Chapter-5

Selection of Suitable Drug Candidates
Urea is a well known adductor of linear long chain organic compounds of diverse nature, which include alkanes, alkenes, alcohols, aldehydes, ketones, carboxylic acid, dicarboxylic acids, amines, ethers, esters, halocarbons, nitriles, thioesters and thioethers, provide their main chain consist of six or more carbon atoms. However, there are no documented reports of urea being used as an adductor for drugs or related moieties. The present work pertains to preparation of co-inclusion compounds of NNAE drugs in urea leading to improvement in dissolution profile, stability and safe handling characteristics of the included drug. Hence, suitable drug candidates for the present study should possess the following characteristics:

- High potency
- Oral activity
- Adequate anchor length
- Poor aqueous solubility
- Dissolution rate limited absorption
- Should preferably belong to BCS class II
- Compliance with steric features
- Unstable
- Ease in estimation
- Preferably a liquid
- Remote possibility of being outdated in near future
- Easy availability

Number of potent poorly soluble and unstable drugs, including those specified in Table 5.1 were screened and following drugs were selected for present study.

- Amiloride hydrochloride
- Enalapril maleate
- Glipizide
- *Cis*-retinoic acid
- Nicorandil
Table 5.1 Characteristics of low dose- drugs (having dose < 100 mg ) confirming to the optimal steric requirements.

<table>
<thead>
<tr>
<th>Name of the Drug</th>
<th>Category</th>
<th>Structure</th>
<th>Dose</th>
<th>Half-life</th>
<th>Melting point</th>
<th>Solubility profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetretin</td>
<td>Retinoid</td>
<td><img src="image" alt="Structure" /></td>
<td>10-25 mg</td>
<td>50 hrs</td>
<td>201°C</td>
<td>Sparingly Soluble in water, Soluble in alcohol</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>Anxiolytic</td>
<td><img src="image" alt="Structure" /></td>
<td>1-3mg</td>
<td>12 hrs</td>
<td>228 °C</td>
<td>Insoluble in water, freely soluble in alcohol</td>
</tr>
<tr>
<td>Amiloride</td>
<td>Potasparingly solubleium</td>
<td><img src="image" alt="Structure" /></td>
<td>20 mg</td>
<td>6.3-6.9 hrs</td>
<td>293.5 °C</td>
<td>Sparingly Soluble in water, Soluble in methanol, Insoluble in acetone</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>CNS Stimulant</td>
<td><img src="image" alt="Structure" /></td>
<td>25 mg</td>
<td>10-13 hrs</td>
<td>B.P. 200 °C</td>
<td>Sparingly Soluble in water, Soluble in ether</td>
</tr>
<tr>
<td>Atenolol</td>
<td>Antihypertensive</td>
<td><img src="image" alt="Structure" /></td>
<td>50-100 mg</td>
<td>6-9 hrs</td>
<td>141 -148 °C</td>
<td>Freely Soluble in alcohol</td>
</tr>
<tr>
<td>Baclofen</td>
<td>Muscle relaxant</td>
<td><img src="image" alt="Structure" /></td>
<td>5 mg</td>
<td>1.5-4 hrs</td>
<td>207 °C</td>
<td>Sparingly Soluble in water and alcohol</td>
</tr>
<tr>
<td>Betaxolol hydrochloride</td>
<td>β -Adrenoreceptor Antagonist</td>
<td><img src="image" alt="Structure" /></td>
<td>10-20 mg</td>
<td>18 hrs</td>
<td>116 °C</td>
<td>Soluble in water and alcohol</td>
</tr>
<tr>
<td>Bisoprolol</td>
<td>β -Adrenoreceptor Antagonist</td>
<td><img src="image" alt="Structure" /></td>
<td>5-10 mg</td>
<td>9-12 hrs</td>
<td>100 °C</td>
<td>Soluble in water, ethanol, methanol and chloroform</td>
</tr>
<tr>
<td>Name of the Drug</td>
<td>Category</td>
<td>Structure</td>
<td>Melting point</td>
<td>Half-life</td>
<td>Dose</td>
<td>Solubility profile</td>
</tr>
<tr>
<td>------------------</td>
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<td>-----------</td>
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<td>-----------</td>
<td>--------------------</td>
<td>------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Brompheniramine Maleate</td>
<td>Antihistaminic</td>
<td>![Structure 1]</td>
<td>132-134 °C</td>
<td>25 hrs</td>
<td>4-8 mg</td>
<td>Soluble in water, sparingly soluble in alcohol, soluble in acetone and alcohol, insoluble in water, soluble in alcohol, sparingly soluble in benzene and ether</td>
</tr>
<tr>
<td>Busulphan</td>
<td>Antineoplastic</td>
<td>![Structure 2]</td>
<td>114-118 °C</td>
<td>2-3 hrs</td>
<td>0.5-2 mg</td>
<td>Freely soluble in water, sparingly soluble in alcohol, soluble in water, methanol, and ethanol, sparingly soluble in benzene and ether</td>
</tr>
<tr>
<td>Captopril</td>
<td>ACE inhibitor</td>
<td>![Structure 3]</td>
<td>152-161 °C</td>
<td>2 hr</td>
<td>25 mg</td>
<td>Freely soluble in water, sparingly soluble in alcohol, soluble in water, methanol, and ethanol, sparingly soluble in benzene and ether</td>
</tr>
<tr>
<td>Carbimazole</td>
<td>Stimulant</td>
<td>![Structure 4]</td>
<td>123.5 °C</td>
<td>3-6 hrs</td>
<td>5-10 mg</td>
<td>Freely soluble in water, sparingly soluble in alcohol, soluble in water, methanol, and ethanol, sparingly soluble in benzene and ether</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>Antihypertensive Agent</td>
<td>![Structure 5]</td>
<td>114 °C</td>
<td>2-3 hrs</td>
<td>12.5 mg</td>
<td>Freely soluble in water, sparingly soluble in alcohol, soluble in water, methanol, and ethanol, sparingly soluble in benzene and ether</td>
</tr>
<tr>
<td>Chlorpheniramine Maleate</td>
<td>Antihistaminic</td>
<td>![Structure 6]</td>
<td>128-136 °C</td>
<td>15-20 hr</td>
<td>40 mg in divided doses</td>
<td>Freely soluble in water, sparingly soluble in alcohol, soluble in water, methanol, and ethanol, sparingly soluble in benzene and ether</td>
</tr>
<tr>
<td>Citalopram hydrobromide</td>
<td>Antidepressant solubilant</td>
<td>![Structure 7]</td>
<td>182-183 °C</td>
<td>35 hrs</td>
<td>20-60 mg</td>
<td>Freely soluble in water, sparingly soluble in alcohol, soluble in water, methanol, and ethanol, sparingly soluble in benzene and ether</td>
</tr>
<tr>
<td>Name of the Drug</td>
<td>Structure</td>
<td>Category</td>
<td>Dose</td>
<td>Melting point</td>
<td>Solubility profile</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
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<td>-----------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Clonazepam</td>
<td><img src="structure1.png" alt="Structure" /></td>
<td>Antiepileptic</td>
<td>4-8 mg</td>
<td>236.5-236.5 °C</td>
<td>Insoluble in water, sparingly soluble in alcohol, sparingly soluble in water and alcohol, soluble in water and alcohol</td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td><img src="structure2.png" alt="Structure" /></td>
<td>Antineoplastic</td>
<td>50 mg</td>
<td>41-45 °C</td>
<td>Insoluble in water, sparingly soluble in alcohol, sparingly soluble in water and alcohol, soluble in water and alcohol</td>
<td></td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td><img src="structure3.png" alt="Structure" /></td>
<td>Antihistaminic</td>
<td>25-50 mg</td>
<td>150-155 °C</td>
<td>Insoluble in water, sparingly soluble in alcohol, sparingly soluble in water and alcohol, soluble in water and alcohol</td>
<td></td>
</tr>
<tr>
<td>Decyclomine</td>
<td><img src="structure4.png" alt="Structure" /></td>
<td>Antimuscarinic Agent</td>
<td>10-20 mg</td>
<td>102-105 °C</td>
<td>Insoluble in water, sparingly soluble in alcohol, sparingly soluble in water and alcohol, soluble in water and alcohol</td>
<td></td>
</tr>
<tr>
<td>Doxepin Hydrochloride</td>
<td><img src="structure5.png" alt="Structure" /></td>
<td>Antidepressingly solubleant</td>
<td>75-100 mg</td>
<td>186 °C</td>
<td>Insoluble in water, sparingly soluble in alcohol, sparingly soluble in water and alcohol, soluble in water and alcohol</td>
<td></td>
</tr>
<tr>
<td>Enalapril Maleate</td>
<td><img src="structure6.png" alt="Structure" /></td>
<td>ACE Inhibitor</td>
<td>2.5-5 mg</td>
<td>144 °C</td>
<td>Insoluble in water, sparingly soluble in alcohol, sparingly soluble in water and alcohol, soluble in water and alcohol</td>
<td></td>
</tr>
<tr>
<td>Retinoid</td>
<td><img src="structure7.png" alt="Structure" /></td>
<td>Retinoid</td>
<td>25 mg</td>
<td>180 °C</td>
<td>Insoluble in water, sparingly soluble in alcohol, sparingly soluble in water and alcohol, soluble in water and alcohol</td>
<td></td>
</tr>
<tr>
<td>Fluoxetine Hydrochloride</td>
<td><img src="structure8.png" alt="Structure" /></td>
<td>Antidepressingly solubleant</td>
<td>20 mg</td>
<td>179-180 °C</td>
<td>Soluble in water and alcohol, sparingly soluble in water, sparingly soluble in alcohol, soluble in water and alcohol</td>
<td></td>
</tr>
<tr>
<td>Category</td>
<td>Name of the Drug</td>
<td>Structure</td>
<td>Melting point</td>
<td>Solubility profile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>------------------</td>
<td>-----------</td>
<td>---------------</td>
<td>-----------------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diuretic</td>
<td>Furosemide</td>
<td><img src="image" alt="Furosemide Structure" /></td>
<td>206 °C</td>
<td>Sparingly soluble in water, Soluble in DMF, Insoluble in water, Soluble in alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutrient</td>
<td>Gamma-Linoleic Acid</td>
<td><img src="image" alt="Gamma-Linoleic Acid Structure" /></td>
<td>B.P. 140 °C</td>
<td>Soluble in 330 parts of alcohol, Soluble in DMF, Insoluble in water, Soluble in alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hygoglycemic</td>
<td>Glibenclamide</td>
<td><img src="image" alt="Glibenclamide Structure" /></td>
<td>172-174 °C</td>
<td>Insoluble in water, Soluble in DMF, Soluble in alcohol, Insoluble in water, Soluble in alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hygoglycemic</td>
<td>Gliclazide</td>
<td><img src="image" alt="Gliclazide Structure" /></td>
<td>181 °C</td>
<td>Insoluble in water, Sparingly soluble in alcohol, Soluble in DMF, Insoluble in alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hygoglycemic</td>
<td>Glipizide</td>
<td><img src="image" alt="Glipizide Structure" /></td>
<td>205 °C</td>
<td>Soluble in water, Soluble in DMF, Insoluble in water, Soluble in alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiovascular Drug</td>
<td>Guanethidine Monosulphate</td>
<td><img src="image" alt="Guanethidine Monosulphate Structure" /></td>
<td>250 °C</td>
<td>Soluble in water, Insoluble in alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sedative-Hypnotic</td>
<td>Haloperidol</td>
<td><img src="image" alt="Haloperidol Structure" /></td>
<td>151 °C</td>
<td>Sparingly soluble in water, Soluble in alcohol, Insoluble in alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antiacne drug</td>
<td>Isotretinoin</td>
<td><img src="image" alt="Isotretinoin Structure" /></td>
<td>175 °C</td>
<td>Insoluble in water, soluble in alcohol, methanol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Half-life</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2 hrs</td>
<td>40-120 mg</td>
</tr>
<tr>
<td>4-6 hr</td>
<td>1-50 mg</td>
</tr>
<tr>
<td>10.4 hrs</td>
<td>5-20mg</td>
</tr>
<tr>
<td>3-5 hr</td>
<td>40-80 mg</td>
</tr>
<tr>
<td>1.5 days</td>
<td>2.5-20mg</td>
</tr>
<tr>
<td>12-36 hrs</td>
<td>0.5-5 mg</td>
</tr>
<tr>
<td>10-20 hrs</td>
<td>10-40 mg</td>
</tr>
<tr>
<td>Solubility profile</td>
<td>Melt point</td>
</tr>
<tr>
<td>--------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Soluble in ether, acetone and chloroform</td>
<td>143 °C</td>
</tr>
<tr>
<td>Soluble in water, dimethylformamide, methanol</td>
<td>166 °C</td>
</tr>
<tr>
<td>Soluble in methanol and alcohol</td>
<td>222 °C</td>
</tr>
<tr>
<td>Soluble in water, ether, DMF, methyl acetate</td>
<td>B.P. 120 °C</td>
</tr>
<tr>
<td>Sparingly Soluble in methylene chloride and alcohol</td>
<td>90 °C</td>
</tr>
<tr>
<td>Soluble in water, ether and ethanol</td>
<td>200-205 °C</td>
</tr>
<tr>
<td>Solubly Soluble in water and alcohol</td>
<td>129-130 °C</td>
</tr>
<tr>
<td>Freely Soluble in water, methanol</td>
<td>107-110 °C</td>
</tr>
<tr>
<td>Name of the Drug</td>
<td>Category</td>
</tr>
<tr>
<td>------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Paroxetine Hydrochloride</td>
<td>Antidepressant</td>
</tr>
<tr>
<td>Pheniramine Maleate</td>
<td>Antihistaminic</td>
</tr>
<tr>
<td>Phytomenadione</td>
<td>Vitamin K</td>
</tr>
<tr>
<td>Pindolol</td>
<td>β-Adrenoreceptor Antagonist</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>NSAID</td>
</tr>
<tr>
<td>Propranolol</td>
<td>β-Adrenoreceptor Antagonist</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>Adrenergic Blocker</td>
</tr>
<tr>
<td>Selegiline Hydrochloride</td>
<td>Dopaminergic</td>
</tr>
</tbody>
</table>

Some other properties:

- **Melting point**
  - 128-138 °C for Paroxetine Hydrochloride
  - 107 °C for Pheniramine Maleate
  - 201-209 °C for Phytomenadione

- **Half-life**
  - 11.1 hrs for Paroxetine Hydrochloride
  - 8-17 hrs for Pheniramine Maleate
  - 2.2 hrs for Phytomenadione

- **Solubility profile**
  - Sparingly Soluble in water, Freely Soluble in alcohol for Paroxetine Hydrochloride
  - Soluble in water and alcohol, Sparingly Soluble in ether and benzene for Pheniramine Maleate
  - Insoluble in water, Soluble in acetone, Sparingly soluble in chloroform, Soluble in DMF for Phytomenadione

- **Dose**
  - 20 mg for Paroxetine Hydrochloride
  - 25-50 mg for Pheniramine Maleate
  - 5 mg for Phytomenadione
  - 10-30 mg for Pindolol
  - 10-20 mg for Piroxicam
  - 20mg-2g for Propranolol
  - 6-16 mg for Salbutamol
  - 10 mg for Selegiline Hydrochloride
<table>
<thead>
<tr>
<th>Name of the Drug</th>
<th>Category</th>
<th>Dose</th>
<th>Half-life</th>
<th>Melting point</th>
<th>Solubility profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamoxiphen Citrate</td>
<td>Antineoplastic Agent</td>
<td>20 mg</td>
<td>4 days</td>
<td>140-142 °C</td>
<td>Sparingly Soluble in water, Soluble in alcohol</td>
</tr>
<tr>
<td>Teprenone</td>
<td>Anti-ulcerative</td>
<td>50 mg</td>
<td>3-4 hrs</td>
<td>B.P. 155-160 °C</td>
<td>Insoluble in water, Soluble in organic liquids</td>
</tr>
<tr>
<td>Terbutaline Sulphate</td>
<td>Bronchodilator</td>
<td>2.5 mg</td>
<td>5.5 hrs</td>
<td>119-122 °C</td>
<td>Soluble in water, Sparingly Soluble in alcohol</td>
</tr>
<tr>
<td>Torasemide</td>
<td>Diuretic</td>
<td>5 mg</td>
<td>2-2.5 hrs</td>
<td>163-164 °C</td>
<td>Soluble in water and ethanol</td>
</tr>
</tbody>
</table>

**Structure**

- [Tamoxiphen Citrate](#)
- [Teprenone](#)
- [Terbutaline Sulphate](#)
- [Torasemide](#)
5.1 Amiloride Hydrochloride (AH)

Amiloride hydrochloride dihydrate is a potassium-conserving diuretic with relatively weak natriuretic and antihypertensive activity. Its primary site of action being in the distal nephron where it inhibits sodium reabsorption while causing retention of potassium and hydrogen ions (Bently, 1968; Benos, 1982). It is mainly indicated in conjunction with kaliuretic diuretic agents to aid in restoration of normal serum potassium levels and/or to prevent development of hypokalemia (Vidt, 1981)

5.1.1 Description (Bodavari, 1996; Mazzo, 1986)

**Chemical Name**

3.5-diamino-N-(diaminomethylene)- 6-chloropyrazinecarboxamide monohydrochloride dihydrate. Also named as 3, 5-diamino-N-(amino-iminomethyl)-6-chloropyrazinecarboxamide monohydrochloride dihydrate.

**Chemical structure**

![Chemical structure of Amiloride Hydrochloride](image)

**Mol. Formula** $\text{C}_6\text{H}_8\text{ClN}_7\text{O.HCl.2H}_2\text{O}$

**Mol. Wt.** 302.12 g/mol

**CAS registry no.** 17440-83-4.

5.1.2 Physicochemical properties

AH occurs as yellow to greenish yellow crystalline powder which is odorless or practically odorless.

**Solubility**

The solubility of AH in a variety of solvents at 25 °C is reported as follows (Mazzo, 1986):

- Acetone: Practically insoluble
- Chloroform: Practically insoluble
- Diethyl ether: Practically insoluble
- Dimethylsulfoxide: Freely soluble
- Ethanol: Very slightly soluble
Selection of Drug Candidates

Ethyl acetate : Practically insoluble
Isopropanol : Slightly soluble
Methanol : Sparingly soluble
Water: Slightly soluble (0.52 g/100 ml)

The pH of a 0.5 % solution in water is 4.2. The solubility of Amiloride hydrochloride in water is typical of an organic base with limited aqueous solubility and increases with a decrease in pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>Solubility (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.8</td>
<td>5.2</td>
</tr>
<tr>
<td>7.6</td>
<td>5.1</td>
</tr>
<tr>
<td>9.4</td>
<td>0.5</td>
</tr>
<tr>
<td>10.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Melting Point

The melting point (with decomposition) of anhydrous AH is 293.5 °C. The dihydrate melts at approximately 288 °C.

Crystal properties

Amiloride hydrochloride dihydrate exists as a crystalline powder. Variations encountered in the X-ray powder diffraction pattern suggest the existence of at least two polymorphic forms and as an anhydrate (Mazzo, 1986). Polymorphism of AH has not been detected by any other physical or chemical measurements techniques (Jozwiakowski et al, 1993). Crystal structure of a methanol hemisolvate has also been reported (Pretscher, 2001).

Hygroscopicity

The monohydrochloride salt of amiloride forms a stable dihydrate and AH, unless specified otherwise, is supplied as such. Amiloride monohydrochloride dihydrate may be converted to the anhydrous form of the salt by drying at 100 °C at pressures <6.6 x 10⁻³ atm for 3 hours. No other stable hydrates of AH have been reported.

Dissociation constant

The dissociation constant of AH as derived from non-aqueous titration indicates that amiloride is a moderately strong organic base with a pKa values approximately 8.7 at 25 °C (Bock et al, 1981).
5.1.3 Pharmacokinetics

It has been shown, using radiolabelled C\textsuperscript{14} AH, that approximately 50% of an oral dose administered to man is absorbed from gastrointestinal tract (Wiess \textit{et al}, 1969; Smith and Smith, 1973). The remainder of the dose is unabsorbed and can be found in feces (Vidt, 1981). Human bioavailability studies comparing 5 mg tablets of AH to an aqueous reference solution showed that the pharmaceutical dosage forms was fully bioequivalent. Absorption of AH is reduced when administered with food (Aue \textit{et al}, 1976).

AH which has been absorbed in man is excreted without metabolism in the urine. No metabolic products have been detected and/or identified. Since, the drug is not metabolized it probably can be administered to patients with hepatic dysfunction, provided that the renal functions are normal. AH is cleared from body by tubular transport and in combination with hydrochlorothiazide, does not alter normal renal potassium excretion or the rate of urinary variable excretion (Vidt, 1981).

Onset of the physiological effect of AH in human is usually noted within 2 hours with peak serum levels being achieved in between 3 and 4 hours. Typical pharmacokinetic half-life range between 6 and 10 hours. Considerable prolongation of half life has been observed in patients with chronic renal failure. Effects of the drug generally subside within 24 hours resulting in urinary levels less than 0.5% of the administered dose. Urinary AH range from 4 \(\mu\)g/ml to 30 \(\mu\)g/ml during diuresis with peak renal clearance of amiloride ranging from 400 ml/min to 600 ml/min. A dose response relationship has been observed in man. Progressively increasing effects of single dose have been noted from 1 mg to 40 mg with a plateau being reached above 40 mg (Wiess \textit{et al}, 1969; Smith and Smith, 1973). Apparent volume of distribution is 380-380 l (5 l/kg).

Dosage 5-10 mg daily up to 20 mg.

5.1.4 Methods of analysis

5.1.4.1 Spectrophotometric analysis

\textit{Direct UV spectrophotometry}

AH exhibits a UV absorption band near 363 nm attributed to the substituted pyrazine ring system. This absorption is the basis for the quantitative determination of the drug. Assay of the compound is based on a comparison of the net absorbance at 363 nm of a sample in 0.1 N HCl with a standard in 0.1 N HCl of known concentration (Mazzo, 1986).
Ultraviolet spectrophotometry via flow injection analysis

UV absorbance at 363 nm is also used as the detection mode for AH determinations by flow injection analysis (FIA). In the case of FIA assay, AH sample and standard solutions are periodically injected into a flowing stream. The resulting changes in UV absorbance at 363 nm of the stream are measured relative to the drug free stream. Calculations of the concentration of AH are made in an identical fashion as direct UV spectrophotometry (Mazzo, 1986).

Liquid liquid extraction with UV spectrophotometry or spectrofluorimetry

AH may be determined in the presence of its degradation products by a liquid-liquid extraction technique followed by quantitation by UV spectrophotometry or spectrofluorimetry. An alkaline aqueous solution of AH is extracted with tributyl phosphate. Amiloride is partitioned into organic layer while its degradates remain in the aqueous phase. Amiloride is then determined by UV spectrophotometry at 363 nm or fluorimetry with an excitation wavelength of ca. 363 nm and emission wavelength of ca. 420 nm. Sensitivity is ~ 0.2 ppm.

5.1.4.2 Chromatography analysis

Thin layer chromatography

Normal phase TLC on silica gel using one of the two developing solvent systems has been employed for AH. In the first system, a developing solvent of 10% n-propanol in chloroform is used to develop a spot resulting from 5 \( \mu \)L of a 1% aqueous solution of the drug. \( R_f \) for the analyte in this system is approximately zero. Detection is by UV absorbance at 250 nm or 363 nm (most sensitive). A sensitivity of 0.01% has been reported. In the second TLC system, 4 parts 3 N aqueous ammonium hydroxide are mixed with 30 parts of tetrahydrofuran to form the developing solvent. A 1 \( \mu \)L spot of a 0.1% aqueous solution of the drug is developed and detected by UV absorbance at 254 nm or 363 nm. \( R_f \) for the analyte is approximately 0.7. Sensitivity under these conditions is in the range of 0.1% to 0.5% of the analyte concentration (USP, 1984).

High Performance Liquid Chromatography

Reversed-phase HPLC is routinely used to determine AH. The method employs a C-2 (300mm x 4.6mm i.d., 10 \( \mu \)m particle size) HPLC column operated at ambient room temperature (~ 25° C). A mobile phase consisting of 85 % aqueous 0.01M sodium hexane
sulphonate (pH = 3.0) in acetonitrile is used to elute amiloride. Flow rate is 2.0 ml/min and detection is by UV absorbance at 280 nm. Under these conditions, amiloride elutes in less than 8 min. (k' ~ 2). Typically 20 μL injections of an approximately 200 μg/mL drug solution are made.

5.1.4.3 Non-Aqueous Titrations

AH can be determined by non-aqueous titration with perchloric acid. The assay involves dissolution of an appropriate amount (~ 450 mg) of AH in 100 ml of glacial acetic acid to which is then added 10 ml of mercuric acetate, 15 ml of dioxane and appropriate amount of crystal violet indicator. The well mixed solution is titrated with 0.1N perchloric acid to a blue end point. Assay results must be corrected for a blank titration. Each equivalent of perchloric acid is equivalent to 26.61 mg of AH (USP, 1984).

5.1.4.4 Determination in biological fluids

Several techniques have been used to determine AH in biological fluids. Among them, liquid scintillation counting for radiolabelled compound, liquid-liquid extraction with measurement of ultraviolet absorbance or fluorescence, thin-layer chromatography with UV or fluorescence detection and high performance liquid chromatography (HPLC) with fluorescence detection have been the most widely used.

Liquid scintillation counting of C-14 labeled amiloride in urine, serum, plasma, tissue and feces has been performed routinely usually with an internal standard used for quantification of the analyte (Baer et al, 1967).

5.1.4.5 Determination in pharmaceuticals

Dissolution testing

The determination of AH in samples resulting from dissolution testing of tablets containing the drug can be accomplished by UV absorbance at 363 nm. Either direct UV-spectrophotometry or UV spectrophotometry vis-à-vis flow injection analysis may be used.

Assay, dosage uniformity and stability testing

HPLC is the technique of choice for determination of AH in tablets for release and/or stability purposes although UV spectrophotometry may be used for determination of dosage uniformity. Reversed phase HPLC with UV detection at 363 nm has been performed using C-18 HPLC column (300nm x 3.9 mm i.d., 10 μm particle size) with a
mobile phase of 25% methanol in 0.05 M phosphate buffer (pH = 3.00. Flow rate was 1.0 ml/min. recently, a fast HPLC method was developed which employs a C-18 mini column (50 nm x 4.6 mm i.d., 5 μm particle size). Using a mobile phase of 20% methanol in 0.02 M phosphate buffer pH = 2 at a flow rate of 4.0 ml/min. Amiloride is eluted in approximately 0.5 min. Detection is again done by UV absorbance at 360 nm (k’~1). This method is routinely used for dosage form uniformity samples (Vincek et al, 1985).

Assay procedure employed for the present studies

A spectrophotometric method of assay for AH was used: the drug content in 0.1N HCl solution was determined by measuring absorbance at $\lambda_{\text{max}}$ of 363 nm using Unicam UV-visible double beam spectrophotometer v1.30. Both urea and oleic acid were determined not to interfere with the analysis at 363 nm. Stock solution of AH in 0.1N HCl with a concentration of 1 mg/ml was prepared. Working standards, to obtain concentrations within a range of 1-15 μg/ml, were prepared by appropriate dilution with 0.1N HCl of the stock solution and used to set up the calibration curve. The calibration curve for AH is presented in Figure 5.1.

Figure 5.1 Calibration curve for amiloride hydrochloride using UV spectrophotometric method of analysis.
5.2 Enalpril maleate

Enalapril maleate is highly effective antihypertensive agent, which act as angiotensin converting enzyme (ACE) inhibitors (Cleary and Taylor, 1986). ACE inhibitor class of drugs prevents conversion of angiotensin I to II. This is reflected by both hemodynamic (decreased blood pressure) and humoral (increased plasma renin, angiotensin I, and decreased angiotensin II) responses to enalapril therapy (Todd and Heel, 1986). They are, therefore, used for the treatment of hypertension, congestive heart failure and to alleviate strain on heart damaged by heart attack (Cieland et al, 1985; Moncloa et al, 1985).

Enalapril acts as a prodrug; following oral administration it is bioactivated by hydrolysis of the ethyl ester to enalaprilate, which is the active ACE inhibitor (SOLVD, 1992). It acts as a prodrug; following oral administration it is bioactivated by hydrolysis of the ethyl ester to enalaprilate, which is the active ACE inhibitor (Cleary and Taylor, 1986; SOLVD, 1992).

5.2.1 Description (Ip and Brenner, 1987; Bodavari, 1997)

Chemical name

(s)-N-(1-ethoxycarbonyl) - 3- phenylpropyl) –L- alanyl) –L- praline, (Z)- butenedioate (1:1) salt.

Chemical structure

Molecular formula C_{28}H_{28}N_{2}O_{5}.C_{4}H_{4}O_{4}

Molecular wt. 492.53

CAS registry number 75095 -16-4
5.3.2 Physicochemical properties

Enalapril maleate is a white to off-white crystalline, odorless powder.

**Solubility**

EM is known to exhibit following solubility profile in various solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>solubility (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>25</td>
</tr>
<tr>
<td>Methanol</td>
<td>200</td>
</tr>
<tr>
<td>Ethanol</td>
<td>80</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>1.4</td>
</tr>
<tr>
<td>Acetone</td>
<td>9.1</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>3.3</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>&gt;400</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.6</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Hexane</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Polyethylene glycol 300</td>
<td>&lt; 0.1</td>
</tr>
</tbody>
</table>

The solubility of enalapril maleate increases with increase in pH.

**Melting point**

145 °C

**Crystal properties**

The compound is polymorphic. Two non-solvated polymorphs have been detected and characterized by high resolution spectroscopic techniques (FTIR, Raman, and solid-state $^{13}$C NMR and X-ray diffraction) and solution calorimetry. However, these polymorphs referred to as form I and Form II are very similar in energy, differing only by 0.6 Kcal.mol. X-ray diffractogram shows exhibits an extra peak at 14.7° 2θ for form I and at 13.0° 2θ for form II (Ip et al, 1986).

**Dissociation constant**

Aqueous acidic/ basic potentiometric titration yields pKa values of 2.97 and 5.35 at 25 °C for enalapril.

**Partition behavior**

At room temperature, enalapril maleate does not partition out of the aqueous media into mineral oil or n-octanol.
Conformational isomers

NMR studies have revealed the existence of two isomers of EM with respect to rotation about the proline amide bond, resulting in a slow equilibrium between two rotational conformers (cis and trans). HPLC methods have provided firm evidence for their existence by demonstrating the chromatographic behavior of EM at various temperatures. Thus, at 10° C, in addition to an early eluting maleic acid peak, the enalapril base peak is resolved into two distinct rotamers peaks. At 80° C, the rotamers peaks coalesced to yield a single symmetrical peak (Salamoun and Slais, 1991).

5.2.3 Pharmacokinetics

Enalapril act as prodrug of the diacid enalaprilat, its active form, which is poorly absorbed by mouth. Following oral administration about 60 % of a dose of enalapril is absorbed from gastro-intestinal tract and peak plasma concentrations are achieved within about 1 hour.

Enalapril, when administered orally, is rapidly absorbed and bioactivated extensively to enalaprilat. The hydrolytic bioactivation appears to be largely post-absorptive and there is no evidence for metabolism of enalapril beyond bioactivation to enalaprilat in man. Peak plasma concentrations of enalaprilate are achieved 3 to 4 hours after an oral dose of enalapril (Macfadyen et al, 1983).

About 60 % of an oral dose of enalapril is excreted in urine, as enalaprilate and unchanged drug, the rest in feces. Enalaprilate is 50-60 % bound to plasma proteins; its elimination is multiphasic but its effective half life for accumulation following multiple dose of enalapril is reported to be about 11 hours in patients with normal renal function. Enalaprilate is removed by haemodialysis and by peritoneal dialysis. An average steady state recovery of enalaprilat is achieved by the third or fourth dose (Till et al, 1984).

5.2.4 Methods of analysis

5.2.4.1 Potentiometric titration

Enalapril maleate can be determined by potentiometric titration with aqueous sodium hydroxide and perchloric acid. With respect to the sodium hydroxide titration, 0.1 N sodium hydroxide is the titrant and a combination pH electrode is employed as the electrode system. The perchloric acid titration employs 0.1 N perchloric acid in glacial acetic acid as the titrant and anhydrous electrode system, e.g. silver/silver chloride.
Selection of Drug Candidates

electrode filled with 0.1N lithium perchlorate in glacial acetic acid (or acetic anhydride), vs a glass electrode is used.

5.2.4.2 Colorimetric/ spectrophotometric

Enalapril maleate is characterized by inflections or shoulders at about 251 and 263 nm and barely discernible maxima at about 257 nm ($A_{1cm}$ 1%, about 15) and about 276 nm (about 8.6). The low intensity and lack of well defined maxima in ultraviolet absorption spectrum of enalapril maleate, typical of an unconjugated phenyl moiety, does not make it useful for characterization or quantification of the compound.

Due to lack of strong UV absorption maxima, more sensitive ways of analysis are needed. One approach has been autoanalyser colorimetric assay procedure. In this approach enalapril maleate forms a yellow dye complex with bromothymol blue in acidic aqueous pH 2/CHCl$_3$ mixture and is then assayed spectrophotometrically at 415 nm after extraction with chloroform. This method is suitable for content uniformity and dissolution assays of enalapril maleate capsules and tablets (Ip and Brenner, 1987).

Analysis of enalapril in pharmaceutical dosage forms via flow injection analysis has been reported (Kato, 1985). Enalapril is determined by extraction as ion-pair with bromothymol blue in unsegmented flow system. The procedure is stability indicating; the degradation products of enalapril and excipients in the dosage forms do not interfere.

5.2.4.3 Chromatographic estimation

Thin layer chromatography

Different solvent systems for the thin layer chromatography of enalapril maleate can be employed to separate enalapril maleate base from its salt forming acid maleic acid and its hydrolysis products. These solvent systems use a silica gel adsorbent containing an ultra violet-fluorescence quench indicator. Detection of components is accommodated by visualization of dried plates under short wavelength (254 nm) UV light. Iodine vapor yields a more intense spot for enalapril base but does not visualize the maleic acid.

In the systems reported above, the low sensitivity of the method (detection limit 1 to 2%) renders it unsuitable for the detection and quantification of low level of impurities in enalapril maleate bulk substance.
**Selection of Drug Candidates**

*High performance liquid chromatography*

Several HPLC methods have been developed which are suitable for quantification of enalapril maleate and process impurities in bulk substances. It has been shown that the peak shape is dependent upon temperature, flow rate and pH. The optimum peak shape is achieved at a lower pH (3) of the mobile phase and either at higher temperature (60 °C) or with the addition of a cationic surfactant and an alkane sulphonate in the mobile phase. Reverse-phase HPLC conditions are employed. Detection is by UV at 210 or 215nm. Flow-rate is normally 1.8 or 2 ml/min. a mobile phase of aqueous phosphate buffer and organic modifier (acetonitrile or a mixture of acetonitrile and methanol) and an oven temperature of 70°C or 80°C provide the selectivity and sensitivity required for satisfactory chromatography (Linda, 1981; Salamoun and Slais, 1991; Trabelsi et al., 2000; Omari et al., 2001).

**Table 5.2 Various HPLC methods employed for estimation of enalapril maleate.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Column</th>
<th>Mobile phase</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk drug</td>
<td>Ultrasphere C8 250 X 4.6 mm, 5 μ</td>
<td>Isocratic 60% A, 40% B A = 0.01M KH₂PO₄ (pH 4.0) B = 80% 1:1 CH₃CN:CH₃OH + 20% A</td>
<td>70°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gradient 70:30 A: B to 30:70 A: B in 40 min. (linear gradient)</td>
<td></td>
</tr>
<tr>
<td>Tablets</td>
<td>Lichrosorb C8, 200 X 4.6 mm, 10 μ</td>
<td>68% A, 32% B A = 0.001M KH₂PO₄ (pH 2.0) B = CH₃CN</td>
<td>80°C</td>
</tr>
<tr>
<td>Tablets</td>
<td>Lichrosorb C8, 200 X 4.6 mm, 10 μ</td>
<td>70% A, 30% B A = 0.02M KH₂PO₄ (pH 4.0) B = CH₃CN</td>
<td>80°C</td>
</tr>
<tr>
<td>Tablets</td>
<td>Endcapped C8 50 X 4.6 mm, 5 μ</td>
<td>80% A, 20% B A = 0.02M KH₂PO₄ (pH 4.0) B = CH₃CN</td>
<td>70°C</td>
</tr>
</tbody>
</table>

**5.2.4.4 Determination in biological fluids**

A number of procedures have been developed for the analysis of enalapril and enalaprilat in biological fluids. Those of primary utility include radiometric, enzyme inhibition and radioimmunology methods.
The physiological disposition and metabolism of enalapril maleate in laboratory animals has been studied with both radiolabeled drug and enzyme-inhibition assay. Following administration of the radioactive drug: radioactivity in urine, feces or plasma samples may be counted by liquid scintillation techniques for total radioactivity. Urinary radioactivity may be separated into enalapril and enalaprilat by thin layer chromatography (Tocco et al, 1982).

Enalaprilat can also be determined by its in vitro inhibition of angiotensin converting enzyme. Plasma, urine and homogenates of feces are placed into XAD-4 columns, washed and then eluted with methanol to yield enalapril and enalaprilat. The enalaprilate component of the mixture is determined by the enzyme assay which uses as a substrate, the tripeptide carbo-benzoxy carbonyl-phenylalanyl-histidyl leucine and converting enzyme isolated from porcine plasma. The product, histidylleucine is then quantified by fluorescence after reaction with o-phthalaldehyde. For the determination of the total drug (enalapril and enalaprilat) samples are hydrolyzed at high pH and reassayed for enalaprilat. Enalapril levels may then be calculated as the difference between the total drug and enalaprilat prior to hydrolysis (Piquillaud et al, 1970).

Assay procedure employed for the present studies

HPLC chromatograms were run on a Shimadzu prominence fitted with LC-20 AD pump, connected to a UV-Vis. detector, SDA-20A set at 215nm. A Phenomex Luna L-7 (5 μm) column (250 mm X 4.6 mm) was utilized. Constant chromatographic column temperature control was maintained at 50 °C with the help of a column oven (CTO-10 AS). The mobile phase used was a mixture of phosphate buffer (pH 2.2) – acetonitrile (75:25) and the flow rate was 1.5 ml/min. The injection volume was 20 μl. Pure water (purified by milli-Q system) was used as solvent. Fig. 5.2 exhibits representative chromatogram for enalapril maleate standard. Retention time for enalapril was found to be ~ 1.7 min.
A stock solution of enalapril maleate having concentration 2 mg/ml was prepared in phosphate buffer 2.2. The solution was appropriately diluted with to contain concentrations in the range of 5 - 50 μg/ml. The calibration curve obtained by plotting integrated peak area against concentration of enalapril maleate in solution is presented in Figure 5.3.

**Figure 5.3 Calibration curve for enalapril maleate using HPLC method.**
5.3 Glipizide

Glipizide (GLP) is a second generation sulphonylurea that lowers the blood glucose level in humans by stimulating the release of insulin from the pancreas and is typically prescribed to treat non-insulin dependent diabetes mellitus (NIDDM) (D'Onofrio et al., 1972; Brogden et al., 1979). GLP, as a shorter acting sulphonylurea, is thought to be more efficacious in enhancing meal-stimulated insulin secretion (Klasseen, 1979, Skillman and Feldman, 1981). Pharmacology suggests that glipizide sensitizes beta cells of pancreas to release insulin only in the presence of elevated serum glucose level (Raptis et al., 1969, Baker and Campbell, 1984, Lawrence et al., 1984).

5.3.1 Description (Bodavari, 1996; Moffat et al., 2003).

Chemical name

Synonym Glyburide

Chemical structure

![Chemical structure of Glipizide]

Molecular formula $\text{C}_{21}\text{H}_{27}\text{N}_5\text{O}_4\text{S}$

Molecular weight 445.54

CAS registry no. 29094-61-9

5.3.2 Physicochemical properties

GLP is available as whitish or almost white crystalline odorless powder

Melting point: 208-209°C

$logP$: 1.763
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**Solubility**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>Practically insoluble</td>
</tr>
<tr>
<td>Alcohol</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Acetone</td>
<td>Sparingly soluble</td>
</tr>
<tr>
<td>Methanol</td>
<td>Soluble</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>Soluble</td>
</tr>
<tr>
<td>0.1N NaOH</td>
<td>Soluble</td>
</tr>
<tr>
<td>Dimethyl formamide</td>
<td>Freely soluble</td>
</tr>
</tbody>
</table>

Glipizide is a weak acid with pKa of 5.9 (Jamzad and Fassihi, 2005). The drug, being insoluble in water, belongs to BCS class 2 (FDA/CDER, 2002) and its dissolution is considered to be a rate-determining step in its absorption from the gastro-intestinal fluids. Recently, attempts have been made to study detailed solubility/dissolution profile of GLP and to improve its dissolution behavior using cyclodextrin inclusion compounds or as solid dispersion with water-soluble carriers (Jamzad and Fassihi, 2006, Aly et al, 2003, Himasankar et al 2002). The crystal structure of GLP and the quantitative energies for interactions between molecules have been determined by determined ab initio using variable temperature laboratory X-ray powder diffraction combined with a direct-space Monte Carlo/simulated annealing methodology (Burley, 2005).

5.3.3 Pharmacokinetics

Glipizide is rapidly and well absorbed from the gastrointestinal tract following oral administration (Balant et al, 1973; Wahlin-Boll et al, 1982). Peak serum concentrations occur between one and six hours of dosing, averaging about 1.5-2 hours, when taken on an empty stomach. Ingestion concurrent with food appears to delay absorption by 40 minutes but has no influence on peak concentration, elimination half life, or bioavailability (Sartor et al, 1978). Glipizide appears to be characterized by a two-compartment open model. The central compartment probably consists of blood and extracellular fluid in highly perfused tissues (e.g. heart and kidney). The peripheral compartment probably consists of less perfused tissues (e.g. muscle, skin, and body fat), which the drug enters more slowly (Pentikainen et al, 1981; Schmidt et al, 1973).

Glipizide is almost completely metabolized by the liver to 2 hydroxycyclohexyl derivatives and N-(2)-acetylaminoethylphenyl sulphonyl- N1-cyclohexylurea (Fuccella
et al., 1973). These metabolites, pharmacologically inactive, are then excreted from the body mainly in the urine. Three percent to ten percent of the parent compound is excreted unchanged in the urine. The accepted terminal plasma half-life for glipizide is two to four hours, but it may range from two to seven hours (Balant, 1981). Renal insufficiency appears not to significantly affect the terminal half-life of the parent drug, but does prolong the terminal half-life of glipizide’s metabolites, also pharmacologically inactive. In adults with normal renal function, the drug’s duration of action is about 10 to 24 hours. In patients with decreased renal function, a more prolonged hypoglycemic effect has been observed (Pentikainen, 1981).

Pharmacokinetic data (Hardman and Limbird, 2001)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral availability</td>
<td>95 %</td>
</tr>
<tr>
<td>Urinary excretion</td>
<td>&lt; 5 %</td>
</tr>
<tr>
<td>Bound in plasma</td>
<td>98.4 %</td>
</tr>
<tr>
<td>Clearance</td>
<td>0.52 ml/min.kg</td>
</tr>
<tr>
<td>Volume of distribution</td>
<td>0.17 litres/kg</td>
</tr>
<tr>
<td>Half life</td>
<td>3.4 hrs</td>
</tr>
<tr>
<td>Peak time</td>
<td>2.1 hrs</td>
</tr>
<tr>
<td>Peak concentrations</td>
<td>465 ng/ml</td>
</tr>
</tbody>
</table>

5.3.4 Methods of analysis

UV analysis

Glipizide exhibits absorption maxima at 276 nm in aqueous system, which has been reported to be sensitive to minor changes in the drug concentration. Hence UV analysis is the most commonly used quantitative method for determination of drug concentration in solution.

HPLC

USP recommends following procedure for assay of drug content:

- Mobile phase: phosphate buffer pH 6: methanol (55: 45)
- Column 15 cm X 3.9 mm column containing 5 µm packing L1.
- Flow rate 1.0 ml/min.
- Detection 225 nm.
Standard concentration stock solution in methanol, followed by dilution in phosphate buffer solution having concentration 0.05 mg/ml.

**Determination in biological samples**

A number of HPLC methods are employed for identification of sulphonyl urea drugs in the blood (Sved et al, 1976; Sener et al, 1995) Some HPLC method based on precolumn derivatization with fluorodinitrobenzene have also been reported (Zecca et al, 1985; Arcelloni et al, 1990)

A method for determination of GLP in breast milk and serum was utilized by Feig et al (Feig et al, 2005). Thus, GLP was extracted from 1-ml samples by liquid-liquid extraction with 5 ml of chloroform. Samples were analyzed by HPLC using a Luna C-8 column (3µm particles, 150 X 4.6 mm) equipped with a C-8 Security Guard cartridge and fluorescence detection (excitation= 470 nm and emission= 530 nm). The HPLC mobile phase consisted of 35% acetonitrile in water and was run at 0.5 ml/min. The limit of detection was 0.005 µg/ml.

Paroni et al compared HPLC method with Capillary electrophoresis (CE) analysis for determination of glipizide in serum samples. (Paroni et al, 2000). Serum samples were acidified HCl, and diluted 1:1 (by volume) with water. The diluted serum samples were loaded onto the solid phase extraction columns and then washed with water, followed by a series of water: methanol mixtures [starting from 95:5 (by volume), 80:20, 70:30, and 60:40]. After air was flushed through the columns, the drugs eluted with methanol: acetonitrile (1:1, by volume) and dried under reduced pressure. The HPLC column used was a Lithosphere 100 RP-18 [25 cm X 4.5 mm (i.d.); 5 µm bead size]. The dried samples were reconstituted with of K$_2$HPO$_4$: methanol (40:60, by volume), and 20–100 µL was injected into the column, using UV detector set at 225 nm. A good compromise between rapidity of analysis and resolution was achieved simply by eluting the column with 600 mL/L methanol in potassium phosphate buffer. To further improve the separation of the first eluting peaks, the analysis was sometimes started at 580 mL/L methanol. CE separation was carried out using equipped with a monochromatic ultraviolet detector set at 200 nm. The method consists of exploiting fused-silica capillary column. The procedure consisted of a prerinse with the running buffer followed by an injection of the sample at low pressure, pressure injection of running buffer, rinse with the washing buffer, followed by 1 min with 0.1 mol/L NaOH. The capillary temperature was maintained at 25 °C. The
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dried samples were reconstituted with 30–60 ml of acetonitrile: water (60:40, by volume) and loaded into the water-cooled autosampler tray.

CE technique was observed to offer several advantages compared with HPLC or mass spectrometry: it is very rapid (analysis time <6 min), relatively robust, inexpensive (low volumes of buffers and reagents), and quite reproducible with the use of a suitable internal standard. Coupling with a diode array detector or with a mass spectrometer may improve the accuracy of the assay.

**Method of analysis for present study:**

Glipizide was estimated by ultraviolet-visible (UV/Vis) spectrophotometric method (Unicam UV-visible double beam spectrophotometer v1.30). Aqueous solutions of glipizide were prepared in phosphate buffer (pH 7.4) and absorbance was measured on UV/Vis spectrophotometer at 276 nm. The method was found to obey Beer’s Law in the concentration range of 5 to 50 μg/mL ($r^2 = 0.999$).

**Fig. 5.4 Standard curve for GLP in phosphate buffer 7.4**

![Graph showing the standard curve for glipizide in phosphate buffer 7.4](image)

**Equation:**

\[ y = 0.023x + 0.031 \]

**$R^2 = 0.999$**
5.4 13-Cis- Retinoic Acid (Isotretinoin)

13-cis retinoic acid, cis-RA (isotretinoin) is a synthetic retinoid, which has found clinical applications by systemic therapy for the treatment of severe calcitrant cystic acne vulgaris and in the cases of persistent and recurrent moderate acne (Jones, 1989; Farrell et al., 1980; Peck et al., 1979). The mechanism of action is believed to involve the inhibition of sebum production and reduction of sebaceous gland size. It also induces changes in skin lipids, follicular keratinization and the inflammation associated with acne (Marsden et al., 1984; Shalita, 1984). In addition, cis-RA has been reported to reverse the process of chemical carcinogenesis by eliminating preneoplastic lesions and has been found useful in prevention of epithelial cancers (Lippman et al., 1993). However, 13-cis retinoic acid is known to have adverse effects typical of chronic hypervitaminosis A and teratogenic effects (Chen, 1985), therefore, use of the drug has been restricted to non-pregnant females and patients unresponsive to conventional acne therapies.

5.4.1 Description (Bodavari, 1997; Dollery, 1998)

Chemical name 3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)2-cis-4-trans-6-trans-8-trans-nonatetraenoic acid.

Synonymous names 13-Z-Retinoic acid; 13-cis-Retinoic acid; 2-cis-vitamin A acid; neovitamin A acid; R0-4-3780

Chemical structure

![Chemical structure of 13-cis Retinoic Acid](image)

Molecular formula $C_{20}H_{28}O_2$

Molecular wt. 300.4

CAS registry number 4759-48-2
5.4.2 Physicochemical properties

Cis-RA occurs as yellow to yellow-orange crystalline powder having characteristic odor.

**Solubility**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>Practically insoluble</td>
</tr>
<tr>
<td>Alcohol</td>
<td>Slightly soluble</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Soluble (10g/100ml).</td>
</tr>
<tr>
<td>Methylene chloride</td>
<td>Soluble</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Soluble</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>Slightly soluble</td>
</tr>
<tr>
<td>Macrogol 400</td>
<td>Slightly soluble</td>
</tr>
<tr>
<td>Polyethylene glycol</td>
<td>Soluble</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>Soluble</td>
</tr>
<tr>
<td>0.1 N NaOH</td>
<td>Soluble</td>
</tr>
</tbody>
</table>

Solubility of cis-RA in a series of oily vehicles (Nankervis et al, 1995)

- Cottonseed oil : 5.1 mg/ml
- Soybean oil : 5.68 mg/ml
- Peanut oil : 6.1 mg/ml
- Miglyol 812 : 12.0 mg/ml

Soybean oil is usually used as a suspension medium for Cis-RA in soft gelatin capsules.

**Melting point** 174-175 °C.

**log P** 6.6 (Nankervis et al, 1993)

**pka** From 6 to 8 depending on concentration and upon solvent composition. Above pka retinoic acid is highly soluble in water. The high value of pka is attributed to intermolecular interactions (Gundersen and Blomhoff, 2001)

**Solid state stability**

Cis-RA is sensitive to air, heat, and light, especially in solution. It should be stored in air-tight containers at a temperature not exceeding 25 °C. It must be protected from light. It is recommended that the contents of an open container should be used as soon as possible and that any unused part may be protected by an atmosphere of an inert gas (Tan et al, 1992; Lucero et al, 1994; Brisaert et al, 1995).
5.4.3 Pharmacokinetics

*Cis*-RA is rapidly absorbed after oral administration to give detection of its concentrations in plasma within 20 to 30 minutes after administration and peak plasma levels between 2-4 hrs after dosing. Drug levels then decline with a half life of approx. 15 hrs. A number of secondary peaks are seen in the blood concentration-time curve owing to enterohepatic recirculation. The drug is almost completely (>99.5 %) bound to plasma proteins. Volume of distribution is 1.5 l/kg. *Cis*-RA is nearly insoluble in water, but soluble in oily or fatty substances. Therefore the plasma concentrations are increased when taken in combination with fatty food.

It is difficult to determine *Cis*-RA's absolute bioavailability, as it is not administered by the intravenous route.

Excretion studies demonstrated that the drug is cleared almost entirely by metabolism with little urinary excretion of intact *cis*-RA and tretinoin. 4-oxo-*cis*-RA is found to be a major metabolite in blood following oral administration; it is biologically active and may contribute to the activity of the parent compound. The drug and its metabolites undergo glucuronidation and metabolic degradation prior to elimination in bile and urine.

As *cis*-RA is known to be teratogenic, pregnancy is an absolute contra-indication to its clinical use.

5.4.4 Methods of analysis

*Colorimetric methods*

These estimation methods are based on reaction of Antimony trichloride (the Carr-Price reagent) with Vitamin A acid, which lead to formation of colored complex with maxima at 565 nm (Jurkowitz, 1962; Nelson et al, 1965). Thus the method involves preparing standard curves covering the range of 0-20 g by making appropriate dilutions of a stock solution of crystalline Vitamin A acid dissolved in chloroform. One ml portions are transferred to a calorimeter tube and 3 drops of acetic anhydride are added. The tube and sample are placed in the calorimeter and 9 ml of antimony chloride reagent (Carr-Price reagent) is added. Absorbancy is to be measured at 565 nm within a period of 35 secs.

Later the formation of a colored complex of Vitamin A acids with TriFlouro Acetic acid (TFA) having absorption maxima at 562 nm was also reported. The method permits the detection of as little as 0.3 µg of vitamin A acids (Wang et al, 1978). Standard solutions
of cis-RA are prepared in chloroform in the concentration range of $3\times 10^{-6}$ to $3\times 10^{-5}$ M. A portion of this solution is placed in cuvette and an equal volume of trifluoreacetic acid is added. The cuvette is immediately covered, and the solution was rapidly mixed by inverting the cuvette twice. In the standard assay, absorbance is measured at 562 nm 10 sec after adding TFA. There is a linear relationship between absorbance and concentration.

**UV determinations**

Cis-RA has electron-rich polyene chain. The polyene chain consists of several carbon–carbon double bonds in conjugation and in cis-RA the polyene chain is extended by a carbonyl group. Thus, very selective and sensitive detection with UV detectors can be achieved. Thus cis-RA exhibits absorption maxima at 354 nm, extinction constant 39750 (solvent ethanol) (Gunderson and Blomhoff, 2001).

**Non-aqueous titration**

The method involves dissolving weighed amount of cis-RA is in acetone and titrating the solution against tetrabutylammonium hydroxide. The end point is determined potentiometrically. (USP)

**Chromatography**

Using liquid gel chromatography, the presence of $^{14}$C retinoic acid as well as other $^{14}$C labeled Vitamin A compounds in rat tissues after a physiological dose of $^{14}$C retinyl acetate was determined (Ito et al, 1974). A clear separation of retinol, retinal and retinoic acid was achieved by liquid-gel partition chromatography on Sephadex LH 20 with solvent mixtures of chloroform and methanol.

**HPLC methods**

Numerous HPLC methods for determination of cis-RA in different kinds of samples have been developed (Vane et al, 1982).

A simple and selective method for determination of cis-RA and tretinoin in human plasma involves separation of retinoids using reverse phase HPLC. The method involves use of ODS column and acetonitrile- 1 % ammonium acetate (65:35) as the eluting solvents. The retinoids are detected by absorbance at 350 nm (Frolik et al, 1978)

A reverse phase HPLC system equipped with two Pecosphere 3 X 3CR ODS cartridge column and fitted with UV –visible detector fixed at 340 nm is used. The mobile phase...
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consists of methanol and water (with 1% ammonium acetate). The gradient program with a flow rate of 1.5 ml/min was as follows: methanol-water 75:25 v/v was eluted for 7 min, followed by an 8 min linear gradient to 100% methanol, followed by a 5 min linear gradient return to methanol-water 75:25 v/v.

Mobile phase consist of MeCN: buffer 80: 20 for 10 min, to 90: 10 (step gradient) buffer was 100mM ammonium acetate adjusted to pH 5.0 with acetic acid. 20 μl of sample is injected into two-reverse phase C18 column in series that were maintained at 40°C throughout the run. Solvent eluting from second column is directed to the diode-array detector and monitored at 360 nm.

Determination in biological samples

Despite continuous development and improvement of analytical equipment and instrumentation, the chromatographic determination of retinoids in biological samples is still among the most challenging tasks in analytical chemistry. This is mainly due to the low concentrations combined with the complexity of the biosamples as well as instability towards several factors such as daylight, high temperatures and oxygen. A generic method for their extraction and separation seems impossible to develop. Depending on the actual sample, its matrix composition, the concentration level of the retinoids present, existing methods need to be modified to obtain the selectivity, sensitivity and reproducibility required for the special application. Thus, a multitude of methods are in use for quantitative and qualitative determination of retinoids in biological samples.

Liquid chromatography continues to be the preferred method for determining retinoids in biological samples. The highly unstable nature of retinoids and the real possibility of artifacts or erroneous results have led to the development of rapid and highly automated protocols for retinoid extraction, separation and detection. Due to strong light absorbance in the ultraviolet region, UV detectors still predominate although mass spectrometric detection has gained increasing popularity (Gundersen and Blomhoff, 2001). Simple protein precipitation or mono-phase extraction directly on a biofluid, after homogenization of tissue or with acetonitrile, 2- propanol, ethanol or acetonitrile–1-butanol (1:1) are used more frequently for extraction of retinoids. Separation of non polar retinoids has been obtained on C_{18} (Wingerath et al, 1997; Hartman et al, 2001) or C_{30} columns with non aqueous mobile phases (Van Breemen et al, 1998) based on,
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acetonitrile, methanol or ethanol modified with chloroform, dichloromethane, 2-propanol, 
1-butanol or MTEB. Separation of polar retinoids including geometrical isomers of 
retinoic acid was achieved on C18 columns either by gradient (Wyss and Bucheli, 1997) 
or isocratic elution (Gundersen et al, 1997; Molander et al, 1999; Sakhi et al, 1998) with 
combinations of acetonitrile–water–ammonium acetate–acetic acid. UV detection is the 
preferred detection technique for retinoids (Wyss and Bucheli, 1997).

Analysis method employed for the present study

HPLC chromatograms were run on a Shimadzu prominence fitted with LC-20 AD pump, 
connected to a UV- Vis. detector, SDA-20A set at 280 nm. A Phenomex Luna C-18 
ODS (5 μm) column (250 mm X 4.6 mm) was utilized. 95 % (v/v) acetonitrile and 5 % 
(v/v) of a 1 % (w/v) aqueous ammonium acetate solution delivered at a flow rate of 1.1 
ml/min. The injection volume was 20 μl. Pure water (purified by milli-Q system) was 
used as solvent. A computer was connected to the detector for data acquisition and peak 
area and retention time calculation. In the chromatogram resulting from this HPLC system, cis-RA was found to elute at the retention time of ~5.477 min. Fig. 5.5 shows 
scan of typical chromatogram for fresh cis-RA sample.

Fig. 5.5 Typical HPLC chromatogram of cis-RA.
A stock solution of 12 mg/ml of cis-RA was prepared in mobile phase. The solution was appropriately diluted to contain a concentration in the range of 20-120 µg/ml. The calibration curve obtained by plotting the integrated peak area against concentration of cis-RA is presented in Fig. 1a in mobile. The calibration curve exhibiting relationship between peak area and concentration of sample is presented in Figure 5.6.

**Figure 5.6 Calibration curve for estimation of cis-RA.**

5.5 Nicorandil

Nicorandil belongs to the class of compounds known as potassium channel activators. Nicorandil has vasodilating properties owing to presence of nitrate group in its chemical structure. The potassium channel activation may also exert direct cytoprotective effects by augmenting normal physiological processes, which protect the heart against ischemic events (Frampton et al, 1992).

5.5.1 Description

*Chemical name* N-(2-nitroxy) ethyl) 3-pyridine carboxamide

*Chemical structure*

\[
\begin{array}{c}
\text{N} \quad \text{H} \\
\text{O} \quad \text{O} \\
\text{O} \quad \text{N} \\
\end{array}
\]
**Molecular formula** C₈H₉N₃O₄

**Molecular weight** 211.18

**CAS registry no.** 65141-46-0

**5.6.2 Physicochemical properties:**
Colorless to off-white crystalline powder.

**Solubility**
- Water: freely soluble
- Ether: poorly soluble
- Ethanol: poorly soluble

**Melting point**: 92-93° C.

**Partition coefficient** Log P (octanol/water) 0.43

**Solid state stability**
In the solid crystalline state, nicorandil is stable under conditions of extreme dryness, but when it is exposed, although for short periods of time and room temperature, even at low humidity, a considerable humidity ensues. The hydrolysis of nicorandil is catalyzed by three factors, each influencing the others, namely, the increased percentage of moisture in the product in powder state, the temperature and the storage period (Veronesi *et al.*, 1996).

**5.5.3 Pharmacokinetics**
Nicorandil is well and rapidly absorbed from gastro-intestinal tract, but food can decrease rate of absorption. After a single dose of 5-20 mg, the peak plasma concentrations (5-250 μg/L) are achieved within 0.5-1 hr (Frampton *et al.*, 1992). Nicorandil has a short elimination half life of 60-80 min. and is 90 % bound to plasma proteins. The drug is not subjected to a significant first pass metabolism; the absolute bioavailability is reported to be 75 % (Frydman *et al.*, 1989).

Nicorandil undergoes biotransformation predominantly by denitration of nicorandil to pharmacologically inactive alcohol metabolite, N-(2-hydroxyethyl)-nicotinamide, followed by side chain degradation to nicotinamide and related metabolites, including nicotinic acid and N-methyl-nicotinic acid and N-methyl nicotinamide. Further metabolism of these products leads to non-toxic water soluble vitamin B complex substances. The denitration occurs primarily in the liver.
The kidney is a major route of elimination with 20% of an administered dose being excreted in urine mainly as metabolites. Less than 2% of the dose is excreted via biliary route. 10% of the administered dose can be found in plasma but it is rapidly excreted.

5.5.4 Methods of analysis

Colorimetric analysis

Some simple, sensitive and selective colorimetric methods for the assay of nicorandil in drug formulations have been reported. The assays are based on exploiting the oxidizing property of the drug due to the presence of nitrate moiety in the chemical structure of the drug. The methods are based on the reaction of drug with phloroglucinol-sulfanilic acid reagent and on the basis of oxidative coupling of MBTH with DL-dopa in the presence of nicorandil in sulfuric acid medium. (Rahman et al, 2004).

UV analysis

A UV scan of an aqueous solution of nicorandil demonstrates absorption maxima at 262 nm, which is sensitive to minor alterations in concentration of the drug in solution.

HPLC analysis

Following are some of the representative HPLC methods reported for nicorandil assay:

Column C8 (250 X 4.6 mm i.d., 5 μm) guard C18, column temperature 30 °C. Mobile phase: methanol: water (15:85), flow rate 1.2 l/min. UV detection at max. 230 nm. Retention time 35.3 min. (Ojha and Pargal, 1999).

Column C18 (300 X 3.9 mm i.d., 10 μm). Mobile phase: acetonitrile:water:borate buffer (0.1 M, pH 8) (15:70:15), flow rate 1 ml/min. UV detection at max. 220 nm. Retention time 8 min. (Tanikawa et al, 1993).

A method for determination of nitrate (NIT) impurity from nicorandil (NIC) and its tablet dosage form was developed. This simple, fast, precise, specific and economical reverse phase liquid chromatographic method was based on application of amino propyl silane (APS) column for determination of NIT in presence of NIC and various excipients using potassium nitrate as a standard. The developed method was applied to evaluate the compatibility study of NIC with various excipients by monitoring the NIT content (Mehta et al, 2006).

Following HPLC method has been reported for stability and pharmacokinetic studies study (Bachert and Fung, 1993)

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HPLC chromatograms were run on a Shimadzu prominence fitted with LC-20 AD pump, connected to a UV-Vis. detector, SDA-20A set at 254 nm. A Phenomex Luna L-3 (5 µm) column (250 mm X 4.6 mm) was utilized. The mobile phase consisted of filtered and degassed 45 % methanol-double distilled water and the flow rate was 1.0 ml/min. The injection volume was 20 µl. Pure water (purified by milli-Q system) was used as solvent. (Nagal et al, 1984)

Determination in biological samples

Several analytical methods have been reported for estimation of nicorandil on biological fluids and/or pharmaceutical formulations including high performance thin layer chromatography, high performance liquid chromatography and gas chromatography coupled with mass spectrometry.

Bhatt et al proposed a rapid and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the estimation of nicorandil in human plasma. Nicorandil was extracted from human plasma using solid-phase extraction technique. Imipramine was used as the internal standard. A Betasil C18 column provided chromatographic separation of analytes followed by detection with mass spectrometry. The method involves a rapid solid-phase extraction from plasma, simple isocratic chromatography conditions and mass spectrometric detection that enables detection at nanogram levels. The proposed method has been validated for a linear range of 1.0-500.0 ng/mL with a correlation coefficient of ≥0.9993. The intra-run and inter-run precision and accuracy was within 10.0%. The overall recovery for nicorandil was 63.81%. The total run time was just 3.0 min (Bhatt et al, 2005).

Schwende and Lewis reported a method for the determination of nicorandil involving solid-phase extraction of the drug and internal standard using Bond-Elut C18 extraction columns, reversed-phase high-performance liquid chromatography on a Zorbax-Phenyl column and detection with photoconductivity and ultraviolet detection in series. Photoconductivity, performed with the Tracor 965 photoconductivity detector, provided a limit of detection of 2 ng/ml in plasma. Ultraviolet detection in series with the photoconductivity detector extended the linear range of the analytical system to 1000 ng/ml (coefficient of variation 4.4%). The utility of the method was demonstrated in a dog pharmacokinetic study in which a 5-mg intravenous dose was compared to a 10-mg
oral solution dose in six beagle dogs. The oral solution was absorbed rapidly, achieving an average maximum concentration of 857 ng/ml in 11.2 min. The absolute bioavailability of nicorandil in dogs in this study was determined to be 84.2%. (Schwende and Lewis, 1990)

HPLC method was developed for the quantification of nicorandil (SG-75) in human plasma samples for routine bioequivalence studies. The sample preparation needs two liquid-liquid extractions, first with CH₃Cl and HClO₄ as denaturation reagent and second with addition of ethyl acetate and Na₂CO₃ (aq). The chromatogram was run under following conditions: Column phenyl (150 X 4.6 mm i.d., 5 μm) mobile phase: ammonium acetate buffer (0.01 M, pH 6.2 with acetic acid); acetonitrile (10:3), flow rate 1.0 ml/min. retention time 6 min. (Andrensek et al, 1999)

Assay method employed for the present investigations

The UV spectrophometric method of assay for NRD was employed for estimation of the drug content. Thus, stock solution of NRD in water having a concentration of 1.2 mg/ml was prepared in water. Working standards, to obtain concentrations within a range of 2-40 μg/ml, were prepared by appropriate dilution of the stock solution and used to set up the calibration curve (Figure 5.7). The drug contents were determined by measuring absorbance at λ_max of 262 nm using Unicam UV-visible double beam spectrophotometer v1.30.

Figure 5.7 Calibration curve used for estimation of nicorandil.
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To conclude, retrofit analysis of literature, led to identification and selection of following five drug candidates, which became the subject of present investigations:

1. **Amiloride hydrochloride (AH):** Amiloride hydrochloride (AH) is a potassium sparing diuretic, with relatively weak natriuretic and anti-hypertensive activity. Though as per USP, Amiloride Hydrochloride is a slightly water soluble drug, but the same has been classified as BCS class I. This may be attributed to the fact that dose of the drug happens to be within the solubility limits. However, complexation with urea is naturally bound to significantly improve its dissolution profile leading to improved bioavailability culminating to immediate relief to a hypertensive patient.

2. **Enalapril maleate (EM):** Enalapril maleate is highly effective antihypertensive agent, which act as angiotensin converting enzyme inhibitors. Though as per BP, Martindale, the Extra Pharmacopoeia, enalapril maleate is a slightly water soluble drug, dose of the drug happens to be within the solubility limits and hence, the drug is classified as BCS class I. However, complexation with urea is bound to significantly improve dissolution profile of the drug, leading to instantaneous release of the drug and to improved bioavailability culminating to immediate relief to a hypertensive patient.

3. **Glipizide (GLP):** A second generation sulphonylurea that lowers the blood glucose level in humans and is typically prescribed to treat non-insulin dependent diabetes mellitus. Glipizide, being insoluble in water, belongs to BCS class II. An attempt has been made in the present study for steep enhancement of dissolution rate of GLP through formation of co-inclusion compounds of GLP in urea using a modified technique.

4. **Cis- Retinoic Acid (isotretinoin):** 13-cis retinoic acid or cis-retinoic acid (cis-RA) is a synthetic retinoid, which has found clinical applications by systemic therapy for the treatment of severe calcitrant cystic acne vulgaris. Cis-RA is a lipophilic drug and is sparingly soluble in water. It is poorly absorbed after oral administration and is advised to be taken with food. In general, retinoids are unstable compounds, being sensitive to oxygen, heat, and light. Their stability is, therefore, of pharmaceutical interest. Moreover, cis-RA is regarded as hazardous...
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drug and requires safe handing by personnel involved with preparation, processing, administration and disposal of the drug. Hence, urea inclusion compound formation is expected to impart safe handling characteristics to cis-RA in addition to simultaneous improvement in the dissolution profile and photostability.

5. *Nicorandil (NRD)*: A potassium channel activators, has coronary vasodilative and coronary vasoconstriction suppressing actions and is useful as a curative for various types of angina. In the solid crystalline state, nicorandil is stable under conditions of extreme dryness, but when it is exposed, although for short periods of time and room temperature, even at low humidity, a considerable humidity ensues. The progressive degradation of nicorandil leads to liberation of nitric acid and N-(2-hydroxyethyl) nicotinamide, a compound that is not pharmacologically active at all considerable dosages. In the present study, possibility of improvement in moisture stability of NRD, a NNAE drug, has been investigated through formation of co-inclusion compounds with urea in presence of suitable RAE.