CHAPTER 3


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</table>
3.1 Introduction:

Earlier, potent antihyperlipidemic activity has been described in a series of N-cyanovinylformamidine\(^{38}\) and compound N-[2-carbethoxy-2-cyano-1-(p-chloroanilino)vinyi]formamidine, \(164p\) was found to be the most potent. This novel compound was found to be two times more potent than the known antihyperlipidemic drug gemfibrozil, in lowering serum cholesterol and triglyceride levels and elevating serum HDL levels. Moreover, subacute toxicity study of this compound has revealed no mortality or behavioural changes in mice even up to the dose of 4500mg/kg p.o. Thus, the compound \(164p\) has potentialities to be developed as novel antihyperlipidemic agent.

3.2 Aim of present work:

As a part of preclinical work, detailed stability and pharmacokinetic study of compound \(164p\) was carried out. This study may also help in elucidating mechanism of action. High Performance Thin Layer Chromatography (HPTLC) method to estimate compound \(164p\) in plasma was developed and validated. Biolavailability study was carried out on Newzealander rabbits.
3.3 Profile of the compound 164p:

1) Chemical name: N-[2-carbethoxy-2-cyano-1-(p-chloroanilino)-
vinyl]formamidine

2) Structure:

3) Molecular formula: C_{13}H_{13}ClN_{4}O_{2}

4) Molecular weight: 292.73

5) Melting point: 207 - 210°C (Dichloromethane - n-Hexane)

6) Physical characteristics: colourless, odourless, fluffy powder

7) Solubility
   Soluble: Benzene, dichloromethane, chloroform, methanol,
           acetonitrile, 0.1N HCl
   Insoluble: n-Hexane, water

8) Thin Layer Chromatography
   a) Mobile phase: Benzene : Methanol (9:1) v/v
   b) Rf value: 0.21

9) ClogP: 2.291

10) pKa (By Potentiometry): pK_1 = 1.78 & pK_2 = 8.32
11) **UV spectra**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\varepsilon$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1N HCl</td>
<td>307.2</td>
<td>34659.2</td>
</tr>
<tr>
<td>0.1N NaOH</td>
<td>256.4</td>
<td>38874.5 (unstable)</td>
</tr>
<tr>
<td>Methanol</td>
<td>316.0</td>
<td>28131.4</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>318.0</td>
<td>26989.7</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>321.8</td>
<td>22774.4</td>
</tr>
</tbody>
</table>

12) **IR spectra (cm$^{-1}$)**

- 3400, 3360, 3300, 3240 (NH), 2200 (CN), 1680 (C=O)

13) **$^1$H NMR spectra**

(CDCl$_3$) :

- $\delta$ 1.25 (t, 3H, CO$_2$CH$_2$CH$_3$), $\delta$ 4.10 (q, 2H, CO$_2$CH$_2$CH$_3$), $\delta$ 5.60 (broad s, 2H, NH$_2$), $\delta$ 6.80 - 7.25 (m, 5H, Ar-H and 2C-H), $\delta$ 14.20 (s, 1H, HN-Ar)

(CDCl$_3$+$D_2$O) :

- $\delta$ 1.25 (t, 3H, CO$_2$CH$_2$CH$_3$), $\delta$ 4.10 (q, 2H, CO$_2$CH$_2$CH$_3$), $\delta$ 6.80 - 7.25 (m, 5H, Ar-H and 2C-H).

14) **Mass spectra**

- 294 (M+2), 292 (M+, 81%), 260, 245 (M-46, 100%),

15) **Stability**

- Compound 164p is stable in polar & nonpolar solvents at room temperature.
- When heated in 0.1N HCl for 6 hr. 164p decomposes to ethyl 3-amino-3-(p-chloroanilino)-2-cyanoacrylate, 195p, an aminal.
- In 0.1N NaOH at room temperature it cyclises to 5-cyano-6-(p-chloroanilino)-pyrimidin-4-one, 200k, within one hour.
3.4 Analytical method development of compound 164p:

3.4.1 Quantitative estimation of compound 164p:

Method: UV spectroscopy
Instrument used: Shimadzu 160 A double beam UV / Visible spectrophotometer
Solvent used: Methanol (analytical grade)
Absorbance measured at: 316 nm

Procedure:

Accurately weighed 10mg of compound 164p was dissolved in methanol in a 100ml volumetric flask and volume made to the mark with methanol. Aliquots of 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2.0, 2.25, and 2.5ml were transferred into 25ml volumetric flask and the volume made to mark with methanol. On dilution, concentrations of 164p varied from 1 to 10 µg/ml. Absorbance of each solution was measured at 316 nm using methanol as blank. Same procedure was repeated three times and linearity curve of absorbance v/s concentration was obtained (Table 3.1; Fig. 1 & 2).

Table: 3.1: Quantitative estimation of compound 164p, (Beer's Curve)
(By UV method)

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Absorbance (at 316 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.071</td>
</tr>
<tr>
<td>2</td>
<td>0.160</td>
</tr>
<tr>
<td>4</td>
<td>0.321</td>
</tr>
<tr>
<td>6</td>
<td>0.491</td>
</tr>
<tr>
<td>8</td>
<td>0.661</td>
</tr>
<tr>
<td>10</td>
<td>0.803</td>
</tr>
</tbody>
</table>
Figure 1: UV spectra of compound 164p and 195p
Figure 2: Beer's curve of compound 164p
3.4.2 Solid phase extraction of compound 164p at different pH:

**Method**: Solid phase extraction

**Cartridge**: RP C-18 solid phase extraction cartridge (E. Merck, India)

**Solvents used**: Acetonitrile (analytical grade), water (triple distilled)

**Buffer solution**: Phosphate buffer solutions of different pH (U.S.P procedure)

**Procedure**:

Stock solution of compound 164p was prepared by dissolving 10mg of the compound in 100ml of acetonitrile. Working standards were prepared by transferring 2.5ml of stock solution to different 25ml volumetric flasks and diluted to mark with phosphate buffer solution of pH 2.3, 4, 6, 6.5, 6.9, 7.4, 8 and 9.1, respectively.

Initially, cartridge was conditioned by washing with 2ml of acetonitrile followed by same volume of water. Care was taken to keep cartridge wet. One ml of standard solution (6µg/ml) was forced slowly through the extraction cartridge so that compound is retained on the cartridge. Cartridge was then washed twice with 1ml of water and dried by a stream of nitrogen through it. After drying, the compound 164p retained was eluted from cartridge with 2ml of acetonitrile. Absorbance of elute was measured at 316 nm. Same procedure was repeated for other buffer solutions.

Absorbance of unknown solution was then compared with standard solution and concentration of drug extracted was calculated by following formula, (Table 3.2).

\[ Cu = \frac{(Au)}{(As)} \cdot Cs \]

\( Cu \) = Concentration of unknown (µg/ml); \( Cs \) = Concentration of standard (µg/ml)

\( Au \) = Absorbance of unknown (nm); \( As \) = Absorbance of standard (nm)

Percentage of drug extracted was then calculated by following formula,

\[ \% \text{ drug extracted} = \frac{(Cu \cdot 100)}{Cs} \]

\( Cu \) = Concentration of unknown (µg/ml); \( Cs \) = Concentration of standard (µg/ml)
Table 3.2: Solid phase extraction of compound 164p in different buffer solutions

<table>
<thead>
<tr>
<th>pH</th>
<th>ABSORBANCE at 316 nm</th>
<th>% of Drug Extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>STANDARD (6 μg/ml)</td>
<td>ELUTE (6 μg/ml)</td>
</tr>
<tr>
<td>6.0</td>
<td>0.504</td>
<td>0.465</td>
</tr>
<tr>
<td>6.45</td>
<td>0.486</td>
<td>0.426</td>
</tr>
<tr>
<td>6.92</td>
<td>0.513</td>
<td>0.456</td>
</tr>
<tr>
<td>7.40</td>
<td>0.524</td>
<td>0.522</td>
</tr>
<tr>
<td>8.00</td>
<td>0.559</td>
<td>0.424</td>
</tr>
<tr>
<td>9.13</td>
<td>0.437</td>
<td>0.420</td>
</tr>
</tbody>
</table>

3.4.3 Development of analytical method for the estimation of compound 164p in human plasma:

3.4.3.1 Plasma spike study by UV method:

Materials:
- Fresh human plasma (Procured from L. G. Hospital blood bank, Ahmedabad.)
- Compound 164p
- Acetonitrile (analytical grade; S. D. Chemicals Ltd., Baroda)
- Anhydrous Sodium sulphate. (S. D. Chemicals Ltd., Baroda)
- Simadzu 160A double beam UV/Visible spectrophotometer
- Standard stock solution [10mg of compound in 100ml acetonitrile (100μg/ml)]
Procedure:

1ml of fresh human plasma was taken in six clean and dry test tubes and 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6ml of standard solution of compound was spiked to get calibration standards of 10, 20, 30, 40, 50 and 60μg/ml. Content of all the test tubes were mixed well and final volume of all the test tubes was made to 6ml by adding required quantity of acetonitrile and mixed well.

Precipitated protein was allowed to settle and the supernatant was decanted from all the test tubes into clean and dry test tubes. About 0.7g of Anhydrous sodium sulphate was added to each test tube to make the solution clear and the solution decanted into a clean and dry test tube. Absorbance of all solutions was measured at 316 nm using blank plasma sample prepared by same process omitting addition of compound. A standard curve of absorbance against the concentration was plotted (Fig. 3).

With a view to achieve the sensitivity and specificity for the detection of compound 164p and its metabolites in serum, specific and sensitive HPTLC method was developed.
Figure 3: Calibration curve of compound 164p in plasma by UV method
3.4.3.2 Plasma spike study by HPTLC method:

Materials:
- Precoated TLC plates (Silica gel 60 F_{254} with thickness of layer 0.2mm, E. Merck, Darmstadt, Germany)
- Fresh human plasma (Procured from L. G. Hospital blood bank, Ahmedabad.)
- Acetonitrile (Analytical grade; S. D. Chemicals Ltd., Baroda)
- Anhydrous sodium sulphate (S. D. Chemicals Ltd., Baroda)
- Benzene (distilled) and Methanol (Analytical grade; S. D. Chemicals Ltd., Baroda)

HPTLC Instruments used:
- Camag Linomat IV autosampler
- Camag Twin through chamber
- Camag TLC Scanner-3 with Camag Cats4 software

Chromatographic conditions:
- Stationary phase: Aluminium TLC plates precoated with silica gel 60 F_{254}, thickness of layer 0.2 mm.
- Mobile phase: Benzene: Methanol (9:1) v/v
- Chamber saturation time: 1hr.
- Distance run by solvent: 50 mm
- Scanning Wavelength: 316 nm

Procedure:
A standard stock solution (100μg/ml) was prepared by dissolving 10mg of compound 164p in 100ml of acetonitrile. Working standard solution (40μg/ml) was prepared by diluting 1ml of stock solution to 25ml with acetonitrile.

0.2 ml of fresh human plasma was taken in five clean dry test tubes, to which working standard solution was added in volumes of 50, 100, 200, 300 and 400 microlitres to get calibration standards of 12.5, 25, 50, 75 and 100ng.
Contents of all the test tubes were mixed thoroughly. Volume of each test tube was made to 1.8 ml by addition of acetonitrile.

Content of all the test tubes was shaken on a shaker for one minute and allowed to settle. Supernatant of each test tube was decanted into another five clean and dry test tubes and 0.25g of anhydrous sodium sulphate was added to each test tube. Clear solution was then decanted into clean dry test tubes. 10 microlitre solution from each test tube was spotted on precoated silicagel 60 F254 plate. Reference standard was spotted separately on TLC plate at the concentration of 12.5, 25, 50, 75 and 100ng using Camag Linomat IV autosampler.

TLC chamber was saturated with solvent system [Benzene : Methanol (9 : 1) v/v] for one hour. Plate was then developed at room temperature. After development the plates were dried completely by passing current of hot air.

Photometric measurements were performed in the absorbance/reflectance mode with Camag TLC scanner-3 using CATS-4 software. The slit dimension was set at 3.0 x 0.3mm. The reflectance absorption spectra of the compound was scanned in situ and maxima 316 nm was chosen for quantification. The purity of chromatographic peaks was confirmed by recording spectra at three different points of each peak: peak start, peak apex and peak end.

Linearity:

Standard curve was plotted for area under curve v/s concentration (Figure 4). Same type of standard curve was plotted on different days (n = 5) to determine the variation in slope and intercept. (Table 3.4)

% Recovery study:

The recovery of compound 164p from plasma was determined by comparing peak areas obtained from spiked plasma at different concentration with calibration curve of standard 164p (Table 3.3). The recovery was quantitative and reproducible.
HPTLC method for the analysis of compound 164p in plasma was found highly sensitive and specific with accuracy near to 100%. Minimum range of detection of the compound 164p was found to be 10ng.

Table 3.3: Plasma spike study by HPTLC method (% Recovery)

<table>
<thead>
<tr>
<th>Concentration of Std. 164p Added (ng)</th>
<th>Concentration Detected (Mean ± S.D.) (n = 4)</th>
<th>% Recovery</th>
<th>% C. V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>11.8 ± 0.9</td>
<td>94.5</td>
<td>7.89</td>
</tr>
<tr>
<td>25</td>
<td>22.4 ± 1.1</td>
<td>89.6</td>
<td>4.51</td>
</tr>
<tr>
<td>50</td>
<td>48.6 ± 5.3</td>
<td>97.3</td>
<td>10.7</td>
</tr>
<tr>
<td>75</td>
<td>66.7 ± 3.0</td>
<td>88.9</td>
<td>4.83</td>
</tr>
<tr>
<td>100</td>
<td>88.2 ± 3.8</td>
<td>88.2</td>
<td>4.31</td>
</tr>
</tbody>
</table>

C. V. = Coefficient of Variation; S. D. = Standard Deviation

Table 3.4: Plasma spike study by HPTLC method (% Accuracy)

<table>
<thead>
<tr>
<th>Concentration Added (ng)</th>
<th>AREA (MEAN ± SEM, n=5)</th>
<th>% C. V.</th>
<th>% ACCURACY</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>1270.9 ± 168.4</td>
<td>13.3</td>
<td>102.1</td>
</tr>
<tr>
<td>25</td>
<td>2170.0 ± 114.6</td>
<td>5.3</td>
<td>100.9</td>
</tr>
<tr>
<td>50</td>
<td>3464.7 ± 146.3</td>
<td>4.2</td>
<td>106.1</td>
</tr>
<tr>
<td>75</td>
<td>4222.0 ± 84.1</td>
<td>2.0</td>
<td>98.4</td>
</tr>
<tr>
<td>100</td>
<td>5412.8 ± 374.8</td>
<td>6.9</td>
<td>92.9</td>
</tr>
</tbody>
</table>

C. V. = Coefficient of variation; SEM = Standard error of mean
Figure 4: Calibration curve of compound 164p in plasma by HPTLC method
3.4.3.3 Stability of compound 164p in fresh human plasma:

Materials and Instruments:
Same as used for plasma spike study by HPTLC method.

Procedure:
A standard stock solution (100μg/ml) was prepared by dissolving 10mg of compound 164p in 100ml of acetonitrile. 0.2 ml of fresh human plasma was spiked with 50μl of standard solution and stored at -20°C for one day. After one day spiked sample was processed by the procedure described earlier. 50μl of the extract was spotted on precoated TLC plate. Standard solution of 164p was spotted on the same plate as external standard.

Plate was then developed in mobile phase Benzene : Methanol ( 9 : 1) v/v and dried by passing current of hot air. Plate was scanned by Camag TLC Scanner 3 at a wavelength of 274 nm*.

*Compound 164p was found to get metabolized to aminal 195p immediately after oral administration so it was not possible to quantify compound at the λ_max of 164p, i.e., 316nm. Scanning and quantification was carried out at λ_max of probable metabolite i.e. 274nm.

Table : 3.5 : Summary of the method validation parameters

<table>
<thead>
<tr>
<th>No</th>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Precision (% C. V.)</td>
<td>4.31 - 7.89</td>
</tr>
<tr>
<td>2</td>
<td>Accuracy (%)</td>
<td>92.98 - 102.06</td>
</tr>
<tr>
<td>3</td>
<td>Minimum detection</td>
<td>10ng/ml</td>
</tr>
<tr>
<td>4</td>
<td>Minimum quantification</td>
<td>12.5ng/ml</td>
</tr>
<tr>
<td>5</td>
<td>Specificity</td>
<td>Specific</td>
</tr>
<tr>
<td>6</td>
<td>Linearity</td>
<td>0.999</td>
</tr>
<tr>
<td>7</td>
<td>Range</td>
<td>12.5 - 100ng/ml</td>
</tr>
</tbody>
</table>
3.4.4. Results:

Quantitative estimation of compound 164p by UV spectroscopy has shown direct correlation between concentration and absorbance. Thus beer's law is obeyed for the range of 1-10μg/ml (Table 3.1). The regression analysis data obtained were \( r = 0.999, \) slope = 0.8225, intercept = -0.0081. The correlation coefficient (r) was found very near to one, which confirms the good correlation between concentration and absorbance (Figure 2).

In solid phase extraction of compound 164p, it was observed that at pH 7.4 nearly 100% of the compound is extracted (Table 3.2).

Plasma spike study of compound 164p by UV method has shown good correlation between concentration and absorbance \( (r = 0.999) \) in the range of 10 - 60μg/ml (Figure 3).

However, HPTLC is more specific and sensitive method for 164p in plasma compared to UV spectroscopy. HPTLC method can detect the drug, 164p, from blood in nanogram quantity. The calibration curve of compound 164p was obtained by plotting the area under the peak against the concentration over the range of 12.5 to 100ng. The correlation was found to be 0.999. (Table 3.3; Figure 4). The accuracy, precision and reliability of the method was confirmed by adding known concentration of drug to the plasma (Table 3.4).

Summary of the validation parameters is given in table 3.5. Accuracy was found nearly 100%. Minimum limits for detection and quantification were observed to be 10ng and 12.5ng, respectively. The method was found to be specific and sensitive (Table 3.5).

Two spots were observed on TLC plate with Rf of 0.21 and 0.47 when compound 164p was spiked in plasma and stored at -20°C for one day. This indicates that compound 164p is not stable in plasma even at temperature of -20°C. The Rf 0.21 is the Rf of compound 164p whereas Rf 0.47 may be of metabolite of compound 164p.
3.5 Pharmacokinetic study of compound 164p:

Animal used: Rabbit (Newzealander strain)
Dose of drug: 100mg/kg of body weight
Route of administration: Oral (In the form of Sodium CMC suspension; 1% w/v)
Method for analysis: HPTLC

Method:

Newzealander rabbits weighing 1.4 - 2.5kg of either sex were selected for the present study. Animals were housed under standard conditions for a week. Each animal acted as its own control.

Rabbits were deprived of food for 12 hrs before administration of the test compound 164p. Compound was given in the form of suspension (sodium CMC; 1% w/v) at a dose of 100mg/kg p.o. Blood samples were collected from the marginal ear vein before administration of drug and at the intervals of 0.5, 1, 2, 4, 6, up to 24 hrs. Collected blood samples were allowed to coagulate for 1 hr and then centrifuged at 5000 RPM for 25 minutes. Serum separated was collected in glass tubes and total plasma protein was precipitated by addition of 2N HCl. All the tubes were allowed to cool to room temperature and pH was adjusted to 7.4 with sodium bicarbonate (10%) solution.

All the serum samples were extracted twice with 1ml of benzene and benzene was collected in separate glass tube. The combined benzene extract was evaporated to dryness at room temperature by flushing with nitrogen gas. The residue was redissolved in acetonitrile and the sample was spotted on TLC plate using Camag Linomat IV autosampler. Same method is used for all the samples.

The TLC plates were developed in a Camag Twin Through glass chamber with solvent system Benzene : Methanol (9 : 1) v/v. After development, TLC plates were dried completely by passing a current of hot air. Photometric measurement was performed in the absorbance or reflectance mode with Camag TLC Scanner and quantification was done at maxima 274nm. Different pharmacokinetic
parameters were calculated from the graph of plasma concentration of drug versus
time (Figure 6).

3.5.1 Results:
Sample of the rabbit plasma after 0.5hr has shown single spot on TLC with
the Rf value of 0.47, while the Rf value of compound 164p is 0.21. This indicate that
the compound 164p may be getting metabolized enzymatically to the compound
having Rf value of 0.47. So it was thought of interest to identify and characterize the
metabolite for further pharmacokinetic studies.

3.6 Identification of probable metabolite of compound 164p:
The probable structure of metabolites formed in plasma for the compound
164p may be formulated as follows.
1) The cyclised pyrimidine: i) 5-cyano-6-(p-chloroanilino)pyrimidin-4-one, 200k, or ii)
5-carbethoxy-4-chloro-6-(p-chloroanilino)pyrimidine, 197o,
2) The decomposition product ethyl 3-amino-2-cyano-3-(p-chloroanilino)acrylate
(aminal), 195p.

Compounds 195p, 197o and 200k were synthesized and characterized by
various spectral analysis. The Rf, m.p. superimposed UV and superimposed IR
were compared for the identification of metabolite.

\[
\begin{align*}
&\begin{array}{c}
R_1 \\
&\begin{array}{c}
\text{NH} \\
&\begin{array}{c}
\alpha \\
197o; R_1 = \text{CO}_2\text{C}_2\text{H}_4; R_2 = \text{Cl}; \text{ i) Dry } \text{HCl}
\end{array}
\end{array}
\end{array}
\end{align*}
\]
3.6.1 Synthesis of probable metabolites of 164p:

1) 5-cyano-6-(p-chloroanilino)pyrimidin-4-one, 200k, was synthesized by refluxing the compound 164p in sodium ethoxide for 4hrs. Compound has m.p. >300°C, Rf value 0.14 and λ_max 305nm. (Chapter 4)

2) 5-carbethoxy-4-chloro-6-(p-chloroanilino)pyrimidine, 197o, was synthesized by stirring compound 164p in dioxane saturated with dry HCl for 2hrs. Compound has m.p. 110-112°C, Rf value 0.38 and λ_max 288nm. (Chapter 4)

3) Ethyl 3-amino-2-cyano-3-(p-chloroanilino)acrylate (aminal), 195p was synthesized by heating the compound 164p in the mixture of methanol : water mixture (50:50) and 2ml of conc. hydrochloric acid for 6hrs. The reaction mixture was cooled to room temperature to get the aminal. The compound has m.p. 173 - 176°C, Rf value 0.47 and λ_max 274nm.

Confirmation of the structure of aminal was received by cyclocondensation of it with formamide to pyrimidin-4-one, 200k (Chapter 4).

\[
\begin{align*}
\text{H}_3\text{C}_2\text{O}_2\text{C} & \quad \text{CN} \\
\text{HN} & \quad \text{NH}_2 \\
\text{R} & \quad \text{CON} \\
\text{HCONNH}_2 & \quad \text{reflux} \\
195p & \quad \rightarrow \quad 200k \\
\text{R} = \text{p-Chloro-C}_6\text{H}_4
\end{align*}
\]

3.6.2 Physical and spectral characteristics of aminal, 195p:

Ethyl 2-amino-3-cyano-2-(p-chloroanilino)acrylate, aminal 195p, is a colourless crystalline powder. It is freely soluble in most of the organic solvents, sparingly soluble in dilute HCl and sodium hydroxide solution. UV spectrum of aminal shows a single peak at λ_max of 274nm.

IR spectrum shows a triplet around 3400 - 3320 cm\(^{-1}\) due to presence of primary and secondary amino groups. Sharp single peak at 2200 cm\(^{-1}\) confirms the presence of cyano group. Absorbance peak at 1670 cm\(^{-1}\) is due to the carbonyl
function of conjugated carbethoxy group.

$^1$H NMR of the aminal has exhibited same proton signals as exhibited by compound 164p except the proton at C-2.

3.6.3 Results:

Interestingly, identical Rf, superimposed UV and IR were observed with the unknown metabolite of vinylamidine 164p in plasma and the synthesized aminal, 195p, While the cyclised pyrimidines have different Rf, UV and IR spectra. These suggest that the compound 164p may undergo enzymatic degradation to its aminal, 195p in the body immediately after oral administration. Therefore, it confirms that the metabolite of compound 164p form in the body is an aminal 195p.
3.7 Profile of compound 195p:

1) Chemical name: Ethyl 3-amino-2-cyano-3-(p-chloroanilino)acrylate

2) Structure:

3) Molecular formula: $C_{12}H_{12}ClN_3O_2$

4) Molecular weight: 265.71

5) Melting point: 173-176°C (Ethanol)

6) Physical characteristics: colourless, odourless, crystalline powder

7) Solubility:
   - Soluble: n-hexane, benzene, chloroform, dichloromethane, methanol, acetonitrile, 0.1N HCl
   - Insoluble: water

8) Thin Layer Chromatography:
   a) Mobile phase: Benzene : Methanol (9:1)v/v
   b) $R_f$ value: 0.47

9) ClogP: 1.4

10) UV spectra:

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\lambda_{max}$ (nm)</th>
<th>$\varepsilon$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1N HCl</td>
<td>273.4</td>
<td>22160.2</td>
</tr>
<tr>
<td>0.1N NaOH</td>
<td>273.8</td>
<td>20220.5</td>
</tr>
<tr>
<td>Methanol</td>
<td>276.6</td>
<td>24843.9</td>
</tr>
</tbody>
</table>
11) IR spectra (cm$^{-1}$) :  
   3400, 3320 (NH), 2200 (CN), 1670 (C=O)

12) $^1$H NMR spectra  
   (CDCl$_3$) :  
   $\delta$ 1.3 (t, 3H, CO$_2$CH$_2$CH$_3$), $\delta$ 4.20 (q, 2H, CO$_2$CH$_2$CH$_3$),  
   $\delta$ 5.65 (broad s, 2H, NH$_2$), $\delta$ 6.80 - 7.3 (m, 4H, Ar-H)  
   $\delta$ 14.00 (s, 1H, HN-Ar)  
   (CDCl$_3$+D$_2$O) :  
   $\delta$ 1.3 (t, 3H, CO$_2$CH$_2$CH$_3$), $\delta$ 4.20 (q, 2H, CO$_2$CH$_2$CH$_3$),  
   $\delta$ 6.80 - 7.30 (m, 4H, Ar-H).

13) Mass spectra (m/e) :  
   267 (M+2), 265(M+), 249, 241, 219, 132, 106

14) Stability :  
   Compound 195p was found stable at room temperature in polar and nonpolar solvent for any length of time.
3.8 Analytical method development for compound, 195p:

Same methods were used for the analytical method development for the compound 195p as applied for the compound 164p. The most sensitive and specific HPTLC method was developed and validated for compound 195p. Summary of the validation parameters are given in table 3.6.

Table : 3.6 : Summary of validation parameters for compound 195p

(HPTLC method)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Precision (% c.v)</td>
<td>2.91 - 5.85</td>
</tr>
<tr>
<td>2</td>
<td>Accuracy (%)</td>
<td>93.83 - 108.15</td>
</tr>
<tr>
<td>3</td>
<td>Limit of detection</td>
<td>10ng/ml</td>
</tr>
<tr>
<td>4</td>
<td>Limit of quantification</td>
<td>20ng/ml</td>
</tr>
<tr>
<td>5</td>
<td>Linearity</td>
<td>0.994</td>
</tr>
<tr>
<td>6</td>
<td>Range</td>
<td>10 - 150ng/ml</td>
</tr>
<tr>
<td>7</td>
<td>Specificity</td>
<td>Specific</td>
</tr>
</tbody>
</table>
Figure 5: Calibration curve of compound 195p in plasma by HPTLC method
3.9 Pharmacokinetic study of aminal, 195p:

The results of the pharmacokinetic study of compound 164p in rabbit revealed that compound 164p gets completely degraded to its aminal 195p within 0.5 hr after its oral administration. So it was not possible to measure compound 164p as such in plasma. So it was thought of interest to carry out the pharmacokinetic study of the metabolite 195p also. Pharmacokinetic study of compound 195p was carried out by the same method as used for compound 164p.

Animal used: Rabbit (Newzealander strain)
Dose of drug: 90.76mg/kg/p.o (molar concentration equivalent to 100mg of compound 164p)
Route of administration: Oral (In the form of Sodium CMC suspension; 1% w/v)
Method used for analysis: HPTLC

Method:

Method used for the pharmacokinetic study of compound 195p was the same as used for pharmacokinetic study of compound 164p. Pharmacokinetic parameters obtained for compound 164p and 195p are given in table 3.7 (figure 6 & 7).

Table: 3.7: Pharmacokinetic parameters of compounds 164p and 195p

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>C_{max} (ng/ml)</th>
<th>t_{max} (hr)</th>
<th>AUC (0-12hr) (ng/ml/hr)</th>
<th>K_{el} (1/hr)</th>
<th>t_{1/2} (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>164p*</td>
<td>5790.8*</td>
<td>8</td>
<td>25479.4*</td>
<td>1.52</td>
<td>5.12</td>
</tr>
<tr>
<td>195p**</td>
<td>288.5</td>
<td>8</td>
<td>1526.8</td>
<td>1.45</td>
<td>4.81</td>
</tr>
</tbody>
</table>

*Number of animals used n = 4
*C_{max}, t_{max} and AUC observed in compound 164p were measured as compound 195p as compound 164p was found to get metabolized to its aminal 195p.
**Number of animals used n = 3
3.10 Results:

Cmax and area under curve (AUC) observed for the compound 164p was very high compared to its aminal 195p. tmax observed for both the compounds was 8hrs. K_d and t½ for both the compounds were found nearly same.

3.11 Discussion:

Various methods were tried for the development of highly specific and selective analytical method for compounds 164p and 195p. HPTLC method was found to be highly sensitive and selective for the analysis of compound 164p as well as for its aminal, 195p. Plasma spike study was done for the validation. Up to 10ng level detection and quantification of the compounds 164p and 195p can be done by this method with the accuracy nearly 100%.

Biological screening of N-cyanovinylformamidine suggests that compound 164p has potent antihyperlipidemic activity. During the pharmacokinetic study it was observed that compound 164p gets metabolized within 0.5hr to its aminal 195p. This suggests that the antihyperlipidemic activity may reside in the aminal 195p. Biological screening of the aminal, 195p has revealed that 195p is less potent than its corresponding N-cyanovinylamidine, 164p (chapter 2).

Pharmacokinetic study 195p has exhibited very low Cmax and AUC compared to compound 164p (Table 3.7). Low Cmax of the compound 195p suggests that it may be poorly absorbed from GIT. In biological screening, compound 195p was found less potent than compound 164p, it may be due to poor absorption of aminal 195p from GIT. Moreover, aminal 195p has low ClogP (1.4) than the vinylamidine 164p (2.3), thereby indicating compound 195p is less lipophilic than 164p and so it is poorly absorbed from GIT.

These results suggests that 164p may be acting as a prodrug which has higher absorption in GIT and getting completely metabolized to its active metabolite aminal, 195p immediately after oral administration.
Figure 6: Time vs concentration curve of compound 164p
Figure 7: Time v/s concentration curve of compound 195p