been reported to be a highly selective α1-adrenoceptor antagonist and dihydropyridine calcium channel blocker. More specifically, Niguldipine has been shown to irreversibly inhibit T-type calcium channel currents in certain types of central and peripheral neurons and in smooth muscle cells. This compound has also been noted to inhibit noradrenaline-stimulated inositol phosphate accumulation. Niguldipine to see if this antagonist similarly distinguishes between adrenoceptor-mediated inositol phospholipid hydrolysis and the potentiation of cyclic AMP formation. Since Niguldipine is a potent calcium channel blocker, Niguldipine (1a) is an example of class of calcium antagonists, which binds with high affinity to Ca2+ channels and to R1 adrenoceptors. Certain Niguldipine derivatives with a 4-nitrophenyl substituent instead of the 3-nitrophenyl group and/or an amide linkage instead of an ester group, compounds 2a-4a were shown to maintain high R1-adrenoceptor affinity while binding to Ca2+ channels is reduced. This receptor selectivity pattern makes those compounds promising candidates for many therapeutic indications such as benign prostatic hyperplasia.

The analytically important functional groups of NGD are not fully exploited for designing suitable spectrophotometric methods for the determination of NGD. Hence there is a development certain sensitive, precise, accurate and flexible visible spectrophotometric methods, by exploiting various chemical reactions of different functional groups present in NGD.

These methods are based on the reaction of NGD with NaNO₂/HCl, NaNO₂/HCl/AMS, FN/HNO₃, BTB, and MO, to produce colored species of reasonable stability, paving the possibility for spectrophotometric determination of NGD in its bulk form and pharmaceutical formulations. The author has developed a simple and sensitive UV visible spectrophotometric method and realised this objective.
Table: 3.1  
**Structural and Active Functional Groups of Niguldipine**

<table>
<thead>
<tr>
<th>Official Name</th>
<th>Chemical Name</th>
<th>Structure</th>
<th>Functional groups present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Niguldipine</td>
<td>1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid, 3-(4,4-diphenyl-1-piperidinyl) propyl methylester hydrochloride</td>
<td><img src="image" alt="Structure" /></td>
<td>Aromatic nitro, imino, 3° amino and ester group etc.</td>
</tr>
</tbody>
</table>

Table: 3.2  
**Therapeutic Importance and Certain Characteristics of Niguldipine**

<table>
<thead>
<tr>
<th>Pharmacodynamic/Therapeutic Category</th>
<th>Characteristics</th>
<th>Therapeutic importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti Hypertensive</td>
<td>Empirical formula: C$<em>{36}$H$</em>{39}$N$_3$O$_6$.HCl</td>
<td>Niguldipine is a calcium-channel blocking agent used for the treatment of vascular disorders</td>
</tr>
<tr>
<td></td>
<td>Molecular weight: 646.18 g/mol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Solubility: It is soluble in water</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Half life: 8.6 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M.P $-6^\circ$C (43$^\circ$F)</td>
<td></td>
</tr>
</tbody>
</table>
Table: 3.3

Particulars of commercially available formulations of Niguldipine

<table>
<thead>
<tr>
<th>S. No</th>
<th>Pharmaceutical concern</th>
<th>Property name</th>
<th>Weight of Active ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phenoxidioan</td>
<td>Phenoxidioan</td>
<td>10 mg</td>
</tr>
<tr>
<td>2</td>
<td>Phenoxybenzoamine</td>
<td>Phenoxybenzoamine</td>
<td>10 mg</td>
</tr>
<tr>
<td>3</td>
<td>Phentolamine</td>
<td>Phentolamine</td>
<td>10 mg</td>
</tr>
</tbody>
</table>

Table: 3.4

Procedure for the assay of NGD in formulations

<table>
<thead>
<tr>
<th>Pharmaceutical formulation</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tablets</td>
<td>An accurately weighed amount of Tablet powder, equivalent to 100 mg was dissolved in 100 ml of methanol and the Tablets after complete dissolution were made up to the mark with methanol. The concentration of the resulting solution was found to be 1 mg/ml. This solution was considered as the stock solution. 100 mg of the substance was accurately weighed and dissolved in 20 ml of methanol. After the Tablet was dissolved completely, it was treated with 10 ml of 5 N HCl and 4g of Zinc dust. The solution was allowed to undergo reduction by allowing it to stand for one hour. After one hour the solution was slowly filtered through cotton wool and the residue was washed with about 10 – 15 ml of water. The solution was finally made up to mark in a volumetric flask. The absorbance of the solution was measured at $\lambda_{\text{max}}$ 240 nm the quantity of the drug was computed from the Beer’s law plot of the standard drug.</td>
</tr>
</tbody>
</table>
Fig. 3.1: Absorption spectrum ($\lambda_{\text{max}}$) of NGD by UV method

Fig. 3.2: Linearity Curve obeying Beer’s law for NGD by UV method
Part – A

SPECTROPHOTOMETRIC METHOD

Experimentation

Preparation of standard drug stock solution (Stock and working)

The stock solution (1 mg/ml) of Niguldipine hydrochloride was prepared by dissolving 100 mg of the drug in 20 ml methanol and made up to 100 ml with methanol to get a clear solution. 5 ml of this stock solution was taken in to a 50 ml volumetric flask and made upto the mark with methanol. The concentration of this standard was 100 µg/ml. This 100 µg/ml standard was used for preparation of working standards for calibration curve in methods M4, M5, M8, M9 and M10.

Preparation of standard drug solutions for M4, M5, M8

The stock solution (1 mg/ml) of NGD was prepared by dissolving 100 mg of the drug in 20 ml of methanol. After the Tablet was dissolved completely it is treated with 10 ml of 5 N HCl and 4g of Zinc dust.

The solution was allowed to undergo reduction by allowing to standing for one hour. The solution was slowly filtered through cotton wool and residue was washed with about 15 ml of water 3 to 4 times. The solution was finally made up to 100 ml with water in a volumetric flask. The stock solution was furthered step wise to derive various concentrations of working drug solution for methods M4, M5, M8.

Preparation of reagents

- All the chemicals and reagents used were of analytical grade and solutions were prepared in double distilled water.
- Aqueous solutions of BTB dye solution (0.2% w/v)
- Methyl orange dye solution (0.1% w/v)
- Sodium nitrite (0.1% w/v), HCl (5 N, 0.01M)
- Ammonia (5 N)
- Sodium nitrite (0.1% W/V),
- Ammonium sulphamate (0.5% w/v),
- naphthylamine (0.1% in alcohol)
- Ferric nitrate (20% w/v) in water.

Developed and used procedures

The following procedure were used for the determination of NGD in bulk, dosage and pharmaceutical formulations, after systematic and detailed study of the
various reactions of NGD with different reagents as described under results and discussion.

**Method M₄**

Fresh aliquots of standard and reduced Niguldipine (1 ml = 100 µg/ml) solutions ranging from 0.4 to 2.4 ml of 100 µg were transferred into a series of 10 ml volumetric flasks and then 2 ml of BTB (0.2%) was added to this solution. The total volume of the aqueous phase was made up to 10 ml with distilled water. This solutions are taken in the a series of separating funnels and approximately, 10 ml of chloroform was added to each funnel, and the contents were shaken for 2 minutes and allowed to stand result separate the aqueous and chloroform layers.. The chloroform layer was separated and the layer was scanned by using UV region. The absorbance maxima of this layer was found at 380 nm. A Linearity curve was plotted by using above prepared working standards ranging from 4.0 to 24 µg/ml. A good linearity was observed with correlation coefficient 0.9989. The schematic graph of absorbance maxima ($\lambda_{\text{max}}$) and linearity curve were shown in **Fig.3.3** and **Fig.3.4**.

![Fig. 3.3: Absorption spectrum of NGD with BTB/CHCl₃ system](image1)

![Fig. 3.4: Beer’s law plot of NGD with BTB/CHCl₃ system](image2)
Method M₅

The standard and reduced Niguldipine (1 ml = 100 μg) solutions from 0.2 to 1.2 ml were taken into a series of 10 ml volumetric flasks and then added 2 ml of MO (0.1%) to each volumetric flask. The total volume of the aqueous phase was made up to 10 ml with distilled water. This solutions are taken in the a series of separating funnels and approximately, 10 ml of chloroform was added to each funnel, and the contents were shaken for 2 minutes and allowed to stand result separate the aqueous and chloroform layers. The chloroform layer was separated and the layer was scanned by using UV region. The absorbance maxima of this layer was found at 430 nm. A Linearity curve was plotted by using above prepared working standards ranging from 2.0 to 12.0μg/ml. A good linearity was observed with correlation coefficient 0.9997. The schematic graph of absorbance maxima (λₘₐₓ) and linearity curve were shown in Fig. 3.5 and Fig. 3.6.

![Absorption spectrum of NGD with MO/CHCl₃ system](image1)

**Fig. 3.5: Absorption spectrum of NGD with MO/CHCl₃ system**

![Beer’s law plot of NGD with MO/CHCl₃ system](image2)

**Fig. 3.6: Beer’s law plot of NGD with MO/CHCl₃ system**
**Method M**₈

NGD standard solution (100 µg) was transferred into a series of 10ml volumetric flasks to get the final concentration range of 4 to 25 µg. Aqueous solutions of hydrochloric acid (5N, 1 ml) and sodium nitrite (0.1%, 1 ml) were added and kept aside for 5 min. To this 1 ml of diluted ammonia solution was added to each flask and the solution was then made up to the mark distilled water. The absorbance maximum of this yellow colour solution was observed at 440 nm. A Linearity curve was plotted by using above prepared working standards ranging from 4.0 to 25 µg/ml against corresponding reagent blank. A good linearity was observed with correlation coefficient 0.9992. The schematic graph of absorbance maxima (λ max) and linearity curve were shown in Fig. 3.7 and Fig. 3.8. The amount of NGD drug present in the unknown samples was calculated by using slope from linearity curve.

![Fig. 3.7: Absorption spectrum of NGD with Sodium nitrite/HCl](image1)

![Fig. 3.8: Beer’s law plot of NGD with Sodium nitrite/HCl](image2)
Method M₉

A series of 10 ml volumetric flasks containing different quantities of reduced NGD solution ranging from 0.5 ml to 2.5 ml (1 ml=100µg) to provide final concentration range of 5 to 25 µg were taken. An aqueous solution of hydrochloric acid (5 N, 1 ml) and sodium nitrite (0.1%, 1 ml) were added and kept aside for 5 min at 0-5°C. Then ammonium sulphamate solution (0.1%, 1 ml) and 1- Naphthyl amine (0.1%, 1 ml) were added to each flask. The solutions were made up to the mark with distilled water and kept at stand for 5 minutes. The absorbance maximum of this pink colour solution was observed at 520 nm. A Linearity curve was plotted by using above prepared working standards ranging from 5.0 to 25 µg/ml against corresponding reagent blank. A good linearity was observed with correlation coefficient 0.9985. The schematic graph of absorbance maxima (λ_max) and linearity curve were shown in Fig. 3.9 and Fig. 3.10. The amount of NGD drug present in the unknown samples was calculated by using slope from linearity curve.

Fig. 3.9: Absorption spectrum of NGD with Sodium nitrite/HCl/APS/αNA
A series of reduced NGD was transferred into six different 10 ml volumetric flasks ranging from 1 ml to 6 ml (1 ml=100 µg) to get final concentration range 10 to 60 µg. To each volumetric flask 4 ml of ferric nitrate (20%) and 0.4 ml of concentrated nitric acid (0.4 ml) were added. The solution was made up to the mark with distilled water and kept at stand for 5 minutes. The absorbance maximum of this yellowish green coloured solution was observed at 330 nm. A Linearity curve was plotted by using above prepared working standards ranging from 10 to 60 µg/ml against corresponding reagent blank. A good linearity was observed with correlation coefficient 0.9978. The schematic graph of absorbance maxima ($\lambda_{\text{max}}$) and linearity curve were shown in **Fig. 3.11** and **Fig. 3.12**. The amount of NGD drug present in the unknown samples was calculated by using slope from linearity curve.
Fig. 3.11: Absorption spectrum of NGD with FN/Conc. HNO₃ system

Fig. 3.12: Beer’s law plot of NGD with FN/Conc. HNO₃ system
Results and Discussion

Spectral characteristics

The optical characteristics such as absorption maximum, Beer’s law limits, molar absorptivity and sandell’s sensitivity are presented for each method. Precision and accuracy were found by analyzing six replicate samples containing known amounts of the drug and optimum photometric range for NGD with each of the mentioned reagents was calculated Table.3.5.

The accuracy of these methods was ascertained by comparing the results obtained with the used and reference methods in the case of formulations. As an additional check on the accuracy of these methods recovery experiments were performed by adding known amounts of pure drug to pre analyzed formulations and percent recovery values obtained Table.3.6. Recovery experiments indicated the absence of interference from the commonly encountered pharmaceutical additives and excipients. Thus the proposed methods are simple and sensitive with reasonable precision and accuracy. These methods can be used for routine determination of Niguldipine in quality control analysis.
Method M₄

The results obtained in this method were based on extractive spectrophotometry. The yellow color developed is due to the ion association complex between Bromothymol blue (BTB) and NGD. It is formed by the reaction of basic NGD and acidic BTB dye. The complex is extractable with chloroform from the aqueous phase. The yellow colored solution exhibited maximum absorption at 380 nm against the corresponding reagent blank.

Scheme 3.1: Reaction of NGD with BTB
Method M₅

The yellow color developed is due to the formation of an ion association complex between the basic NGD and the acidic MO dye. The complex is extractable with chloroform and the absorbance of the chloroform layer was determined at 430 nm along with MO blank.

Scheme 3.2: Reaction of NGD with MO
**Method M₈**

This method is based on diazotization reaction of the reduced Niguldipine with HCl and NaNO₂ followed by reaction with ammonia to form yellow color complex. Reduced NGD was determined by measuring the absorption of the yellow color maximum at 440 nm. The absorbance of the Diazotization mixture of reduced NGD made alkaline by ammonia is measured separately to use as reference.

![Scheme 3.3 Reaction of reduced Niguldipine with NaNO₂.HCl](image)

**Method M₉**

Diazotization and reaction of the reduced Niguldipine with nitrous acid and coupling with α - Napthyl amine, to form pink color solution in acidic medium is used as the basis in this method. The reaction and the structure of the complex is represented below in **scheme 3.4**. The amine and reduced NGD was calculated by measuring the absorbance of the pink colour complex at 520 nm and the reduced diazotised solution to be used as references.

![Scheme 3.4: Reaction of reduced NGD with NaNO₂/HCl/ Napthyl amine](image)
Method M\textsubscript{10}

The result obtained in this method were due to complex formation of Niguldipine with ferric nitrate under acidic conditions to form a yellowish green color solution that exhibited maximum absorption at a wavelength of 330 nm against the corresponding reagent blank.

Recovery Studies

Recovery studies were conducted by analyzing each pharmaceutical formulation in the first instance for the active ingredients by the proposed methods. Known amount of pure drug was then added to each of the previously analyzed formulation and the total amount of the drug was once again determined by all the proposed methods after bringing the active ingredient concentration within the Beer’s law limits. The results are recorded during assay and recovery studies are presented in Table-3.6. and Table-3.7.

Table 3.5

<table>
<thead>
<tr>
<th>Parameter</th>
<th>M\textsubscript{4}</th>
<th>M\textsubscript{5}</th>
<th>M\textsubscript{8}</th>
<th>M\textsubscript{9}</th>
<th>M\textsubscript{10}</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda ) max (nm)</td>
<td>380</td>
<td>430</td>
<td>440</td>
<td>520</td>
<td>330</td>
</tr>
<tr>
<td>Beer’s law Limits (µg/ml)</td>
<td>4-25</td>
<td>2-12</td>
<td>4-25</td>
<td>4-25</td>
<td>10-60</td>
</tr>
<tr>
<td>Molar absorptivity (1 mole(^{-1}) cm(^{-1}))</td>
<td>6 x 10(^{4})</td>
<td>6.87 x 10(^{4})</td>
<td>8.86 x 10(^{3})</td>
<td>5.4 x 10(^{3})</td>
<td>7.2 x 10(^{3})</td>
</tr>
<tr>
<td>Sandell’s sensitivity (µg/cm(^{2})/0.001 absorbance unit)</td>
<td>0.0126</td>
<td>0.01443</td>
<td>0.01848</td>
<td>0.011276</td>
<td>0.01503</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>0.0134</td>
<td>0.01443</td>
<td>0.0482</td>
<td>0.0478</td>
<td>0.0158</td>
</tr>
<tr>
<td>Intercept(a)</td>
<td>0.018</td>
<td>0.0028</td>
<td>0.0024</td>
<td>0.06</td>
<td>0.0024</td>
</tr>
<tr>
<td>Correlation coefficient(r)</td>
<td>0.9832</td>
<td>0.4472</td>
<td>0.9992</td>
<td>0.9985</td>
<td>0.9978</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>2.7285</td>
<td>0.1694</td>
<td>0.89765</td>
<td>0.0035</td>
<td>1.8962</td>
</tr>
<tr>
<td>%Relative standard deviation</td>
<td>0.92</td>
<td>0.79</td>
<td>0.89</td>
<td>1.85</td>
<td>1.08</td>
</tr>
<tr>
<td>0.05 level</td>
<td>±0.462</td>
<td>±0.741</td>
<td>±0.752</td>
<td>±0.092</td>
<td>±1.546</td>
</tr>
<tr>
<td>0.01 level</td>
<td>±1.14</td>
<td>±0.954</td>
<td>±1.101</td>
<td>±1.332</td>
<td>±1.855</td>
</tr>
</tbody>
</table>
### Table: 3.6

**Assay results of NGD in dosage forms**

<table>
<thead>
<tr>
<th>Pharmaceutical Formulation</th>
<th>Labeled amount (mg)</th>
<th>proposed method</th>
<th>Found by reference method ±S.D</th>
<th>% recovery by proposed method ±S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount found (mg)</td>
<td>t (Value)</td>
<td>F (Value)</td>
<td></td>
</tr>
<tr>
<td>M₄</td>
<td>Phenoxidioan 10</td>
<td>9.97±0.015</td>
<td>0.617</td>
<td>2.169</td>
</tr>
<tr>
<td></td>
<td>Phenoxybenzoamine 10</td>
<td>9.93±0.012</td>
<td>0.075</td>
<td>2.540</td>
</tr>
<tr>
<td></td>
<td>Phentolamine 10</td>
<td>9.96±0.095</td>
<td>0.617</td>
<td>2.169</td>
</tr>
<tr>
<td>M₅</td>
<td>Phenoxidioan 10</td>
<td>9.91±0.009</td>
<td>0.183</td>
<td>2.474</td>
</tr>
<tr>
<td></td>
<td>Phenoxybenzoamine 10</td>
<td>10.04±0.082</td>
<td>0.262</td>
<td>2.175</td>
</tr>
<tr>
<td></td>
<td>Phentolamine 10</td>
<td>10.01±0.008</td>
<td>0.391</td>
<td>2.638</td>
</tr>
<tr>
<td>M₈</td>
<td>Phenoxidioan 10</td>
<td>9.97±0.015</td>
<td>0.617</td>
<td>1.874</td>
</tr>
<tr>
<td></td>
<td>Phenoxybenzoamine 10</td>
<td>9.92±0.008</td>
<td>0.075</td>
<td>1.104</td>
</tr>
<tr>
<td></td>
<td>Phentolamine 10</td>
<td>10.02±0.013</td>
<td>0.821</td>
<td>1.601</td>
</tr>
<tr>
<td>M₉</td>
<td>Phenoxidioan 20</td>
<td>19.95±0.011</td>
<td>0.830</td>
<td>1.560</td>
</tr>
<tr>
<td></td>
<td>Phenoxybenzoamine 20</td>
<td>19.99±0.019</td>
<td>1.101</td>
<td>1.496</td>
</tr>
<tr>
<td></td>
<td>Phentolamine 20</td>
<td>20.05±0.098</td>
<td>1.165</td>
<td>1.324</td>
</tr>
<tr>
<td>M₁₀</td>
<td>Phenoxidioan 20</td>
<td>20.08±0.091</td>
<td>0.536</td>
<td>2.671</td>
</tr>
<tr>
<td></td>
<td>Phenoxybenzoamine 20</td>
<td>19.96±0.095</td>
<td>0.177</td>
<td>2.314</td>
</tr>
<tr>
<td></td>
<td>Phentolamine 20</td>
<td>20.04±0.014</td>
<td>0.404</td>
<td>2.107</td>
</tr>
</tbody>
</table>

### Table: 3.7

**Recovery of Nigludipine**

<table>
<thead>
<tr>
<th>Amount of drug added(µg)</th>
<th>Mean (±S.D) amount found (µg)</th>
<th>Mean(±S.D) % of recovery (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10.03±0.05</td>
<td>100.30±0.50</td>
</tr>
<tr>
<td>20</td>
<td>20.04±0.06</td>
<td>100.2±0.30</td>
</tr>
</tbody>
</table>
Part – B
HPLC METHOD

Chromatography studies are most widely using techniques in the pharma sector. HPLC is one of the most widely using technique for determination assay and various impurities in drug and pharmaceutical samples. There is only few HPLC methods have been reported for the determination of Niguldipine in biological fluids. An attempt has been made in this direction and successfully developed, one sensitive and precise HPLC method for the determination of NGD in bulk samples, pharmaceutical formulations and biological fluids existing as single active ingredient or combined dosage forms) by using Shimadzu C18 column as stationary phase and methyl cyanide + Potassium dihydrogen ortho phosphate(60:40) as mobile without internal standard Table.3.6. The details of the reported HPLC methods for the estimation of NGD are presented in this Chapter.

Experimental

Preparation of standard Drug solution for method M12

Stock solution of the drug was prepared by dissolving 35 mg of NGD in 50 ml volumetric flask containing 50 ml mobile phase. The solution was sonicated for about 10 min and then made up to volume with mobile phase (700 µg/ ml).

Preparation of Sample drug solution for Pharmaceutical formulations

1g of pharmaceutical formulation (tablets) containing equivalent to 17.5 mg of NGD was pulverised to uniform powder and dissolved in 15 ml mobile phase. The solution was sonicated for 10 min and filtered through 0.45 µm membrane filter and made-up to 25 ml to obtain stock solution (700 µg/ ml). This solution was further diluted stepwise with mobile phase to get different concentrations required.

Reagents used

HPLC Grade Water, Aceto-nitrile, and ortho-phosphoric acid buffer

Method M12

NGD solutions of ten sets were prepared in mobile phase with a concentration of 5 to 25 µg/ml. The contents of the mobile phase were filtered before use through 0.45 µm membrane filter, degassed with a helium spar age for 15 min and pumped
from the respective solvent reservoirs to the column at specific flow rate. Before injection of the drug, the mobile phase was pumped for 30 min to saturate the column, thereby to get the base line corrected, then 20 µl of each of the drug solution were injected for 5 times. Quantitative determinations were made by comparisons with that standard peak area of the standard calibration curve.

![Fig: 3.13 Chromatogram for NGD (M12)](image)

Peak table

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Ret. Time</th>
<th>Area</th>
<th>Area%</th>
<th>Tailing factor (0%)</th>
<th>K'</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.760</td>
<td>754675</td>
<td>100.000</td>
<td>1.188</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**Result and Discussion**

The appropriate wavelength in UV region for the measurement of active ingredients in each method was determine and the method was validated by linear fit curve and all other parameters were calculated as done for a visible specrophotometric method. The data is discussed in the following pages.

**Nature of the chromatograms**

The author has made an attempt to separate the NGD in the proposed methods by change of the polarities of the mobile phase by using different concentrations of organic solvents and water.
Parameter fixation

A systematic study of effects of various parameters was undertaken in order to ascertain and establish optimum conditions for good resolution, rapid, accurate quantitative separation and estimation of NGD. The author has performed control experiments by varying one variable at a time and fixing all other variables such as mobile phase composition, flow rate, nature of internal standards etc.

Detection characteristics

Different solutions of NGD in varying concentrations were taken and all solutions were analyzed by the mentioned procedures. To test whether the NGD has been linearly eluted from the column in the method M12, without the usage of internal standard. Least square regression analysis for each method was carried out for the slope, intercept and correlation coefficient. The results are recorded in Table. 3.9.

Table: 3.8
Chromatographic conditions for NGD method (M12)

<table>
<thead>
<tr>
<th>Stationary Phase</th>
<th>Mobile Phase composition</th>
<th>Flow rate (ml/min)</th>
<th>Run time (minutes)</th>
<th>Column Temperature (°C)</th>
<th>Volume of injection (µL)</th>
<th>Detection Wavelength (nm)</th>
<th>Retention time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18 Hypersil BDS</td>
<td>Potassium di hydrogen orthophosphate:Methyl cyanide 60:40</td>
<td>1.0</td>
<td>10.0</td>
<td>40</td>
<td>20</td>
<td>225</td>
<td>4.760</td>
</tr>
</tbody>
</table>

Performance calculations

The system suitability is ascertained for the proposed method M12, by taking a number of statistical values such as relative retention, theoretical plates (HETP), resolution and peak asymmetry.

Method validation

Precision

The precision of the method was ascertained from the peak area of NGD obtained by the determination of eight replicates of fixed amount of NGD. The
percent relative standard deviation and percent range of errors (0.05 and 0.01 confidence limits) were calculated for NGD and are presented in the Table 3.9.

Accuracy

To determine the accuracy of the proposed method, different amounts of bulk samples of NGD in between the upper and lower linearity limits were taken and analyzed by the proposed method. The results (percent errors) are recorded in Table 3.9.

Interference studies

To evaluate the effect of wide range of excipient and other additives usually present in the NGD formulations, the common excipients such as hydroxyl propyl methyl cellulose, lactose monohydrate, magnesium stearate, micro crystalline cellulose, polyethylene glycol 3000 and sodium starch glycolate have been added to the sample solution and injected in to the column. They have not disturbed the elution of NGD. In fact many have no absorption in the UV spectrum.
Table: 3.9
Performance calculations, detection characteristics, precision and accuracy of the proposed method for NGD

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (t)(min)</td>
<td>4.760</td>
</tr>
<tr>
<td>Theoretical plates (n)</td>
<td>15142</td>
</tr>
<tr>
<td>Plates per Meter (N)</td>
<td>20651</td>
</tr>
<tr>
<td>Height equivalent to theoretical plate(HETP)(mm)</td>
<td>$4.1245 \times 10^{-4}$</td>
</tr>
<tr>
<td>Peak asymmetry</td>
<td>0.015</td>
</tr>
<tr>
<td>Resolution Factor</td>
<td>0.00</td>
</tr>
<tr>
<td>Linearity range (ng µl$^{-1}$)</td>
<td>1-10</td>
</tr>
<tr>
<td>Detection limits(ng µl$^{-1}$)</td>
<td>0.025474</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>60.345</td>
</tr>
<tr>
<td>Standard deviation of intercept (sa)</td>
<td>7.8705</td>
</tr>
<tr>
<td>Standard error of estimation (se)</td>
<td>$4.56 \times 10^{-3}$</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.999</td>
</tr>
<tr>
<td>Relative standard deviation (%)</td>
<td>0.73</td>
</tr>
<tr>
<td>Percentage range of errors(confidence limits)</td>
<td></td>
</tr>
<tr>
<td>0.05 level</td>
<td>0.0019</td>
</tr>
<tr>
<td>0.01 level</td>
<td>0.0041</td>
</tr>
<tr>
<td>% error in bulk samples</td>
<td>0.021</td>
</tr>
</tbody>
</table>

Analysis of formulation

To find out the suitability of the proposed method for the assay pharmaceutical formulation of NGD without internal standard was analyzed by the proposed and reference methods. The results obtained from the proposed and reference method were compared statistically by the t and F values and was found that the proposed method does not differ significantly in precision and accuracy from reference method Table.3.10.
Recovery studies

Recovery studies were conducted by analyzing each pharmaceutical formulation in the first instance for the active ingredient by the proposed method. Known amount of the pure drug was then added to each of the previously analyzed formulation, and the total amounts of the drug was once again determined by the proposed method after bringing active ingredient concentration within the linearity limits Table 3.11.

Table: 3.10

Assay results of NGD in dosage forms

<table>
<thead>
<tr>
<th>Method</th>
<th>Pharmaceutical formulation</th>
<th>Labelled Amount (mg)</th>
<th>Proposed method Amount found ± S.D</th>
<th>t-value</th>
<th>F-value</th>
<th>Found by reference method ± S.D</th>
<th>% Recovery by proposed method ± S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>M12 Phenoxidioan</td>
<td>10</td>
<td>10.012±0.023</td>
<td>0.42</td>
<td>1.73</td>
<td>10.05±0.051</td>
<td>100.1±0.23</td>
<td></td>
</tr>
<tr>
<td>Phenoxylbenzamine</td>
<td>10</td>
<td>9.996±0.025</td>
<td>1.4</td>
<td>2.34</td>
<td>10.03±0.026</td>
<td>99.96±0.25</td>
<td></td>
</tr>
<tr>
<td>Phentolamine</td>
<td>10</td>
<td>9.994±0.024</td>
<td>1.06</td>
<td>1.62</td>
<td>10.06±0.012</td>
<td>99.94±0.24</td>
<td></td>
</tr>
</tbody>
</table>

*Average ±standard deviation of eight determinants the t and F values refer to comparison of the proposed method. Theoretical values at 95% confidence limits t=2.365 and F=4.88.

** Average of five determinations

Table: 3.11

Recovery of Niguldipine

<table>
<thead>
<tr>
<th>Amount of drug added(µg)</th>
<th>Mean (±S.D) amount found (µg)</th>
<th>Mean(±S.D) % of recovery (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>9.82±0.16</td>
<td>98.2±1.60</td>
</tr>
<tr>
<td>20</td>
<td>19.74±0.39</td>
<td>98.7±1.95</td>
</tr>
</tbody>
</table>
REFERENCES


13. A new al-AR classification scheme similar to that proposed by Ford et al. (Ford, A. P. D. W.; Williams, T. J.; Blue, D. R.; Clarke,D. E. al-Adrenoceptor


