Drugs under study

The drugs chosen for the thesis work are as follows:

- Felbamate
- Cevimeline HCl
- Lenalidomide
- Clofarabine
- Saxagliptin HCl
- Ambrisentan
- Bepotastine Besilate
- Conivaptan HCl

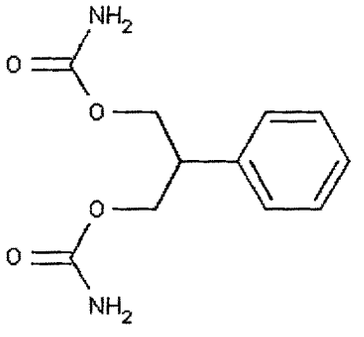
The present thesis covers the drug profiles, the synthetic schemes which are used for the synthesis of the drug substances, the HPLC stability indicating analytical methods developed exclusively to separate all the identified and unidentified impurities, structure elucidation and characterization experiments along with the degradation studies for each of the drugs and the proposed pathways. All the relevant samples like drug substance, drug product and the analytical instrumentation is provided by Apicore LLC and Apicore Pharmaceuticals Pvt Ltd.

FELBAMATE

FELBAMATE

Felbamate (2-phenyl-1,3-propanediol dicarbamate) was approved for the treatment of several forms of epilepsy. Felbamate is a modulator of NMDA (N-Methyl-D-asparate) receptor function and a glycine site antagonist. Felbatol® (felbamate) is an antiepileptic available as 400 mg and 600 mg tablets and as a 600 mg/5 mL suspension for oral administration. Its chemical name is 2-phenyl-1, 3-propanediol dicarbamate.

Felbamate is a white to off-white crystalline powder with a characteristic odor. It is very slightly soluble in water, slightly soluble in ethanol, sparingly soluble in methanol, and freely soluble in dimethyl sulfoxide.



The inactive ingredients for Felbatol® (felbamate) tablets 400 mg and 600 mg are starch, microcrystalline cellulose, croscarmallose sodium, lactose, magnesium stearate, FD&C Yellow No. 6, D&C Yellow No. 10, and FD&C Red No. 40 (600 mg tablets only). The inactive ingredients for Felbatol® (felbamate) suspension 600 mg/5 mL are sorbitol, glycerin, microcrystalline cellulose, carboxymethylcellulose sodium, simethicone, polysorbate 80, methyl paraben, saccharin sodium, propyl paraben, FD&C Yellow No. 6, FD&C Red No. 40, flavorings, and purified water.

Synthesis

Several known literature procedures are available in literature for synthesis of Felbamate drug substance. For example, US Patents 4982016 and 5091595 report the preparation of Felbamate by

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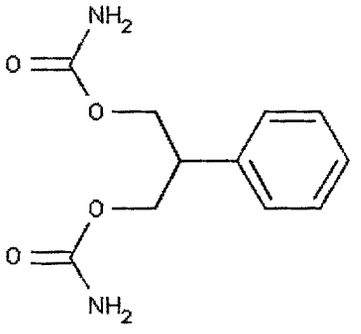

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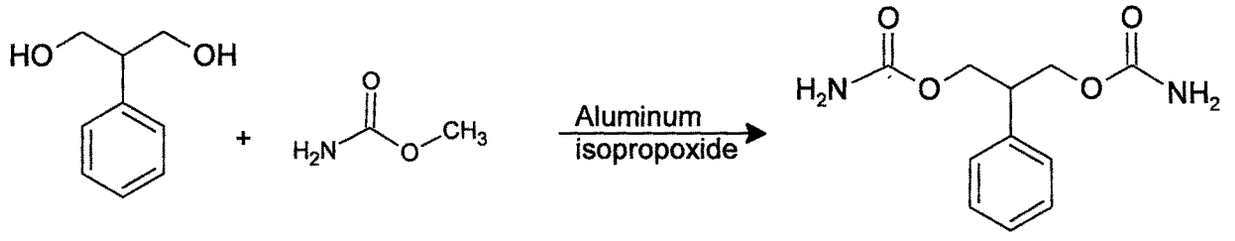
Synthesis

Several known literature procedures are available in literature for synthesis of Felbamate drug substance. For example, US Patents 4982016 and 5091595 report the preparation of Felbamate by

urethane exchange or by a modified phosgene method. Also B.J. Ludwig et al., (J.Med. Chem., Vol. 12(3), 1969, PP 462-472)[10] report various procedures for converting diols to dicarbamates. However, the most viable and effective procedure remains to be the synthetic pathway using 2-phenyl-1, 3-propanediol and methyl carbamate as starting materials. Hence, we have synthesized Felbamate using the same procedure following US Patent 2884444.

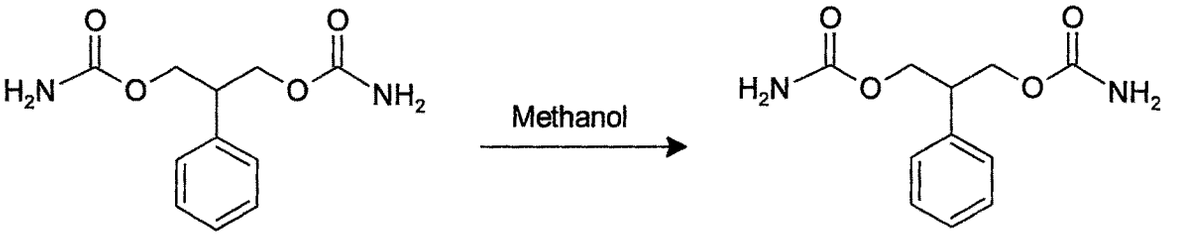
The scheme of synthetic procedure is as follows:

FELBAMATE STAGE-I: SYNTHESIS OF FELBAMATE TECHNICAL



2-Phenyl-1,3-Propane diol Methylcarbamate Felbamate Technical

FELBAMATE STAGE-II: SYNTHESIS OF FELBAMATE DRUG SUBSTANCE



Felbamate Technical Felbamate API

Process related impurities

The impurity profiling study has been conducted for the above synthetic process. The review of the manufacturing procedure reveals that methanol will be the by-product which is generated as the reaction progresses and was distilled as it forms in the reaction. So methanol is the residual solvent impurity which can be found in the Felbamate as per the process which forms as a by-product and has also been used in the purification procedure of felbamate in the second stage.

Theoretically, substitution at one end of the alcohol in the 2-phenyl 1,3-propanediol with the monocarbamate will lead to a process impurity which could be formed if the reaction does not go to completion.

Residual Aluminum metal ion (Heavy Metal) could be the possible process impurity which could arise due to the presence of Aluminum isopropoxide in the reaction.

Any residual unreacted 2-phenyl-1,3-Propanediol and methyl carbamate could be the process impurities and/or degradation impurities.

Also Isopropyl carbamate will be the process impurity which could get carried over from the starting materials, which will be a residual content of Isopropyl Carbamate present in the methyl carbamate.

Degradation Impurities

Since the drug substances and drug products will be stored for long durations in the pharmaceutical companies and the pharmacy retail outlets before they are consumed by the patient, it becomes imperative and prudent to study the degradation products which could be formed due to various stress factors on the drug substance either during shipment or storage. So to establish the degradation pathways and the degradation products we have performed several studies on felbamate by applying external stress factors like acid, base, heat, light etc. and characterized the structures of the same.

STRESS STUDIES (INDUCED DEGRADATION)

The Felbamate drug substance was subjected to induced degradation under the following stress Conditions:

HYDROLYTIC DEGRADATION WITH HYDROCHLORIC ACID

Felbamate drug substance 101.4 mg was accurately weighed and transferred into a 100 mL volumetric flask. 10mL of 1N Hydrochloric acid was added and heated at 80°C for 2 hours on a water bath. The solution was cooled to ambient temperature. 10mL of 1N sodium hydroxide was added to neutralize acid. 10 mL of acetonitrile was added and sonicated for about 1 minute. 50 mL of diluent was added and sonicated for 5 minutes. It was diluted to volume with diluent and mixed well.

PREPARATION OF THERMAL DEGRADATION SAMPLE

Felbamate drug substance 100.0 mg was accurately weighed and transferred into a 100 mL volumetric flask and heated at 110°C for 4 hours in an oven. The sample was cooled to ambient temperature; 10 mL of acetonitrile was added and sonicated for about 1 minute with occasional shaking. 50 mL of diluent was added and sonicated for 5 minutes. It was diluted to volume with diluent and mixed well. The treated sample solution was analyzed as per the proposed method.

PREPARATION OF PHOTOLYTIC DEGRADATION SAMPLE

Felbamate drug substance 1.0 gm was exposed to UV radiation for about 168 hours on a petri-dish. 99.9 mg from the above sample which is subjected to photolytic degradation was accurately weighed and transferred into a 100 mL volumetric flask. 10 mL of acetonitrile was added and sonicated for about 1 minute with occasional shaking. 50 mL of diluent was added and sonicated for 5 minutes. It was diluted to volume with diluent and mixed well. The treated sample solution was analyzed as per the proposed method.

PREPARATION OF SUNLIGHT DEGRADATION SAMPLE

Felbamate drug substance 99.4 mg was accurately weighed and transferred into a 100 mL volumetric flask. The flask was kept in sun light for 7 hours after which 10 mL of acetonitrile was added and sonicated for about 1 minute with occasional shaking. 50 mL of diluent was added and sonicated for 5 minutes. It was diluted to volume with diluent and mixed well. The treated sample solution was analyzed as per the proposed method.

OXIDATIVE DEGRADATION WITH HYDROGEN PEROXIDE

Felbamate drug substance 99.1 mg was accurately weighed and transferred into a 100 mL volumetric flask. 10 mL of 10% v/v Hydrogen peroxide was added and heated at 80°C for 2 hours on a water bath. The solution was cooled to ambient temperature. 10 mL of acetonitrile was added and sonicated for about 1 minute with occasional shaking. 50 mL of diluent was added and sonicated for 5 minutes. It was diluted to volume with diluent and mixed well.

HYDROLYTIC DEGRADATION WITH ALKALI (BASE)

Felbamate drug substance 100.3 mg sample was accurately weighed and transferred into a 100 mL volumetric flask. 10mL of 1N sodium hydroxide was added and heated at 80°C for 2 hours on a water bath. The solution was cooled to ambient temperature. 10mL of 1N Hydrochloric acid was added to neutralize sodium hydroxide. 10 mL of acetonitrile was added and sonicated for about 1 minute with occasional shaking. 50 mL of diluent was added and sonicated for 5 minutes. It was diluted to volume with diluent and mixed well.

The following analytical method was developed and found to be stability indicating method which separates all the impurities which are formed during the stress studies along with the process impurities.

ANALYTICAL METHOD

1.0 ASSAY (BY HPLC)

1.1 Instrumentation

- A High Performance Liquid Chromatograph equipped with Gradient elution capability, Ultraviolet Spectrophotometer as detector and an auto sampler.
  (Shimadzu LC 2010 system or waters 2690 or equivalent).

- **Data handling system**
  LC Solution Software or equivalent chromatographic software
- A stainless steel column of length 150 mm, internal diameter 4.6 mm and filled with Octadecylsilane chemically bonded to porous silica particles of 5 µ diameter.

1.2 Chromatographic conditions

Column	: Peerless Basic C18, (150 x 4.6 mm), 5µm
Flow rate	: 1.8 mL/min
Detection	: UV at 210 nm
Injection volume	: 20 µL
Column temperature	: 30 °C
Sample temperature	: 25 °C
Elution mode	: Isocratic
Retention time	: About 5 min for Felbamate.
Runtime	: 20 min

1.3 Reagents

1) Process Water
2) Acetonitrile (HPLC grade)
3) Methanol (HPLC grade)

1.4 Mobile phase

A mixture of Acetonitrile, Methanol and Water in a ratio of 126:84:790 (v/v/v) was prepared. The mobile phase was filtered and degassed through 0.45µ Nylon membrane filter.

1.5 Diluent (For standard stock solution)

A mixture of Acetonitrile, Methanol and Water in a ratio of 222:148:630 (v/v/v) was prepared which is to be used as diluent.

1.6 System suitability solution

Felbamate related compound "A" standard 25.0 mg and 100.0 mg of Felbamate standard was accurately weighed and transferred into a 50 mL volumetric flask, the components were dissolved and diluted to volume with mobile phase and mixed well.

Transferred 5.0 mL of above solution into a 50 mL volumetric flask and diluted to volume with mobile phase and mixed.

1.7 Standard stock solution

50.0 mg of Felbamate standard was accurately weighed and transferred into a 50 mL volumetric flask. 5 mL of methanol was added. The solution was sonicated and shaked to completely dissolve and diluted to volume with diluent and mixed.

1.8 Standard solution

10.0 mL of standard stock solution was transferred into a 50 mL volumetric flask and diluted to volume with mobile phase and mixed.

1.9 Sample stock solution (prepare in duplicate)

Felbamate sample 50.0 mg was accurately weighed and transferred in to a 50 mL volumetric flask. 5 mL of methanol was added. The solution was sonicated and shaked to completely dissolve and it was diluted to volume with diluent and mixed.

1.10 Sample solution

10.0 mL of each sample stock solution was transferred into a 50 mL volumetric flask and diluted to volume with mobile phase and mixed.

1.11 Procedure

After equilibrating the column, separately injected equal volumes of mobile phase as a blank, system suitability, standard and sample solutions in to the Chromatographic system

as per the below sequence. Recorded the chromatograms. Integrated only Felbamate peak in the standard and sample solutions.

Injection sequence

Solution details	# Injections
Blank (Mobile phase)	1
System suitability solution	1
Standard solution	5
Sample solution-1	1
Sample solution-2	1

1.12 System suitability – Acceptance criteria

For system suitability solution

USP resolution between peaks due to Felbamate related compound A and Felbamate should be NLT 2.0

For standard solution

i) USP tailing factor for Felbamate peak should be NMT 2.0.

ii) RSD for the peak areas of Felbamate from five replicate injections should be NMT 1.0%

1.13 Calculations

The assay of Felbamate (%w/w) was calculated on dried basis by using the below formula

$$\text{Assay on dried basis (\% w/w)} = \frac{rU}{rS} \times \frac{Cs}{Cu} \times 100$$

Where,

rU = Peak response of Felbamate from the sample solution.

rS = Mean peak response for Felbamate obtained from five replicate injections standard solution.

C_S = Concentration of Felbamate in the standard solution (mg/mL)

C_u = Concentration of Felbamate in the sample solution (mg/mL)

$$\text{Where, Cs} = \frac{W1}{50} \times \frac{10}{50} \times \frac{p}{100}$$

$$Cu = \frac{W2}{50} \times \frac{10}{50} \times \frac{100 - LOD}{100}$$

W1	=	Weight of Felbamate standard in mg.
W2	=	Weight of Felbamate sample in mg.
p	=	Potency / Assay of Felbamate standard in percentage
LOD sample	=	Loss on drying of Felbamate sample in percentage

1.14 Reporting results

Report the average value of assay obtained from both sample solution.

1.15 Specification

NLT 98.0% and NMT 102.0% (% w/w on the dried basis)

2.0 RELATED SUBSTANCES (BY HPLC)

2.1 Organic impurities (early eluting)

2.1.1 **Instrumentation**

- A High Performance Liquid Chromatograph equipped with Gradient elution capability, Ultraviolet Spectrophotometer as detector and an auto sampler.
  (Shimadzu LC 2010 system or waters 2690 or equivalent)

- **Data handling system**
  LC Solution Software or equivalent chromatographic software

- A stainless steel column of length 150 mm, internal diameter 4.6 mm and filled with Octadecylsilane chemically bonded to porous silica particles of 5 µ diameter.

4.0 Chromatographic conditions

Column	: Peerless Basic C18, (150 x 4.6 mm), 5µm
Flow rate	: 1.8 mL/min
Detection	: UV at 210 nm
Injection volume	: 20 µL
Column temperature	: 30 °C
Sample temperature	: 25 °C
Elution mode	: Isocratic
Retention time	: About 5 min for Felbamate.
Runtime	: 20 min

2.1.2 Reagents

1) Process water
2) Acetonitrile (HPLC grade)
3) Methanol (HPLC grade)

2.1.3 Mobile phase

A mixture of Acetonitrile, Methanol and Water in a ratio of 126:84:790 (v/v/v) was prepared. The mobile phase was filtered and degassed through 0.45µ Nylon membrane filter.

2.1.4 Diluent (For standard stock solution)

A mixture of Acetonitrile, Methanol and Water in a ratio of 222:148:630 (v/v/v) was prepared to be used as diluent.

2.1.5 System suitability solution

Felbamate related compound "A" standard 5.0 mg was weighed along with 20 mg of Felbamate standard and transferred into a 100 mL volumetric flask. Mobile phase was added to the flask and dissolved the content and diluted to volume with mobile phase and mixed.

2.1.6 **Standard stock solution**

50.0 mg of Felbamate standard was accurately weighed and transferred into a 50 mL volumetric flask. 5 mL of methanol was added, the solution was sonicated to completely dissolve. It was diluted to volume with diluent and mixed.

2.1.7 **Standard solution**

1.0 mL of standard stock solution was transferred in to a 100 mL volumetric flask and diluted to volume with mobile phase and mixed.

Further 5.0 mL of this solution was transferred into a 50 mL volumetric flask and diluted to volume with mobile phase and mixed.

2.1.8 **Sample solution**

Felbamate sample 50.0 mg was accurately weighed and transferred into a 50 mL volumetric flask. The components were dissolved and diluted to volume with mobile phase and mixed.

2.1.9 **Procedure**

After equilibrating the column, separately injected equal volumes of mobile phase as a blank, system suitability, standard and sample solutions in to the chromatographic system as per the below sequence. Recorded the chromatograms and peak areas. Examined blank chromatogram for any extraneous peaks and disregarded the corresponding peaks observed in the chromatograms of sample solution.

2.1.10 **Injection sequence**

Solution details	**# Injections**
Blank (Mobile phase)	1
System suitability solution	1
Standard solution	6
Sample solution	1

2.1.11 **System suitability – Acceptance criteria**

For system suitability solution

USP resolution between peaks due to Felbamate related compound A and Felbamate should be NLT 2.0

For standard solution

i) USP tailing factor for Felbamate peak should be NMT 2.0.

ii) RSD for the peak areas of Felbamate from six replicate injections should be NMT 10%.

2.1.12 **RRT and RRF table**

Components	**~RRT**	**~RRF**
Phenylpropanediol	0.43	1.7
Felbamate related compound-A	0.65	1.3
Felbamate	1.0	--
N-Aminocarbonyl Felbamate	1.43	0.89
Felbamate related compound-B*	2.23	--
Individual unspecified impurity	--	1.0

*Which is quantified in the test for Organic impurities, Late Eluting

2.1.13 **Calculation**

Note: Disregard peaks due to late eluting organic impurity i.e. Felbamate related compound B which elutes at RRT about 2.2 in sample solution.

Calculate the content of each impurity (%w/w) for sample solution using the following formula

$$\text{Impurity content for known or unknown (\%w/w)} = \frac{rU}{rS} \times \frac{Cs}{Cu} \times \frac{1}{F} \times 100$$

Where,

rU = Peak response of respective impurity from the sample solution.

rS = Mean peak response for Felbamate obtained from six replicate injections standard solution.

C_S = Concentration of Felbamate standard in the standard solution (µg/mL)

C_u = Concentration of Felbamate in the sample solution (µg/mL)

F = Relative response factor for respective impurity.

$$\text{Where, Cs} = \frac{W1}{50} \times \frac{1}{100} \times \frac{5}{50} \times \frac{p}{100} \times 1000$$

$$Cu = \frac{W2}{50} \times 1000$$

W1 = Weight of Felbamate standard in mg.

W2 = Weight of Felbamate sample in mg.

p = Potency/Assay of Felbamate standard in percentage

2.1.14 **Reporting results**

Report the value of each known impurity *(i.e.* Phenylpropanediol, Felbamate related compound A and N-Aminocarbonyl Felbamate*)*, individual unspecified impurity and total impurities (Sum of Early and Late impurities) obtained from sample solution

2.1.15 **Specification limits**

Organic Impurities (Early eluting) by HPLC (%w/w)

a. Phenylpropanediol	:	NMT 0.05 (% w/w)
b. Felbamate related compound A	:	NMT 0.05 (% w/w)
c. N-Aminocarbonyl felbamate	:	NMT 0.15 (% w/w)
d. Individual unspecified impurity	:	NMT 0.1 (% w/w)

2.2 Organic impurities (Late eluting)

2.2.1 Instrumentation

- A High Performance Liquid Chromatograph equipped with Gradient elution capability, Ultraviolet Spectrophotometer as detector and an auto sampler.
  (Shimadzu LC 2010 system or waters 2690 or equivalent)

- **Data handling system**
  LC Solution Software or equivalent chromatographic software
- A stainless steel column of length 150 mm, internal diameter 4.6 mm and filled with Octadecylsilane chemically bonded to porous silica particles of 5 µ diameter.(Use Symmetry C18 (150 x 4.6 mm), 5µ or equivalent).

2.2.2 Reagents

1) Process water
2) Acetonitrile (HPLC grade)
3) Methanol (HPLC grade)

2.2.3 Mobile phase

A mixture of Acetonitrile, Methanol and Water in a ratio of 222:148:630 (v/v/v) was prepared. The solution was filtered and degassed through 0.45µ Nylon membrane filter.

2.2.4 Diluent

Mobil Phase

2.2.5 Standard stock solution

Felbamate standard 50.0 mg was accurately weighed and transferred into a 50 mL volumetric flask, the compound was dissolved and diluted to volume with mobile phase and mixed.

Transferred 1.0 mL of this solution in to a 100 mL volumetric flask and diluted to volume with mobile phase and mixed.

2.2.6 Standard solution

5.0 mL of standard stock solution was transferred into a 50 mL volumetric flask and diluted to volume with mobile phase and mixed.

2.2.7 **Impurity stock solution**

5.0 mg of Felbamate related compound B standard was accurately weighed and transferred into a 100 mL volumetric flask, dissolved and diluted to volume with mobile phase and mixed.

2.2.8 **System suitability solution**

Transferred each of 1.0 mL of Impurity stock solution and 5.0 mL of standard stock solution in to a 50 mL volumetric flask and diluted to volume with mobile phase and mixed.

2.2.9 **Sample solution**

50.0 mg of Felbamate sample was weighed and transferred in to a 50 mL volumetric flask, dissolved and diluted to volume with mobile phase and mixed.

2.2.10 **Procedure**

After equilibrating the column, separately injected equal volumes of mobile phase as a blank, system suitability, standard and sample solutions in to the chromatographic system as per the below sequence. Recorded the chromatograms and peak areas. Examined blank chromatogram for any extraneous peaks and disregarded the corresponding peaks observed in the chromatograms of sample solution.

2.2.11 **Injection sequence**

Solution details	**# Injections**
Blank (Diluent)	1
System suitability solution	1
Standard solution	6
Sample solution	1

2.2.12 **System suitability criteria**

For system suitability solution

USP resolution between peaks due to Felbamate related compound B and Felbamate should be NLT 3.

For standard solution

iii) USP tailing factor for Felbamate peak should be NMT 2.0.

iv) RSD for the peak areas of Felbamate from six replicate injections should be NMT 10%.

2.2.13 **RRT and RRF table**

Components	**~RRT**	**~RRF**
Felbamate	1.0	--
Felbamate related compound-B	1.9	1.29
Felbamate dimer	9.1	1.0
Individual unspecified impurity	--	1.0

2.2.14 **Calculation**

Note: Disregarded peaks due to early eluting organic impurities *i.e.* Phenylpropanediol, Felbamate related compound A and N-Aminocarbonyl felbamate in sample solution (RRTs of these impurities will be confirmed from specificity study).

Calculate the content of each impurity (%w/w) for sample solution using the following formula

$$\text{Impurity content for known or unknown } (\%w/w) = \frac{rU}{rS} \text{ x } \frac{Cs}{Cu} \times \frac{1}{F} \times 100$$

Where,

rU = Peak response of respective impurity from the sample solution.

rS = Mean peak response for Felbamate obtained from six replicate injections standard solution.

C_S = Concentration of Felbamate standard in the standard solution (µg/mL)

C_u = Concentration of Felbamate in the sample solution (µg/mL)

F = Relative response factor for respective impurity.

$$\text{Where, } Cs = \frac{W1}{50} \times \frac{1}{100} \times \frac{5}{50} \times \frac{p}{100} \times 1000$$

$$Cu = \frac{W2}{50} \times 1000$$

W1 = Weight of Felbamate standard in mg.

W2 = Weight of Felbamate sample in mg.

p = Potency/Assay of Felbamate standard in percentage

2.2.15 **Reporting results**

Report the value of each known impurity (i.e. Felbamate related compound B and Felbamate dimer), individual unspecified impurity and total impurities (Sum of Early and Late eluting impurities) obtained from sample solution.

2.2.16 **Specification**

Organic Impurities (Late eluting) by HPLC (%w/w)

a. Felbamate related compound-B : NMT 0.15 (% w/w)

b. Felbamate dimer : NMT 0.15 (% w/w)

c. Individual unspecified impurity : NMT 0.1 (% w/w)

d. Total impurities

(Sum of Early and Late eluting impurities) : NMT 0.75 (% w/w)

3.0 CONTENT OF METHYLCARBAMATE BY HPLC

3.1 Instrumentation

- A High Performance Liquid Chromatograph equipped with Gradient elution capability, Ultraviolet Spectrophotometer as detector and an auto sampler.
  (Shimadzu LC 2010 system or waters 2690 or equivalent)

- **Data handling system**
  LC Solution Software or equivalent chromatographic software
- A stainless steel column of length 250 mm, internal diameter 4.6 mm and filled with Octadecylsilane chemically bonded to porous silica particles of 5 µ diameter. (Use Symmetry Shield RP18 (250 x 4.6 mm), 5µ or equivalent).

3.2 Reagents

Process Water

3.3 Mobile phase

Water is used as mobile phase. Filter and degas through 0.45µ Nylon membrane filter.

3.4 Chromatographic conditions

Column	: Symmetry Shield RP18 (250 x 4.6 mm), 5µ
Flow rate	: 1.0 mL/min
Detection	: UV at 200 nm
Injection volume	: 50 µL
Column temperature	: 30 °C
Sample temperature	: 25 °C
Elution mode	: Isocratic
Retention time	: About 5 min for Methylcarbamate
Runtime	: 15 min

3.5 Diluent

Water

3.6 Standard solution

10 mg of Methylcarbamate was accurately weighed and transferred into a 100 mL of volumetric flask and water was added, the flask was sonicated to dissolve and diluted to volume with water and mixed.

3.7 Sample solution

1.0 g of Felbamate was suspended in 5 mL of water, and mixed on a vortex mixture for 1 min followed by sonication for 5 min. The slurry was filtered, and used as the sample solution.

3.8 Procedure

After equilibrating the column, separately injected equal volumes of diluent as a blank, standard and sample solutions in to the chromatographic system as per the below sequence. Recorded the chromatograms and peak areas of Methylcarbamate. Examined blank chromatogram as there should not be any interference at the RT of Methylcarbamate peak in sample solution.

Injection sequence

Solution details	**# Injections**
Blank (Diluent)	1
Standard solution	6
Sample solution	1

3.9 System suitability criteria

- USP tailing factor for Methylcarbamate peak in standard solution should be NMT 2.0
- RSD for the peak areas of Methylcarbamate from six replicate injections of standard solution should be NMT 10 %.

3.10 Calculation

Calculated the content of Methylcarbamate, in %w/w, using the below formula

$$\text{Methyl carbamate } (\%w/w) = \frac{A_{Sample}}{A_{std}} \text{ x } \frac{W_{std}}{100} \times \frac{5}{W_{Sample}} \times P$$

A_{sample} = Peak area of Methylcarbamate in sample solution

A_{std} = Mean peak area of six replicate injections of Methylcarbamate peak in standard solution.

W_{sample} = Weight of sample in mg

W_{std} = Weight of Methylcarbamate standard in mg

P = Potency of Methylcarbamate in percentage (on as is basis)

3.11 Reporting Results

Report the value of Methylcarbamate content obtained from sample solution

3.12 Specification

NMT 0.05 (% w/w)

% DEGRADATION AND PEAK PURITY TABLE

TABLE – Peak Purity Table

Condition	% Purity	% Degradation	Purity Angle	Purity Threshold
Control Sample	100.00	-	0.520	1.003
Acid hydrolysis-1N. HCl-10 mL-80°C-2hrs.	96.33	3.67	0.119	1.004
Base hydrolysis-1N NaOH-10 mL-80°C-2hrs	16.36	83.64	0.041	1.027
Peroxide-10%-10 mL-80°C-2hrs.	95.91	4.09	0.122	1.007
Thermal-110°C-4 hrs.	99.79	0.21	0.109	1.004
Photolytic-UV light-168 hrs.	99.53	0.47	0.105	1.007
Sun light-7 hrs.	99.81	0.19	0.105	1.005

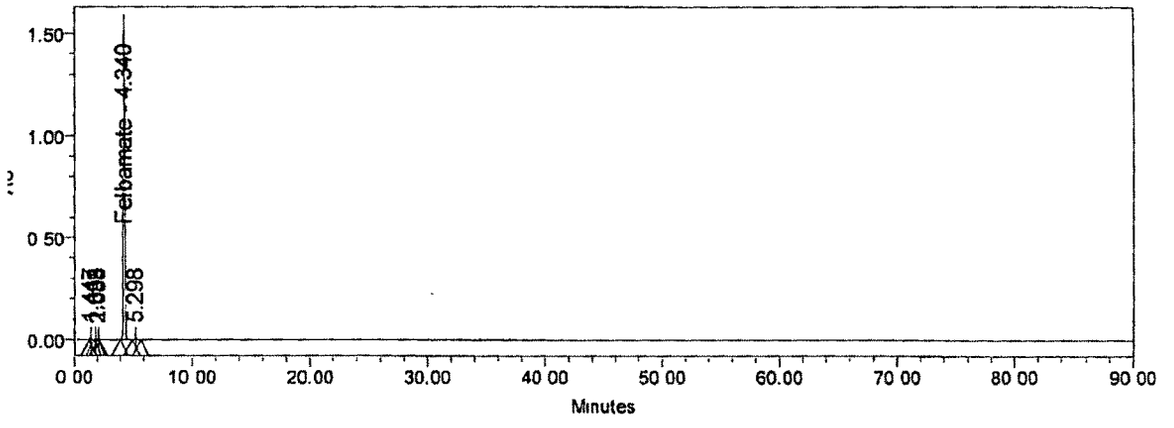
Purity angle should be less than the Purity Threshold for the peak to be considered as pure with no co-elution of the impurities.

This information points to the stability indicating nature of the HPLC method developed for estimating the impurity content in felbamate drug substance or drug product.

The peak purity data given in the table and the peak purity plots shown indicate that there are no co-eluting peaks at the retention time of Felbamate and the peak is homogeneous. This illustrates the capability of the proposed method to separate degradation products from Felbamate, hence the specificity of the method. The analytical method developed was used to calculate and understand the extent of degradation and also to detect the degradation impurities which are formed in small percentages as well.

The HPLC chromatograms are depicted below:

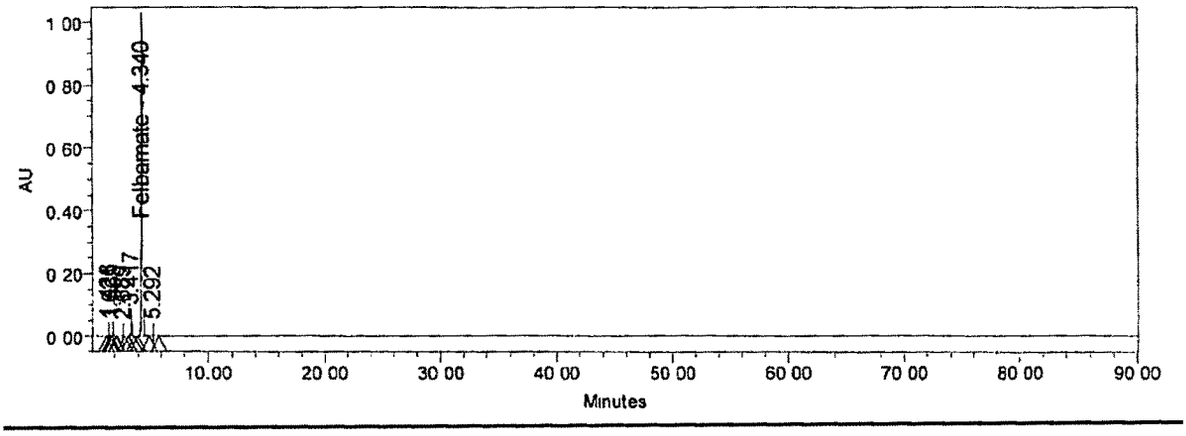
Control sample Chromatogram



Peak Results

	Name	RT	Area	% Area	
1		1.447	19222	0.15	Diluent
2		1.853	11093	0.09	Diluent
3		2.088	1893	0.02	Diluent
4	Felbamate	4.340	12517235	99.52	
5		5.298	27623	0.22	Diluent

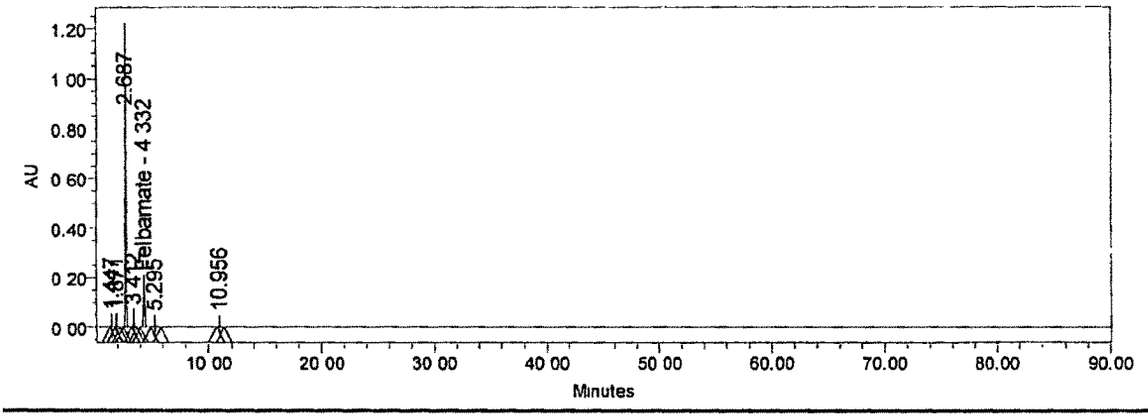
Acid degradation sample



Peak Results

	Name	RT	Area	% Area	
1		1 428	43614	0.52	Diluent
2		1.621	3978	0.05	
3		1.869	26671	0.32	Diluent
4		2.689	17656	0.21	
5		3.417	298519	3.57	
6	Felbamate	4.340	7949641	94.96	
7		5 292	31847	0.38	Diluent

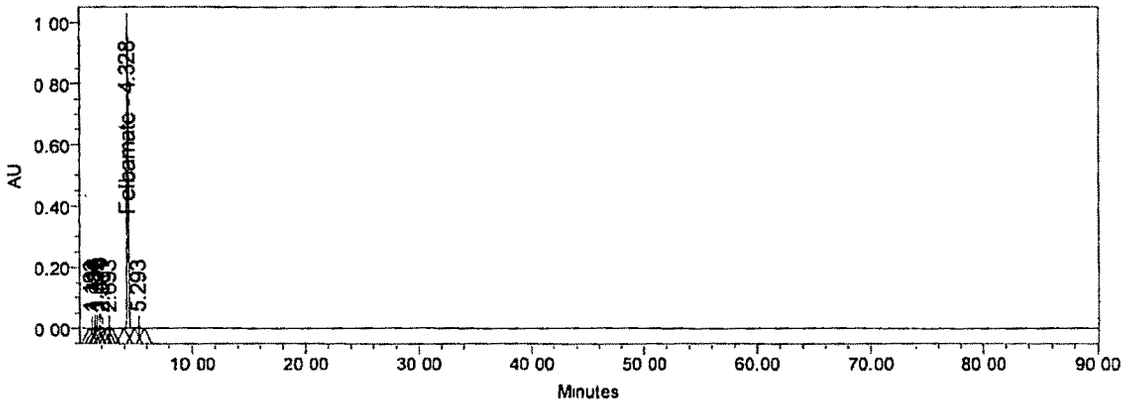
Base degradation chromatogram



Peak Results

	Name	RT	Area	% Area	
1		1.447	55685	0.70	Diluent
2		1.871	36127	0.46	Diluent
3		2.687	6348806	80.08	
4		3.412	165065	2.08	
5	Felbamate	4.332	1276272	16.10	
6		5.295	37538	0.47	Diluent
7		10.956	8717	0.11	

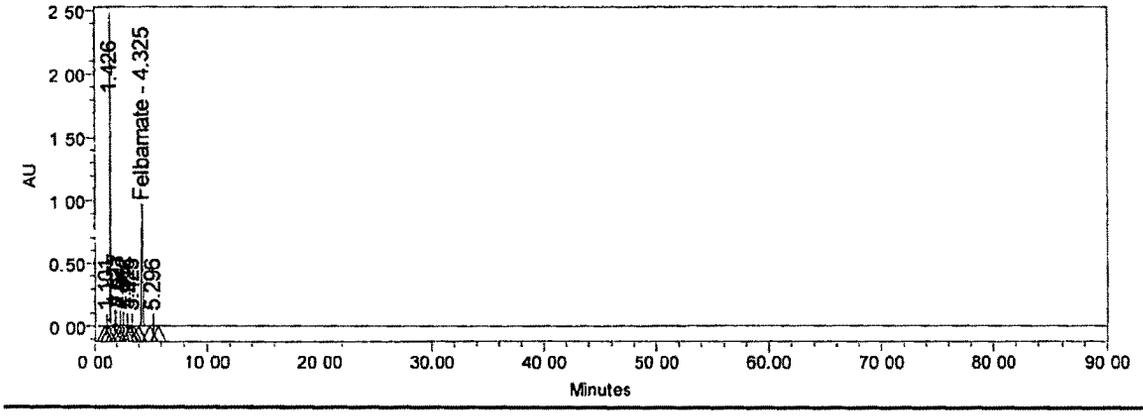
Thermal Degradation chromatogram



Peak Results

	Name	RT	Area	% Area	
1		1.193	6860	0.09	
2		1.436	34330	0.43	Diluent
3		1.629	9532	0.12	
4		1.870	37792	0.47	Diluent
5		2.081	2058	0.03	Diluent
6		2.693	10453	0.13	
7	Felbamate	4.328	7841291	98.33	
8		5.293	31828	0.40	Diluent

Oxidative degradation chromatogram



Peak Results

	Name	RT	Area	% Area
1		1.101	7307	0.04
2		1.426	11059333	59.92
3		1.877	141386	0.77
4		2.073	101690	0.55
5		2.347	146865	0.80
6		2 682	110809	0.60
7		3.028	19845	0.11
8		3.423	6304	0.03
9	Felbamate	4.325	6830588	37.01
10		5.296	31922	0.17

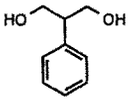
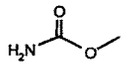
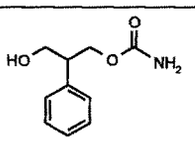
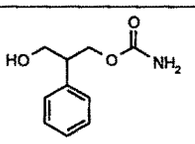
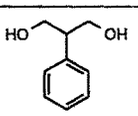
Diluent + Peroxide

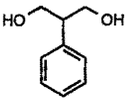
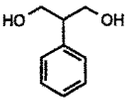
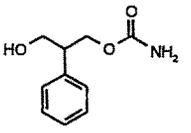
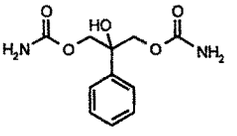
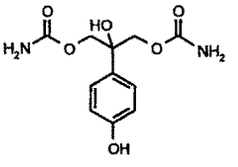
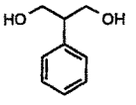
Diluent

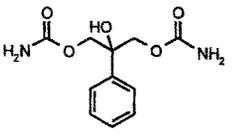
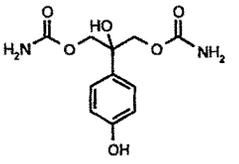
Diluent

Diluent

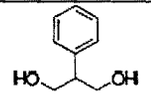
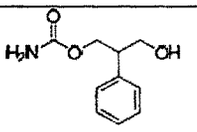
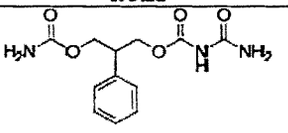
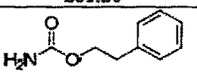
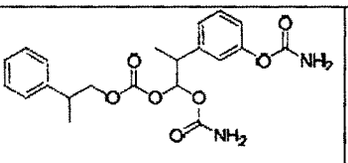
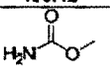
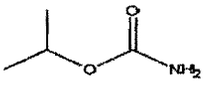
FELBAMATE IMPURITY PROFILE

No.	IMPURITY TYPE		STRUCTURE	MW	RRT
A	**STARTING MATERIALS**				
	1.	2-Phenyl-1,3-propanediol (ImpurityA)		152	0.63
	2.	Methyl carbamate (Impurity C)		75	By TLC
B	**BY PRODUCTS**				
	2-PHENYL-1,3-PROPANEDIOL MONOCARBAMTE (Impurity B)			195	0.79
C	**DEGRADATION**				
	1.	**Acid hydrolysis**			
		2-PHENYL-1,3-PROPANEDIOL MONOCARBAMTE (Impurity B)		195	0.79
		2-Phenyl-1,3-propanediol (ImpurityA)		152	0.63

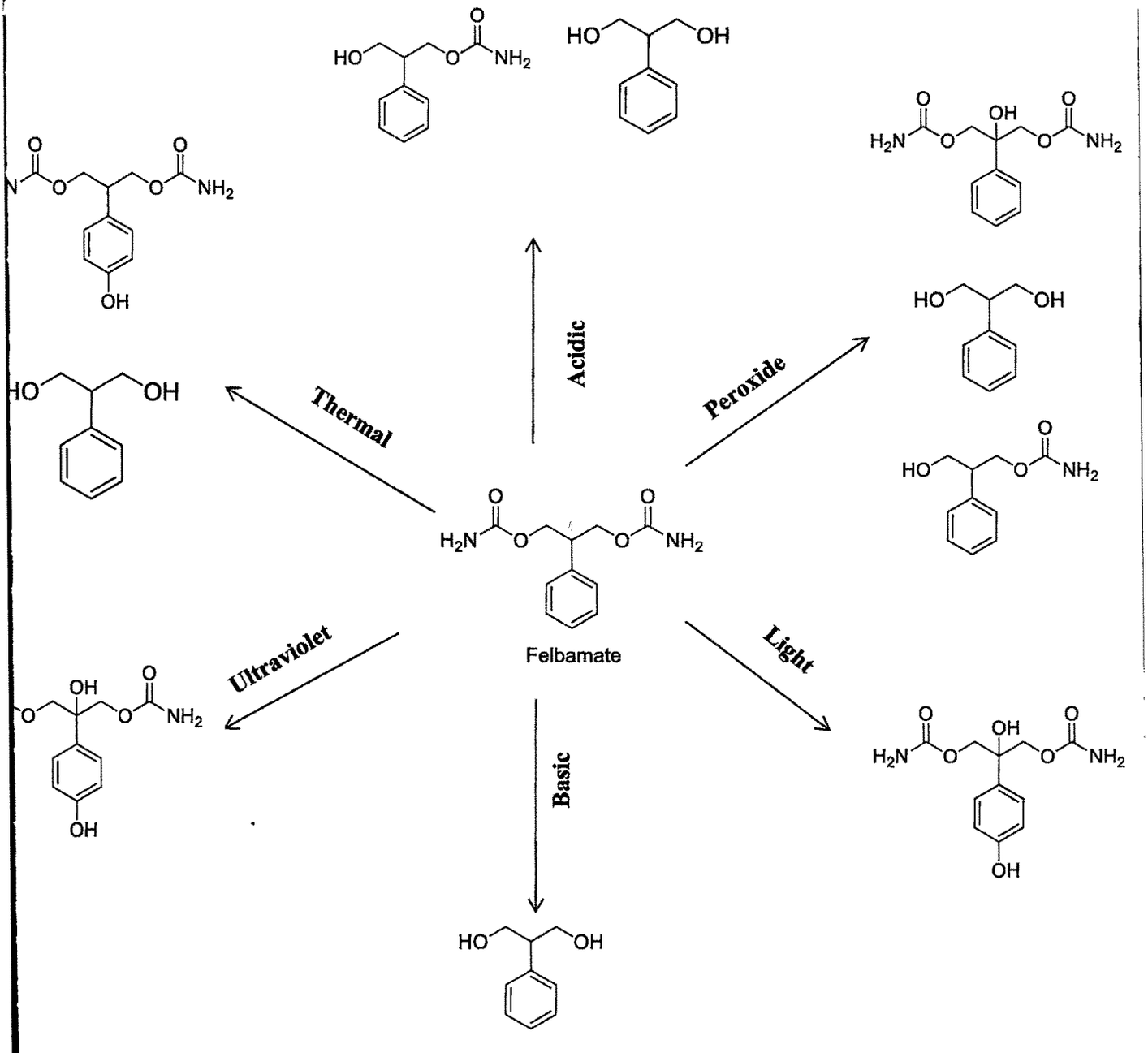
	2.	**Base hydrolysis**			
		2-Phenyl-1,3-propanediol (ImpurityA)		152	0.63
	3	**H_2O_2 degradation**			
		2-Phenyl-1,3-propanediol (ImpurityA)		152	0.63
		2-Phenyl-1,3-propanediol monocarbamate (Impurity B)		195	0.79
		2-Hydroxy Felbamate		254	0.52
	4.	**UV Light**			
		2-(p-hydroxy phenyl)-2-hydroxy-1,3—propanediol dicarbamate		270	0.25
	5	**Thermal**			
		2-Phenyl-1,3-propanediol (ImpurityA)		152	0.63

		p-Hydroxy Felbamate		254	0.37
	6	**Sunlight**			
		2-(p-hydroxy phenyl)-2-hydroxy-1,3—propanediol dicarbamate		270	0.25

List of impurities:

Name	Chemical Name, Structure, Molecular Formula and Molecular Weight	Source	Limits
Phenylpropanediol	 2-phenylpropane-1,3-diol $C_9H_{12}O_2$ 152.19	Process Impurity	Not more than 0.05%
Felbamate related compound-A	 3-hydroxy-2-phenylpropylcarbamate $C_{10}H_{13}NO_3$ 195.22	Process Impurity	Not more than 0.05%
N-Aminocarbonyl felbamate	 3-carbamoyloxy-2-phenylpropyl allophanate $C_{12}H_{15}N_3O_5$ 281.26	Process Impurity	Not more than 0.15%
Felbamate related compound-B	 Phenethyl carbamate $C_9H_{11}NO_2$ 165.19	Process Impurity	Not more than 0.15%
Felbamate dimer	 3,3'-carbonylbis(oxy)bis(2-phenylpropane-3,1-diyl) dicarbamate $C_{21}H_{24}N_2O_7$ 416.42	Process Impurity	Not more than 0.15%
Methyl carbamate	 Methyl carbamate $C_2H_5NO_2$ 75.07	Reagent	Not more than 0.05%
Isopropyl Carbamate	 Isopropyl Carbamate C4H9NO2 103.12	Reagent	Not more than 0.42 ppm

DEDRADATION PATHWAYS FOR FELBAMATE:



IMPURITY ISOLATION

2-Phenyl-1,3-propanediol Monocarbamate

During the first stage reaction which involves methyl carbamate and 2-phenyl-1,3-propanediol in presence of Aluminum isopropoxide, the mother liquor collected after the filtration has 2-Phenyl-1,3-propanediol Monocarbamate as major component. So the mother liquor is concentrated on a buchi roto-evaporator and the residue obtained is applied onto the silica column and eluted the pure fraction of 2-phenyl-1,3-propanediol monocarbamate with different ratios (starting from 1% ethyl acetate to 35% with increments like 1%, 5%, 10%, 20% and 35% in Hexane) of ethyl acetate and hexane. The collected fraction was tested on HPLC to confirm the impurity and its purity. This fraction is concentrated and solid is isolated.

Characterization and structure elucidation of impurities

Characterization of impurities has been carried out mainly by LC/MS/MS either by direct infusion if the impurity is available as pure compound and many other cases the Liquid chromatography mass chromatography has been done by eluting the compounds through the HPLC column and analyzing by mass detector. In cases where the impurities formed are very small with 0.1% to 5% levels with respect to the drug, the mass is picked up from the LC/MS/MS run and in combination with the HPLC retention times (based on polarity) were predicted and confirmed by daughter ion analysis. The isolated impurities were also analyzed by NMR for structure confirmations.

Mass spectrometer:

Micromass Quattro-LC triple quadruple mass spectrometer equipped with Electro spray ionization source with Z-spray technology. System capable of performing both positive and negative ion scans. Argon is used as a collision gas. Masslynx data acquisition system from Micromass, Version 3.5 is used as a data system.

CHARACTERIZATION AND STRUCTURE ELUCIDATION

All the possible process and degradation individual impurities either synthesized or isolated by enriching the impurity in the mother liquors are characterized by various analytical techniques and confirmed the structure of the impurities. Later these impurities were spiked and confirmed the retention times (RT's) and relative retention times (RRT's). The full physico-chemical characterization of each individual impurity is provided below.

PHYSICO-CHEMICAL CHARACTERIZATION OF METHYL CARBAMATE

1.0 INTRODUCTION

This report describes the physico-chemical characterization of methyl carbamate. The physico-chemical characterization of methyl carbamate was established by analytical techniques such as HPLC, FT-IR, MASS, NMR, TGA and DSC analysis. The chromatographic purity of methyl carbamate standard was determined by HPLC.

2.0 PHYSICAL PROPERTIES

2.1 APPEARANCE

TABLE-[1]: Appearance results for methyl carbamate

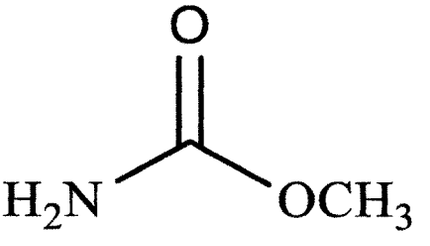
Name	Appearance
Methyl Carbamate	White crystalline solid

3.0 CHEMICAL PROPERTIES:

3.1 Chemical Name: Methyl Carbamate

3.2 Common Name: Methyl urethane

3.3 Structural Formula:



3.4 Molecular Formula: $C_2H_5NO_2$

3.5 Molecular Weight: 75.07

4.0 CHROMATOGRAPHIC PURITY BY HPLC:

The chromatographic purity of methyl carbamate standard was determined by High performance liquid chromatograph (HPLC) using the test procedure developed for the same. The results are depicted in the **Table-2**. The chromatograms are depicted in **Exhibits below**. The potency of methyl carbamate was derived by subtracting all possible organic as well as inorganic impurities from 100.

TABLE-[2]: Results of chromatographic purity by HPLC of methyl carbamate

Name	Chromatographic purity by HPLC	Potency
Methyl Carbamate	91.59%	91.22 %

5.0 CHARACTERIZATION BY INFRARED SPECTRUM:

The Infrared spectra of Methyl Carbamate was obtained by using FT-IR instrument and is depicted in **Exhibits below.** Interpretation of functional groups is presented in **Table-3.**

TABLE-[3]: FT-IR Frequency table of Methyl Carbamate

Bond	Approx. Frequency (cm^{-1})	Intensity and range
$-NH_2$	3446.79, 3336.85	3450 - 3200
-C=O	1691.57	1760 – 1650

Conclusion: The IR frequencies are in-line with the functional groups.

6.0 CHARACTERIZATION BY MASS SPECTROMETRY

The Mass spectrum of methyl carbamate was performed for the parent ion scans using infusion-MS and infusion-MS/MS techniques on Quattro-LC mass spectrometer (Micromass-LC/MS/MS system). The result is depicted in Table 4. The mass spectrum is depicted in **Exhibits below.**

TABLE-[4]: Table: Molecular ion data for Methyl Carbamate by infusion-MS Technique

Name	m/z value +Na	Molecular weight of Methyl Carbamate
Methyl Carbamate	97.97	75.07

Conclusion: Confirms the Mass of methyl carbamate

7.0 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER (BRUKER 400 MHz, NMR SYSTEM)

The NMR of methyl carbamate was performed by 400 MHz Bruker Nuclear magnetic resonance spectrometer by preparing sample in DMSO. The ^{1}H NMR & ^{13}C NMR spectra are depicted in **Exhibits below** and the interpretation is provided in Table-5&6.

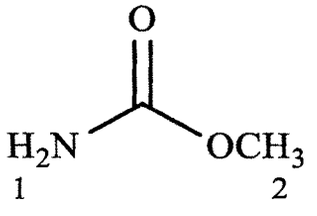


TABLE-[5]: Proton (^{1}H) NMR:

Position	1H	Methyl Carbamate	
		δ (ppm)	Multiplicity
1	2H	6.456	Singlet
2	3H	3.372-3.477	Singlet

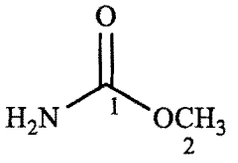


TABLE-[6]: Carbon (^{13}C) NMR:

Position	δ (ppm)
1	50.86
2	157.19

Conclusion: Proton and carbon NMR confirms the structure of methyl carbamate

8.0 CHARACTERIZATION BY DIFFERENTIAL SCANNING CALORIMETER [DSC]

The DSC of methyl carbamate was performed and the DSC data presented in **Exhibits** has the melting range 57.75°C. The observed peak is sharp and compound melts completely at the melting range of methyl carbamate. Partial melting of the crystals was not observed before melting temperature is attained.

9.0 CHARACTERIZATION BY THERMO GRAVIMETRIC ANALYSIS [TGA]

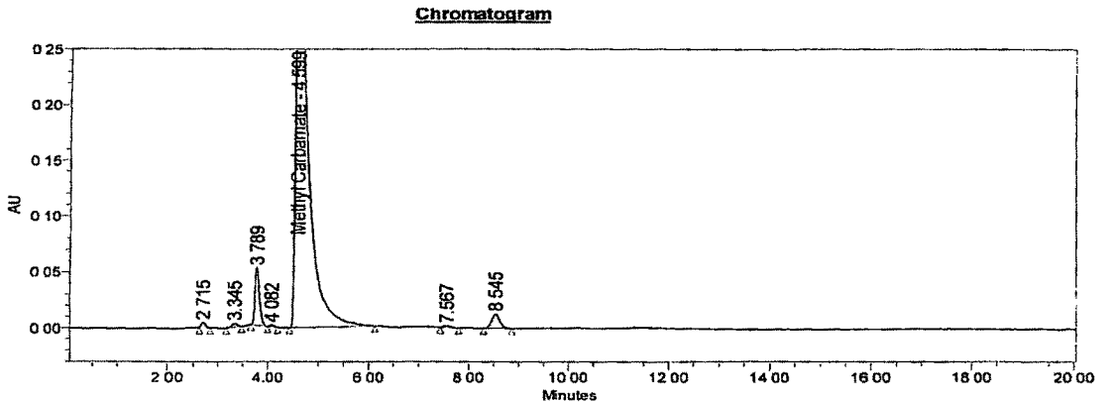
The TGA of methyl carbamate was performed and the TGA data presented in **Exhibit-7** depicts only residual moisture present in the sample. There is no hydrate form observed as there is no bounded moisture found in the sample. Also the methyl carbamate sample does not exist as any solvate either. The compound does not lose any weight more than approximately 3.15% to 14.40 % until the melting range temperature is attained.

10.0 STORAGE CONDITION

To be stored in a well closed container at room temperature.

EXHIBITS

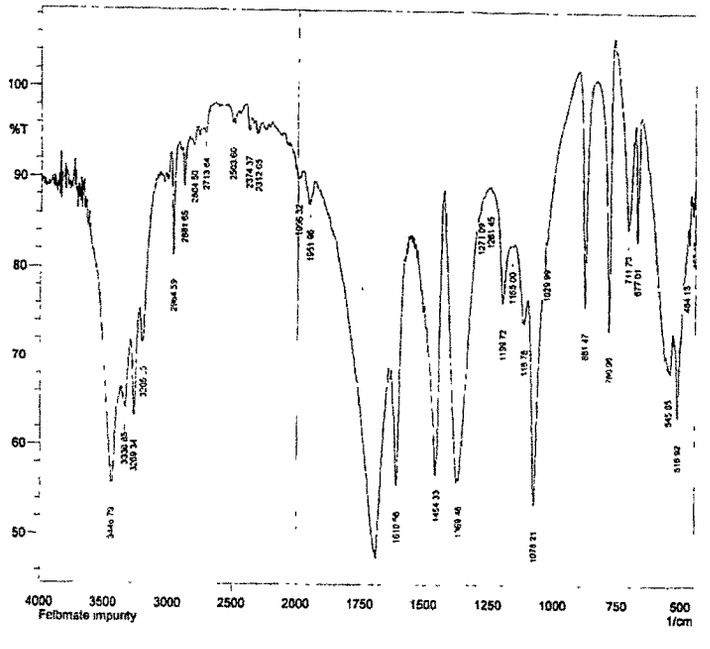
HPLC chromatogram of Methyl Carbam



Peak Table

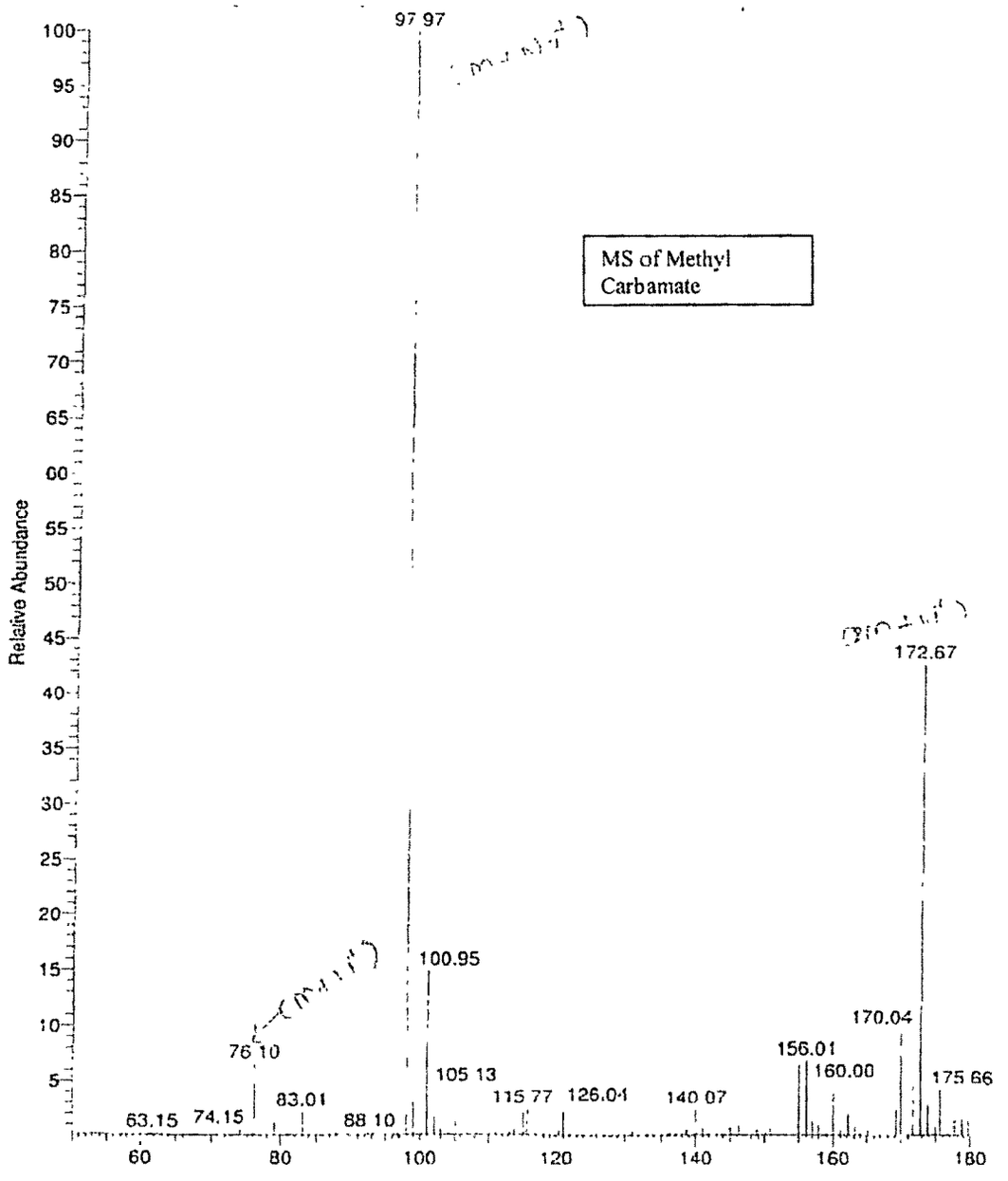
	Peak Name	RT	Area	% Area
1		2.72	25674	0.39
2		3.34	23921	0 36
3		3.79	317533	4.84
4		4 08	8106	0.12
5	Methyl Carbamate	4.60	6028450	91 88
6		7.57	14960	0 23
7		8.55	142615	2.17
Sum			6561259	100.00

Infrared Spectrum of Methyl Carbamate

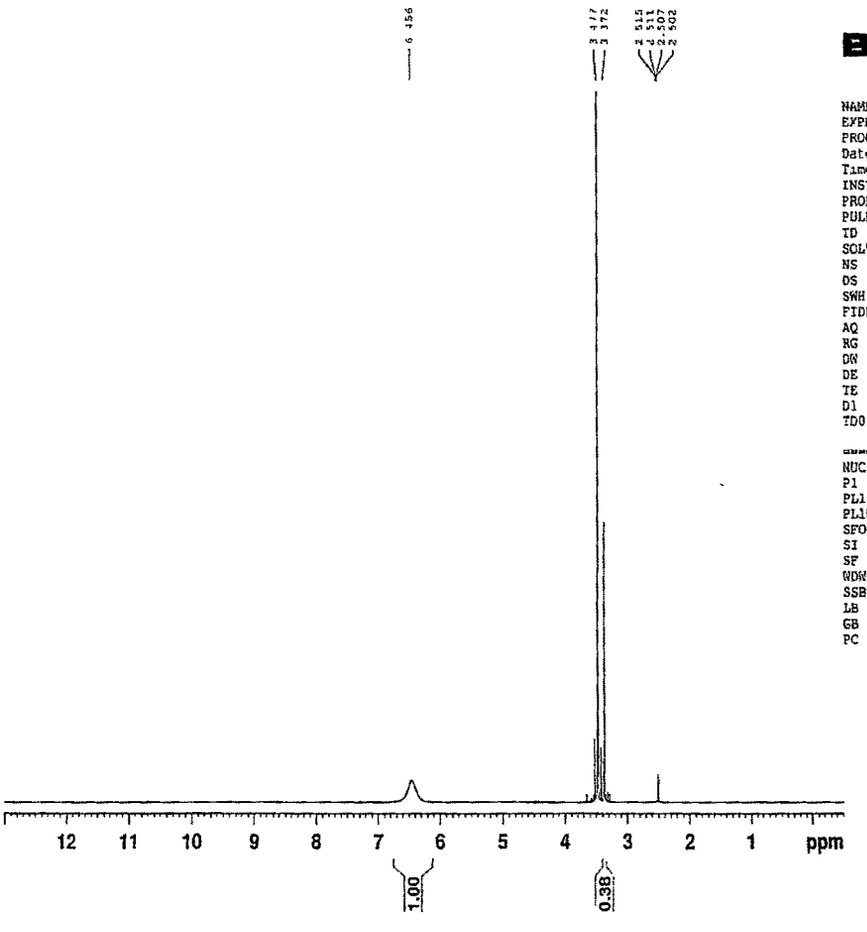


No.	Peak	Intonsity	Area
1	457 13	86 27	0 876
2	484 13	81 063	0 772
3	516 92	63 258	5 787
4	545 85	68 338	3 665
5	877 01	83 157	0 891
6	711 73	84 515	2.146
7	788 96	73 142	1 856
8	881 47	75 807	1 703
9	1029 99	82 915	2 991
10	1078 21	53 527	11 56
11	1116 78	73 685	5 265
12	1165	82 598	1 114
13	1199 72	76 157	8 311
14	1261 45	88 766	0 489
15	1271 09	88 019	0 82
16	1369 46	56 226	13 003
17	1454 33	57 969	3 604
18	1610 56	55 548	8 835
19	1691 57	47 493	10 438
20	1951 96	87 103	2 288
21	1996 32	89 923	1 435
22	2312 65	94 836	0 975
23	2374 37	95 164	0 653
24	2503 6	96 062	0 456
25	2713 84	94 908	0 997
26	2804 5	93 496	1 632
27	2881 65	89 031	1 964
28	2964 59	81 366	3 107
29	3205 69	71 614	11 079
30	3269 34	63 269	11 324
31	3336 85	64 057	12 811
32	3446 79	55 722	11 31

Mass Spectra of Methyl Carbamate



Proton NMR Spectra of Methyl Carbamate



BRUKER

NAME	060513 Apicore
EXPNO	7
PROCNO	1
Date_	20130508
Time	16.27
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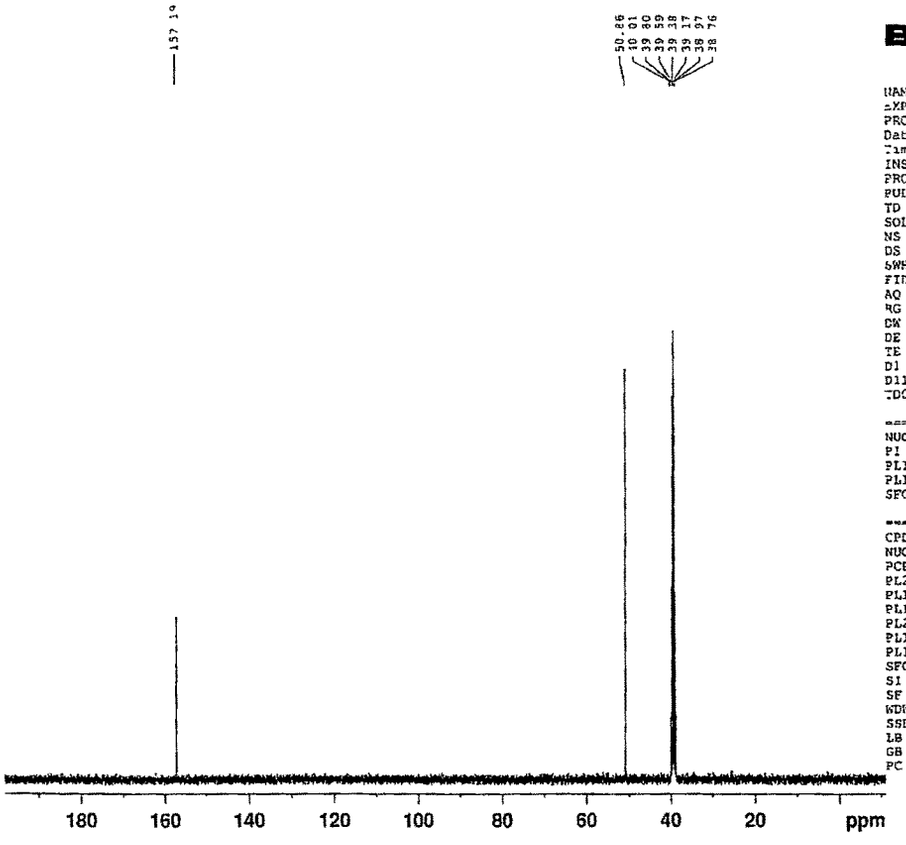
PROBHD	5 mm PABBO BB-
PULPROG	zg30
TD	65536
SOLVENT	DMSO
NS	16
DS	0
SWH	8223.685 Hz
FIDRES	0.125483 Hz
AQ	3.9846387 sec
RG	50.8
DW	60.800 usec
DE	6.50 usec
TE	296.3 K
D1	1.00000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	1H
P1	11.20 usec
PL1	0.00 dB
PL1W	17.35713577 W
SFO1	400.1328009 MHz
SI	32768
SF	400.1300000 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

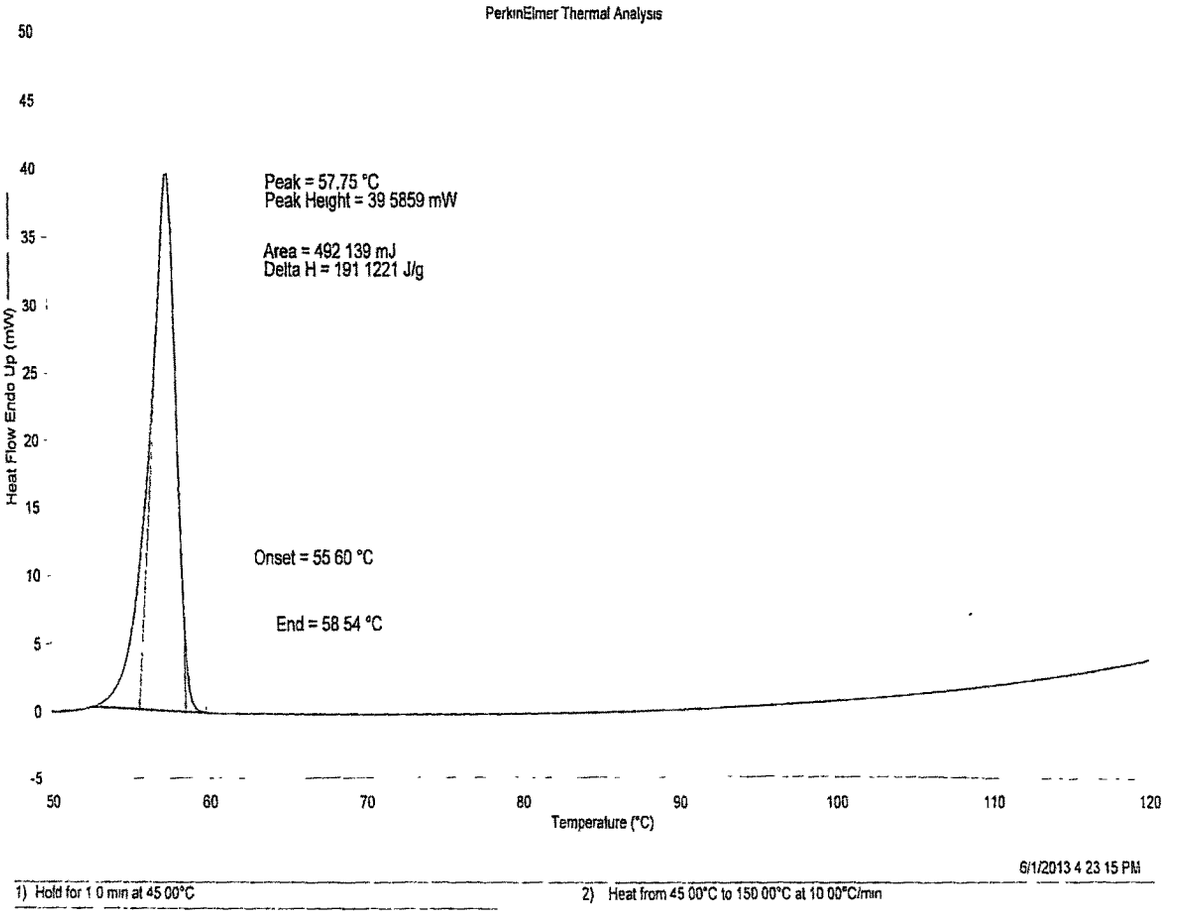
Carbon NMR Spectra of Methyl Carbamate



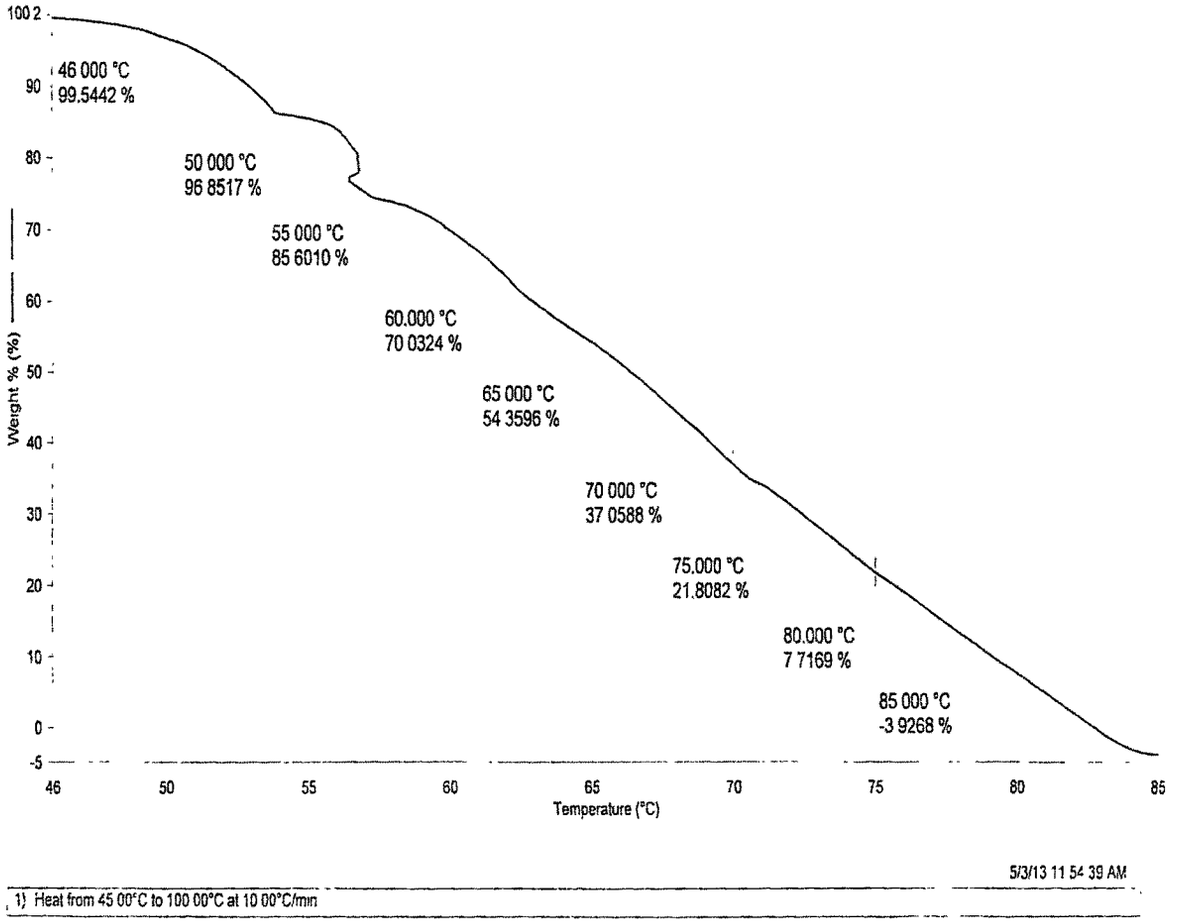
BRUKER

NAME	060513 Apicore	
EXPNO	8	
PROCNO	1	
Date_	20130508	
Time	16 32	
INSTRUM	spect	
PROBHD	5 mm PABBO BB-	
PULPROG	zgpg30	
TD	65536	
SOLVENT	DMSO	
NS	50	
DS	4	
SWH	26041.666	Hz
FIDRES	0.397364	Hz
AQ	1.2583412	sec
RG	203	
DW	19.200	usec
DE	6.50	usec
TE	297.1	K
D1	2.00000000	sec
D11	0.03000000	sec
TD0	1	
======== CHANNEL f1 ========		
NUC1	13C	
P1	6.00	usec
PL1	1.60	dB
PL1W	63.25595093	W
SFO1	100.6228298	MHz
======== CHANNEL f2 ========		
CPDPRG2	waltz16	
NUC2	1H	
PCPD2	90.00	usec
PL2	0.00	dB
PL12	17.87	dB
PL13	20.87	dB
PL2W	17.35713577	W
PL12W	0.28345099	W
PL13W	0.14206201	W
SFO2	400.1316005	MHz
SI	32768	
SF	100.6126193	MHz
WDW	EM	
SSB	0	
LB	1.00	Hz
GB	0	
PC	1.40	

DSC report of Methyl Carbamate



TGA report of Methyl Carbamate



PHYSICO-CHEMICAL CHARACTERIZATION OF N-AMINOCARBONYL FELBAMATE

The Physico-chemical characterization of N-amino carbonyl felbamate was established by analytical techniques such as HPLC, FT-IR, MASS, NMR, TGA and DSC analysis. The Chromatographic purity of N-amino carbonyl felbamate Standard was determined by HPLC.

1.0 PHYSICAL PROPERTIES

1.1 APPEARANCE

TABLE-[1]: Appearance results for N-amino carbonyl felbamate

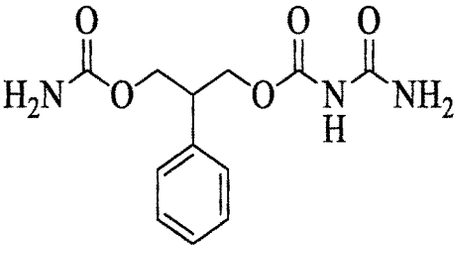
Name	Appearance
N-AMINO CARBONYL FELBAMATE	Off-white crystalline powder

2.0 CHEMICAL PROPERTIES

2.1 Chemical Name: N-amino carbonyl felbamate OR 3-carbamoyloxy-2-phenylpropyl allophanate

2.2 Common Name: N-amino carbonyl felbamate

2.3 Structural Formula



2.4 Molecular Formula: $C_{12}H_{15}N_3O_5$

2.5 Molecular Weight: 281.26

3.0 CHROMATOGRAPHIC PURITY BY HPLC

The chromatographic purity of N-amino carbonyl felbamate was determined by High performance liquid chromatograph (HPLC) using developed test procedure. The results are depicted in the Table-1. The chromatograms are depicted in **Exhibits below**. The potency

of N-amino carbonyl felbamate was derived by subtracting all possible organic as well as inorganic impurities from 100.

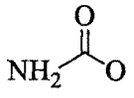
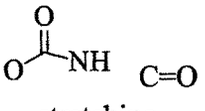
TABLE-[2]: Results of chromatographic purity by HPLC of N-amino carbonyl felbamate

Name	Chromatographic purity by HPLC	Potency
N-AMINO CARBONYL FELBAMATE	92.49%	91.34

4.0 CHARACTERIZATION BY INFRARED SPECTRUM

The Infrared spectra of N-amino carbonyl felbamate was obtained by using FT-IR instrument and is depicted in **Exhibit-2.**

TABLE-[3]: FT-IR Frequency table of Felbamate Related compound N-amino carbonyl felbamate

Bond	Approx. Frequency (cm^{-1})	Intensity and range
	3184-3334	3200-3300
 stretching	1693-1732	1650 – 1750
Aromatic ring C-C stretching	1415-1496	1500- 1400

Conclusion: The IR frequencies are in-line with the functional groups.

5.0 CHARACTERIZATION BY MASS SPECTROMETRY

The –ve Mass spectrum of N-amino carbonyl felbamate was performed for the parent ion scans using infusion-MS and infusion-MS/MS techniques on Quattro-LC mass spectrometer (Micromass-LC/MS/MS system). The result is depicted in Table-4. The mass spectrum is depicted in **Exhibits below.**

TABLE-[4]: Molecular ion data for N-amino carbonyl felbamate by infusion-MS Technique

Name	m/z value+ Na	m/z value	N-amino carbonyl Felbamate
N-amino carbonyl felbamate	304.15	281.89	281.28

Conclusion: Confirms the Mass of N-amino carbonyl felbamate.

6.0 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER (BRUKER 400 MHz, NMR SYSTEM)

The NMR of N-amino carbonyl felbamate was performed by 400 MHz Bruker Nuclear magnetic resonance spectrometer by preparing sample in $CDCl_3$. The ^1H NMR & ^{13}C NMR spectra are depicted in **Exhibits below** and the interpretation is provided in Table 5&6.

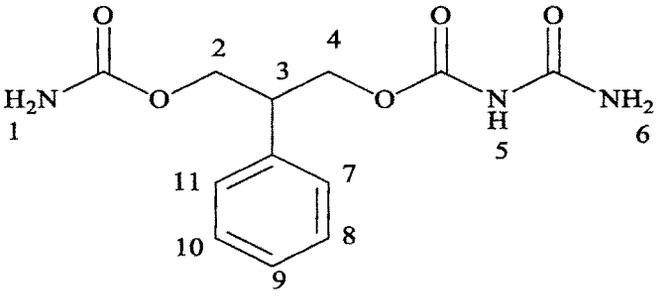


TABLE-[5]: Proton (^{1}H) NMR:

Position	1H	N-AMINO CARBONYL FELBAMATE	
		δ (ppm)	**Multiplicity**
1	NH2	NONE	NONE
2	2H	4.184-4.213	dd(doublet of doublet)
3	1H	3.200-3.269	multiplet
4	2H	4.313-4.339	dd(doublet of doublet)
5	NH	NONE	NONE
6	NH2	NONE	NONE
7,8,9,10&11	5H	7.138-7.228	Multiplet

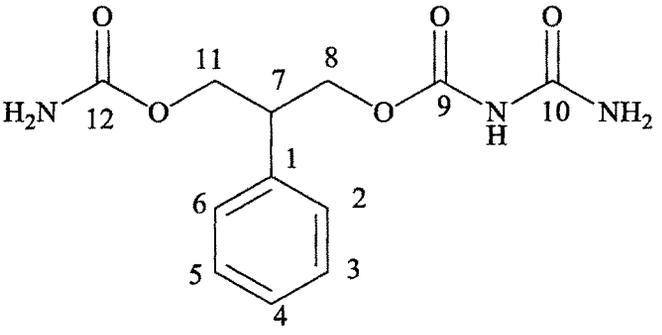


TABLE-[6]: Carbon (^{13}C) NMR:

Position	δ (ppm)
1	139.89
6&2	129.29
3&5	129.72
4	128.43
12	159.64
11	67.39
7	45.71
8	66.16
9	156.88
10	155.85

Conclusion: Proton and carbon NMR confirms the structure of N-amino carbonyl Felbamate.

7.0 CHARACTERIZATION BY DIFFERENTIAL SCANNING CALORIMETER [DSC]

The DSC of N-amino carbonyl felbamate was performed and the DSC data is presented in **Exhibits** has the melting point 182.452°C. The observed peak is sharp and compound melts completely at the melting range of N-amino carbonyl felbamate. Partial melting of the crystals was not observed before melting temperature is attained.

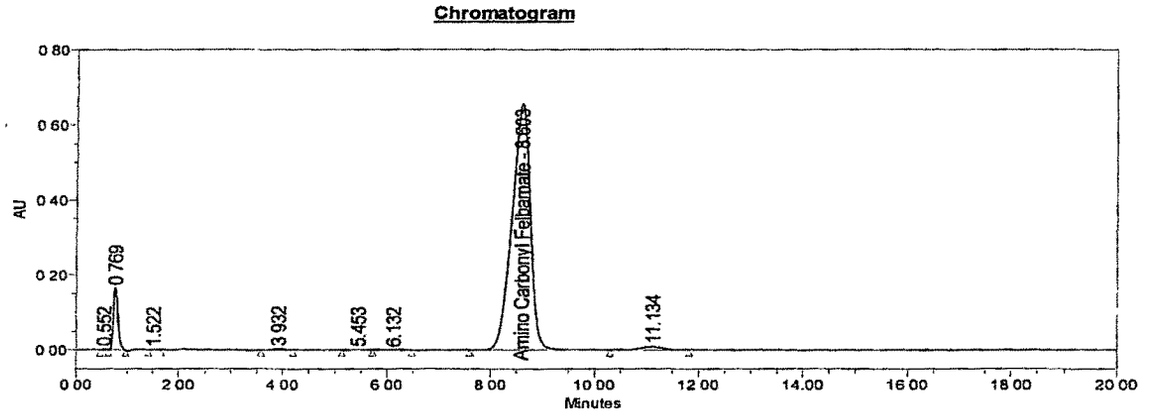
8.0 CHARACTERIZATION BY THERMO GRAVIMETRIC ANALYSIS [TGA]

N-amino carbonyl felbamate was analyzed by TGA and the data presented in **Exhibits** depicts only residual moisture present in the sample. There is no hydrate form observed as there is no bounded moisture found in the sample. Also the N-amino carbonyl felbamate related compound sample does not exist as any solvate either. The compound does not lose any weight more than approximately 1.8% until the melting range temperature is attained.

9.0 STORAGE CONDITION

Store in a well closed container at room temperature.

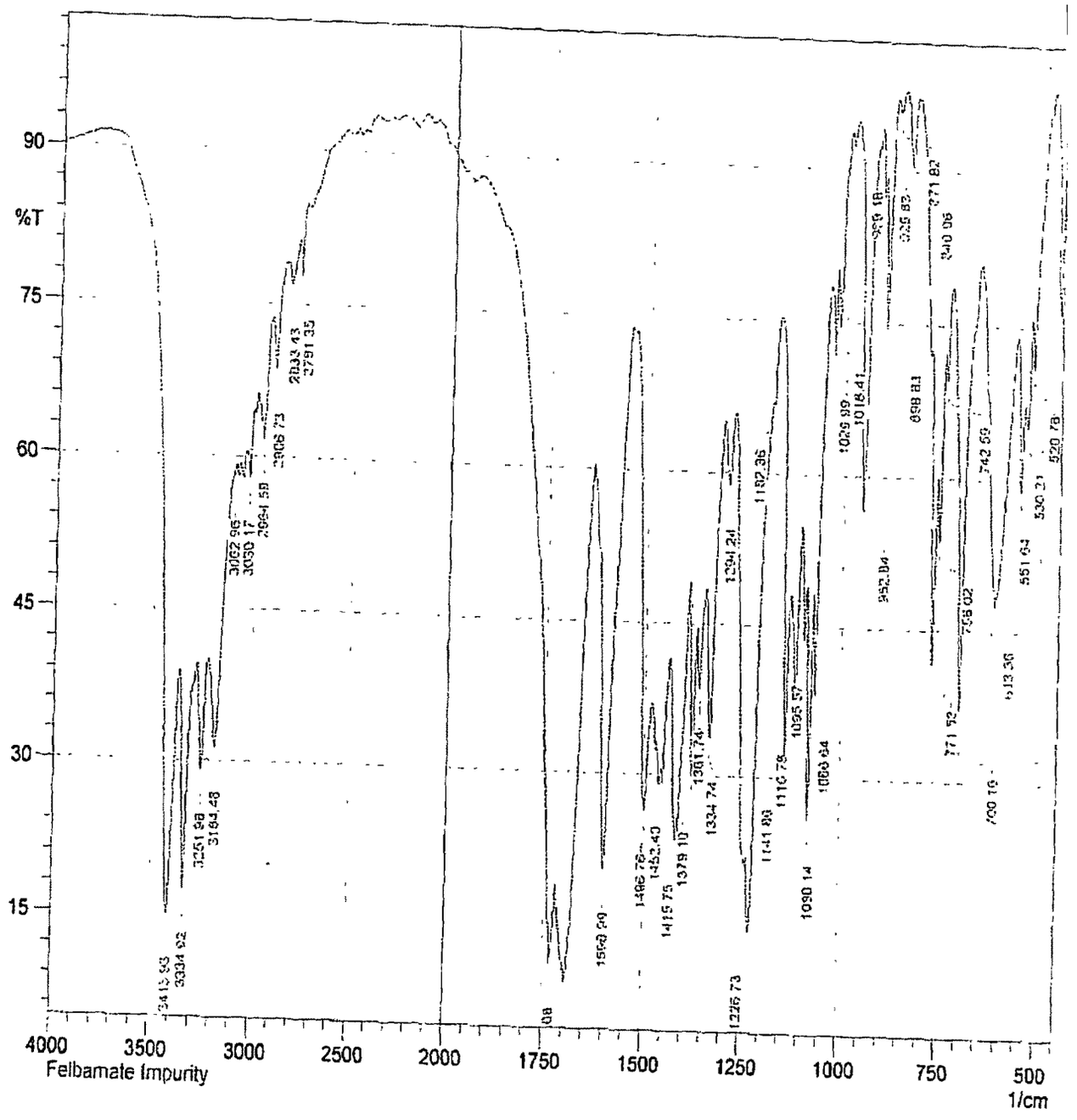
HPLC chromatogram of N-amino carbonyl felbamate



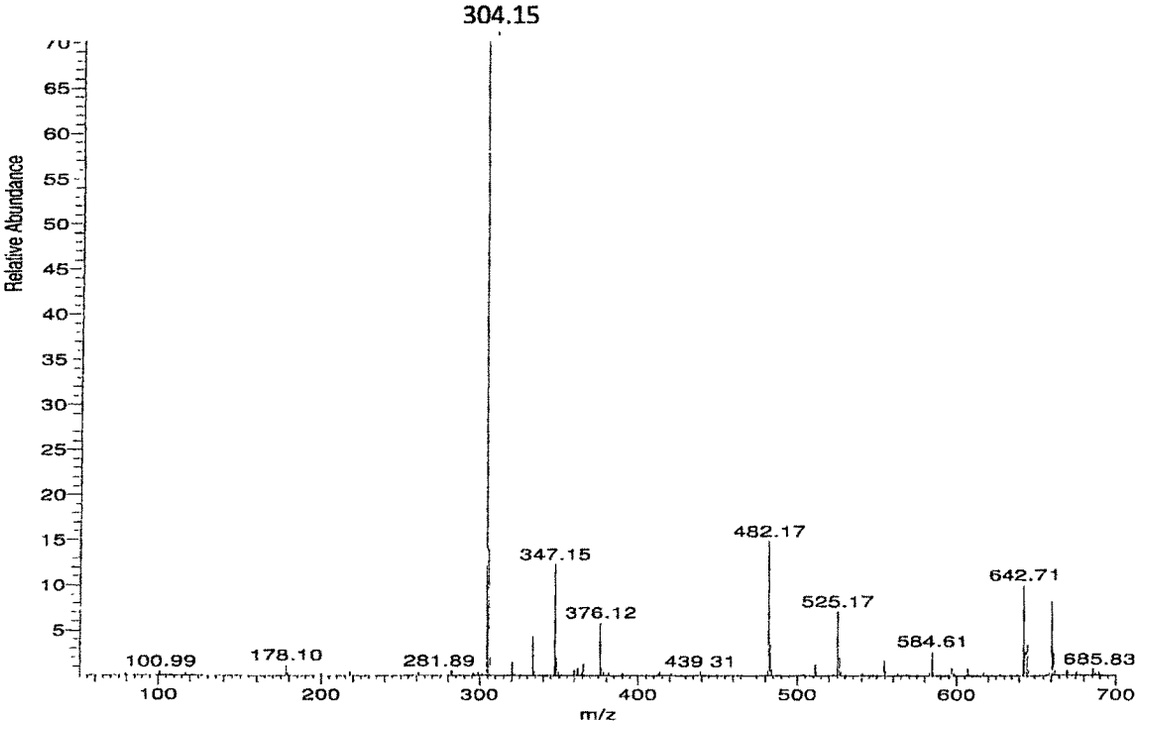
Peak Table

	Peak Name	RT	Area	% Area
1		0.55	8794	0.05
2		0.77	1020205	5.89
3		1.52	2718	0.02
4		3.93	38045	0.22
5		5.45	4899	0.03
6		6.13	12627	0.07
7	Amino Carbonyl Felbamate	8.60	15978331	92.19
8		11.13	266955	1.54
Sum			17332574	100.00

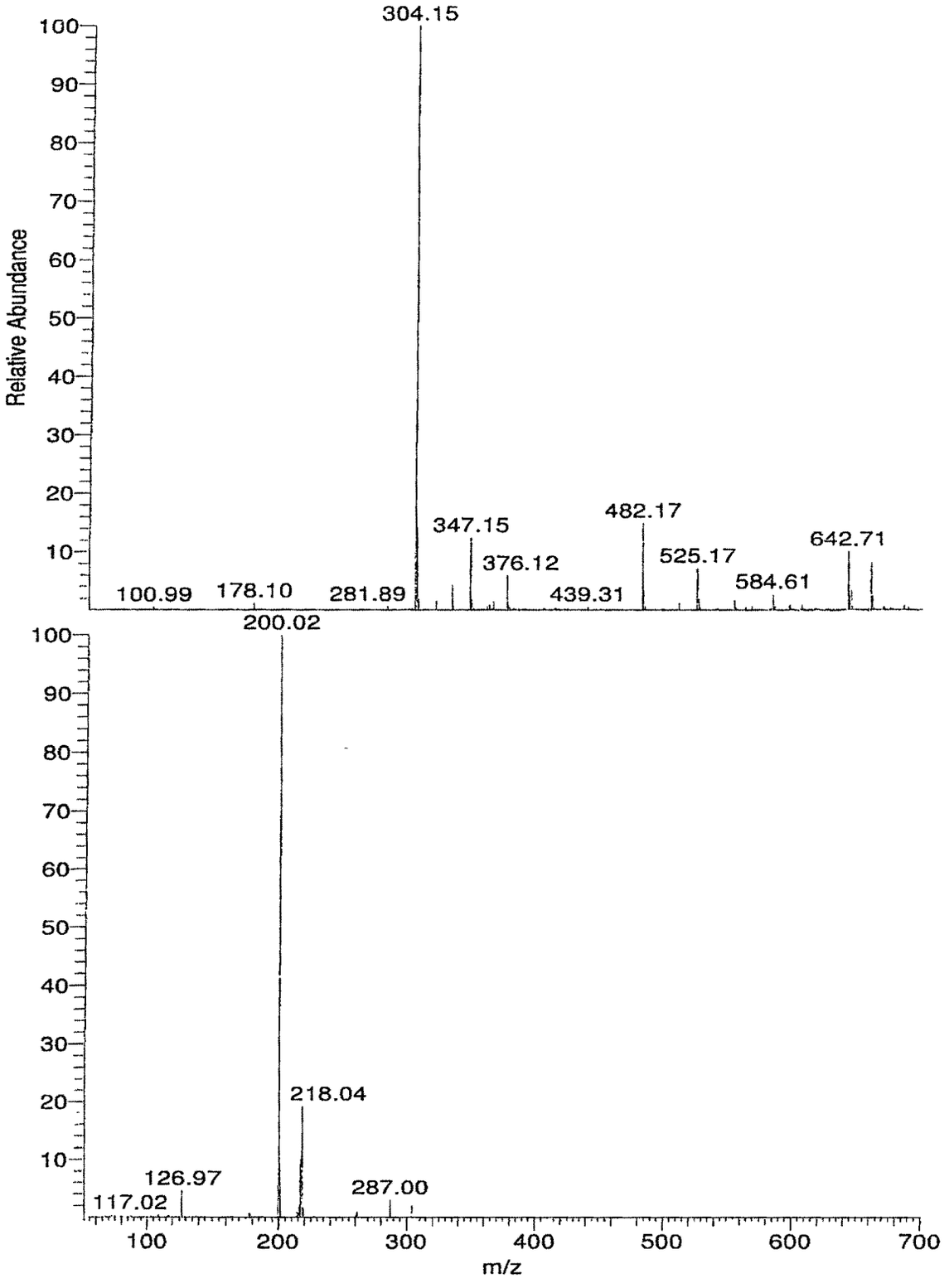
Infrared Spectrum of N-amino carbonyl Felbamate



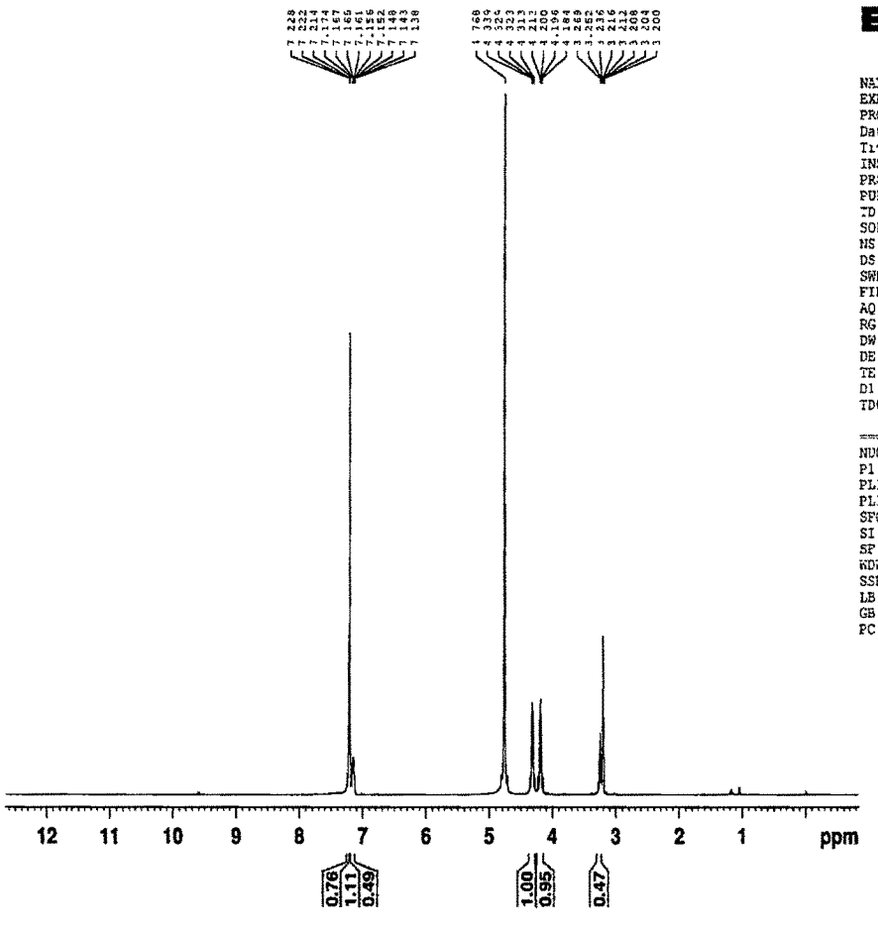
Mass Spectra of N-amino carbonyl Felbamate



Mass Spectra (MS/MS) of N-amino carbonyl Felbamate



Proton NMR Spectra of N-amino carbonyl Felbamate



BRUKER

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PROCNO	1
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PULPROG	zg30
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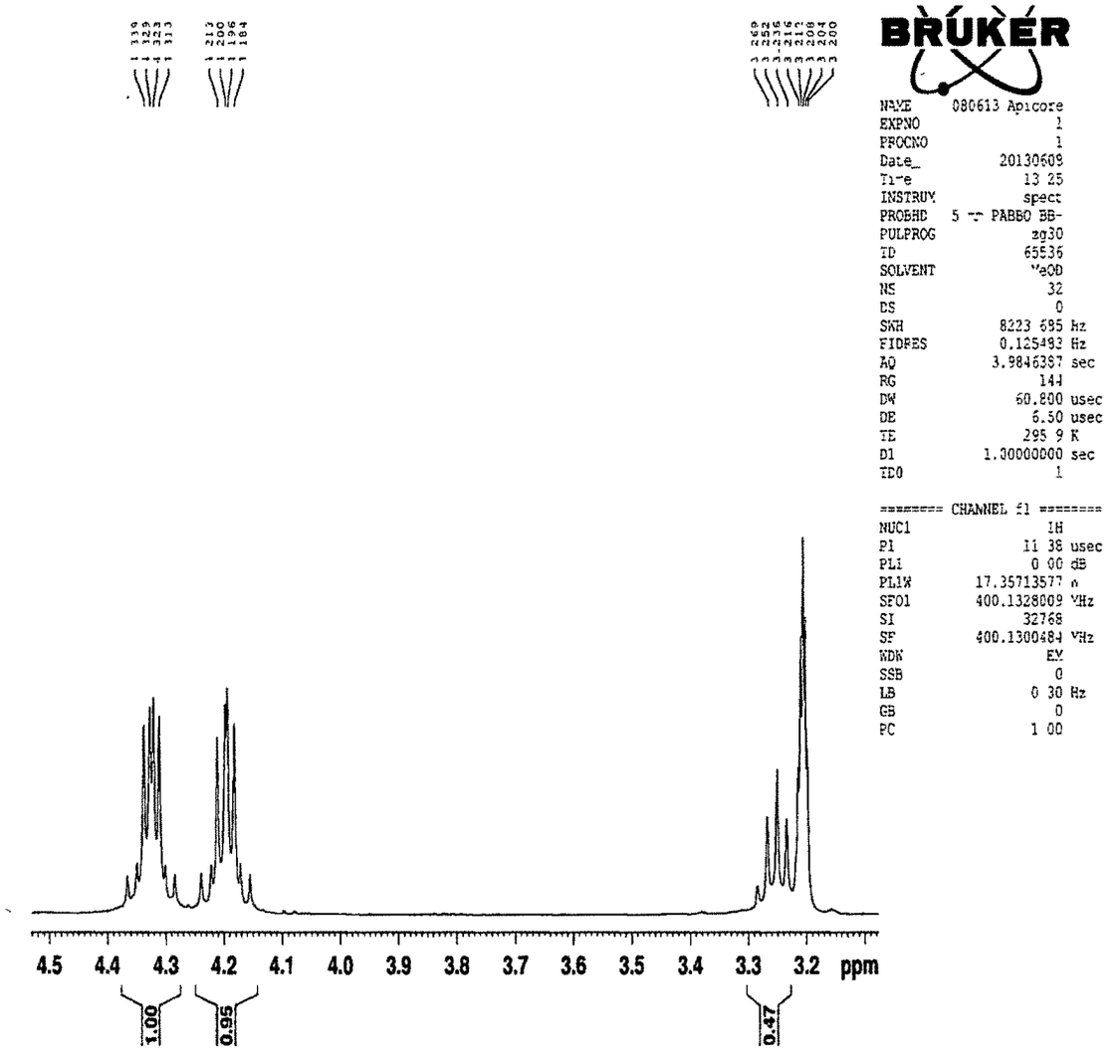
SOLVENT	MeOD
NS	32
DS	0
SWH	8223.685 Hz
FIDRES	0.125483 Hz
AQ	3.9846387 sec
RG	144
DW	60.800 usec
DE	6.50 usec
TE	295.9 K
D1	1.00000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	1H
P1	11.38 usec
PL1	0.00 dB
PL1W	17.35713577 W
SFO1	400.1328009 MHz
SI	32768
SF	400.1300484 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00



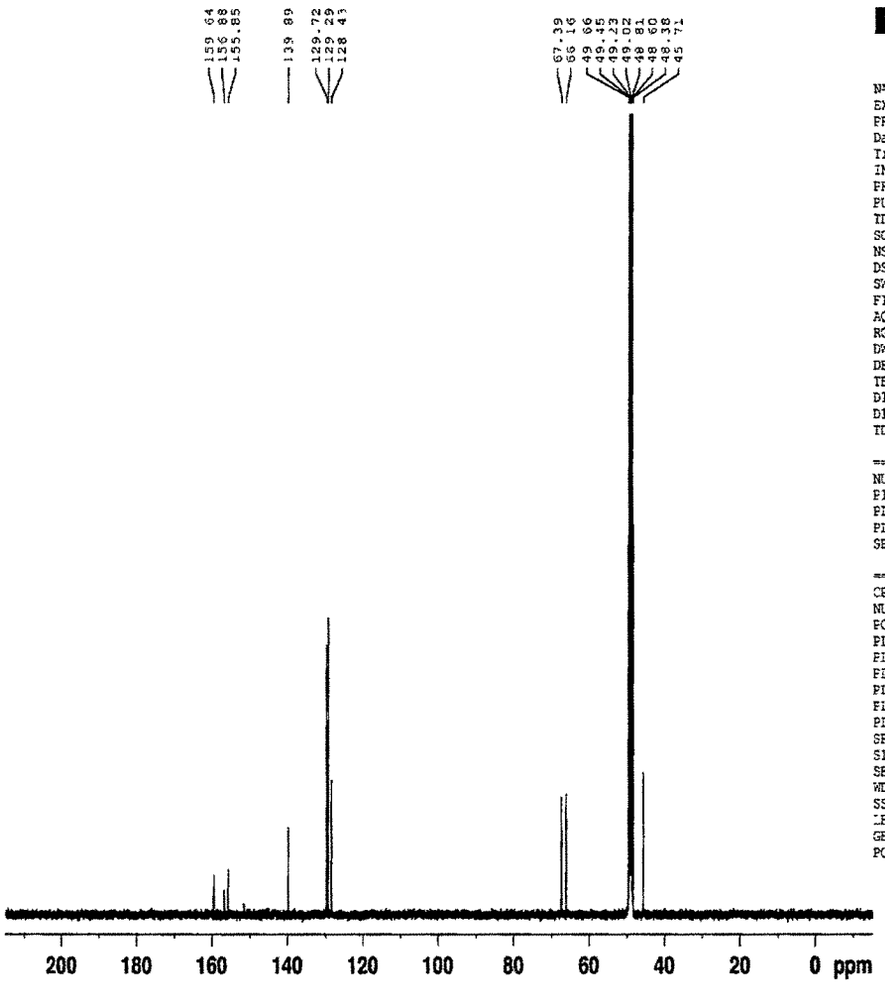
BRUKER

NAME	080613 Apicore
EXPNO	1
PROCNO	1
Date_	20130609
Time	13 25
INSTRUM	spect
PROBHD	5 mm PABBO BB-
PULPROG	zg30
TD	65536
SOLVENT	MeOD
NS	32
DS	0
SWH	8223 685 Hz
FIDRES	0.125493 Hz
AQ	3.9846387 sec
RG	144
DW	60.800 usec
DE	6.50 usec
TE	295 9 K
D1	1.00000000 sec
TD0	1

======== CHANNEL f1 ========

NUC1	1H
P1	11 38 usec
PL1	0 00 dB
PL1W	17.35713577 W
SFO1	400.1328009 MHz
SI	32768
SF	400.1300484 MHz
WDW	EM
SSB	0
LB	0 30 Hz
GB	0
PC	1 00

Carbon NMR Spectra of N-amino Carbonyl Felbamate



BRUKER

NAME	080613 Apicore
EXPNO	2
PROCNO	1
Date_	20130608
Time	14 21
INSTRUM	spect
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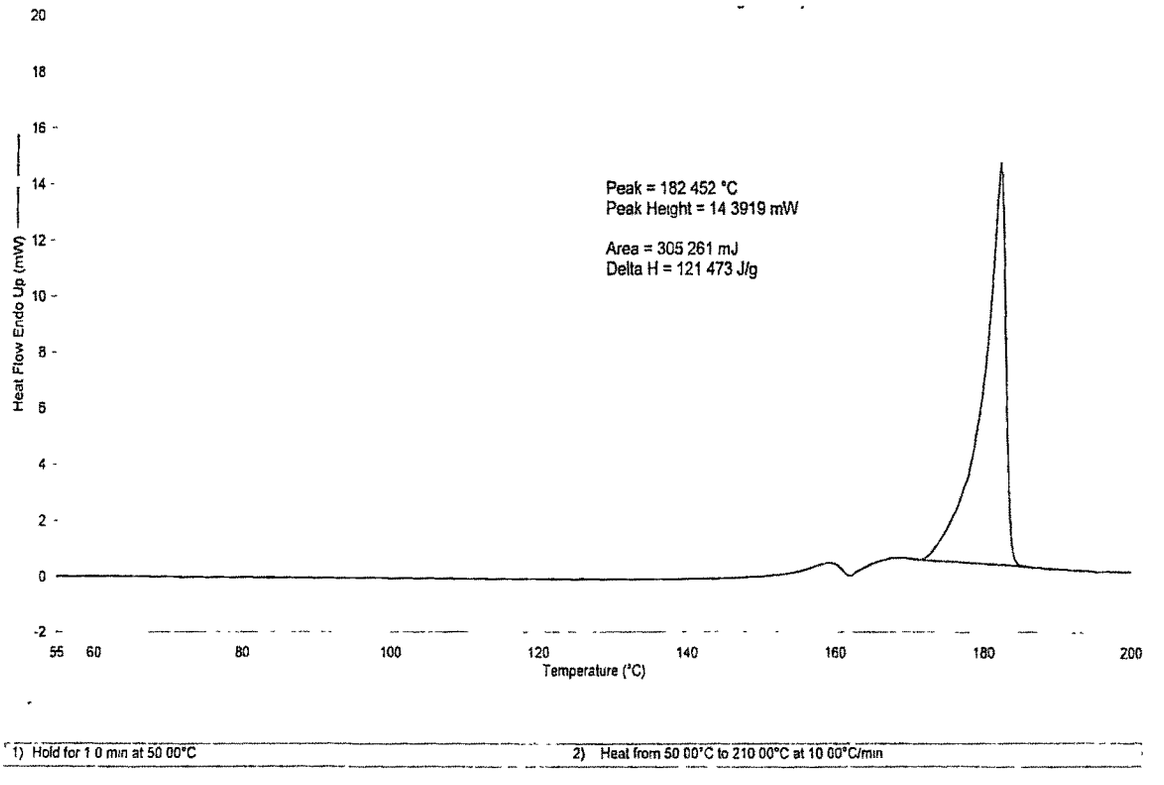
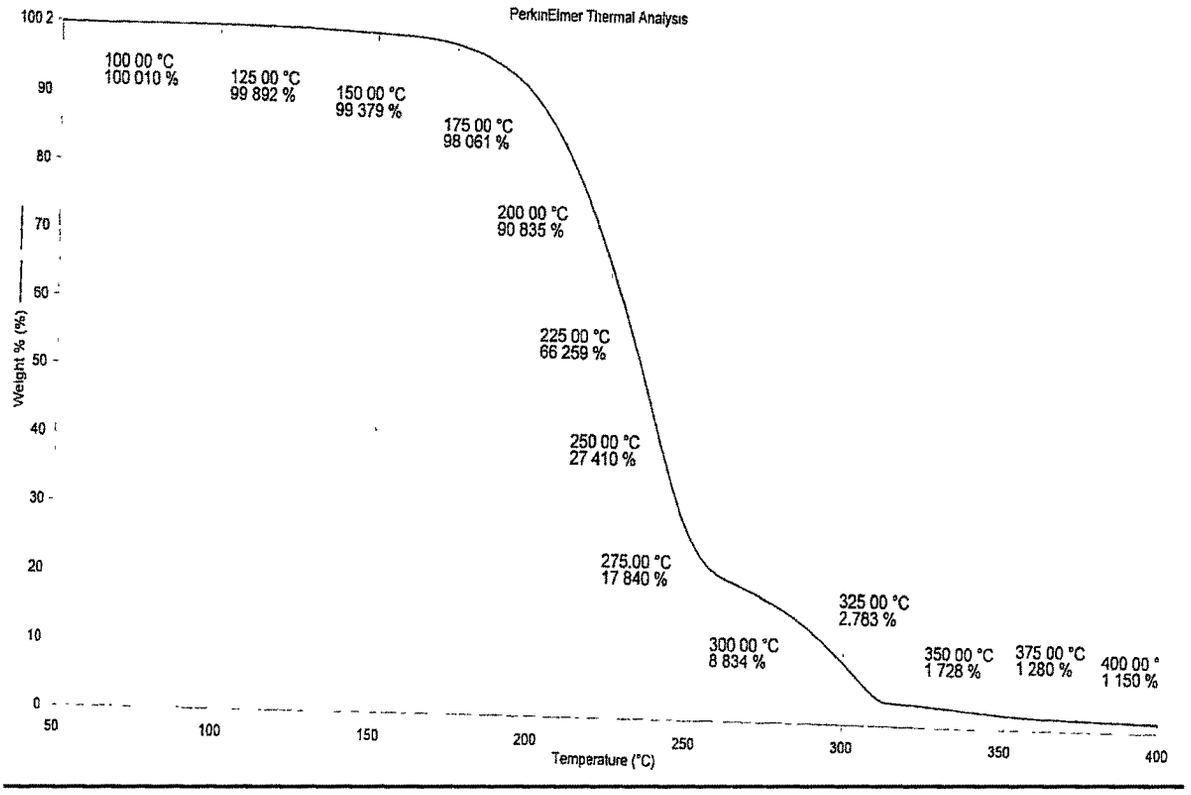
PULPROG	zgpg30
TD	65536
SOLVENT	MeOD
NS	993
DS	4
SWH	26041.666 Hz
FIDRES	0.397364 Hz
AQ	1.2583412 sec
RG	203
DW	19.200 usec
DE	6.50 usec
TE	297.5 K
D1	2.00000000 sec
D11	0.03000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	13C
P1	8.00 usec
PL1	1.60 dB
PL1W	63.25595093 W
SFO1	100.6228298 MHz
======== CHANNEL f2 ========	
CPDPRG2	waltz16
NUC2	1H
PCPD2	90.00 usec
PL2	0.00 dB
PL12	17.96 dB
PL13	17.96 dB
PL2W	17.35713577 W
PL12W	0.27763751 W
PL13W	0.27763751 W
SFO2	400.1316005 MHz
SI	32768
SF	100.6126261 MHz
WDW	EM
SSB	0
LB	1.00 Hz
GB	0
PC	1.40

DSC report of N-amino carbonyl Felbamate



TGA report of N-amino carbonyl Felbamate



1) Heat from 50.00°C to 410.00°C at 10.00°C/min

PHYSICO-CHEMICAL CHARACTERIZATION OF 2-PHENYL PROPANE-1,3-DIOL

The Physico-chemical characterization of 2-phenyl propane-1,3-diol was established by analytical techniques such as HPLC, FT-IR, MASS, NMR, TGA and DSC analysis. The chromatographic purity of 2-phenyl propane-1,3-diol Standard was determined by HPLC.

1.0 PHYSICAL PROPERTIES

1.1 APPEARANCE

TABLE-1 Appearance results for 2-phenyl propane-1,3-diol

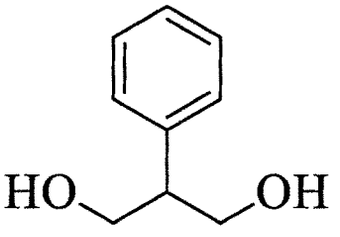
Name	Appearance
2-phenyl propane-1,3-diol	White crystalline powder

2.0 CHEMICAL PROPERTIES

2.1 Chemical Name: 2-phenyl propane-1,3-diol

2.2 Common Name: Phenylpropanediol

2.3 Structural Formula:



2.4 Molecular Formula: $C_9H_{12}O_2$

2.5 Molecular Weight: 152.19

3.0 CHROMATOGRAPHIC PURITY BY HPLC

The chromatographic purity of 2-phenyl propane-1,3-diol was determined by High performance liquid chromatograph (HPLC) using the test procedure developed. The results are depicted in the Table-2. The chromatograms are depicted in **Exhibits**. The potency of 2-phenyl propane-1,3-diol was derived by subtracting all possible organic as well as inorganic impurities from 100.

TABLE-2 Results of Chromatographic purity by HPLC of 2-phenyl propane-1,3-diol

Name	Chromatographic purity by HPLC	Potency
2-phenyl propane-1,3-diol	99.96%	99.49 %

4.0 CHARACTERIZATION BY INFRARED SPECTRUM

The Infrared spectra of 2-phenyl propane-1,3-diol was obtained by using FT-IR instrument and is depicted in **Exhibits.**

Table-3 FT-IR Frequency table of 2-phenyl propane-1,3-diol

Bond	Approx. Frequency (cm^{-1})	Intensity and range
OH stretching	3292 cm^{-1}	3200-3500
Aromatics	1450.97-1494.83cm^{-1}	1400-1500

Conclusion: The IR frequencies are in-line with the functional groups.

5.0 CHARACTERIZATION BY MASS SPECTROMETRY

The –ve Mass spectrum of 2-phenyl propane-1,3-diol was performed for the parent ion scans using infusion-MS and infusion-MS/MS techniques on Quattro-LC mass spectrometer (Micromass-LC/MS/MS system). The result is depicted in Table-4. The mass spectrum is depicted in **Exhibits.**

Table-4 Molecular ion data for 2-phenyl propane-1,3-diol by infusion-MS Technique

Name	[m/z value]$^-$	m/z value	Molecular weight of 2-phenyl propane-1,3-diol
2-phenyl propane-1,3-diol	150.96	152.19	152.19

Conclusion: Confirms the Mass of 2-phenyl propane-1,3-diol.

6.0 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER (BRUKER 400 MHz, NMR SYSTEM)

The NMR of 2-phenyl propane-1, 3-diol was performed by 400 MHz Bruker Nuclear magnetic resonance spectrometer by preparing sample in DMSO. The ^{1}H NMR & ^{13}C NMR spectra are depicted in **Exhibits** and the interpretation is provided in Table-[5].

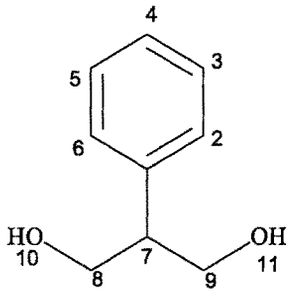


TABLE-[1]: Proton (^{1}H) NMR

Position	1H	2-phenyl propane-1,3-diol	
		δ (ppm)	**Multiplicity**
2,3,4,5 & 6	5H	7.162-7.296	Multiplet
8 & 9	4H	3.568-3.728	Triplet
10 & 11	2H	4.541-4.567	Triplet
7	1H	2.77-2.836	Multiplet

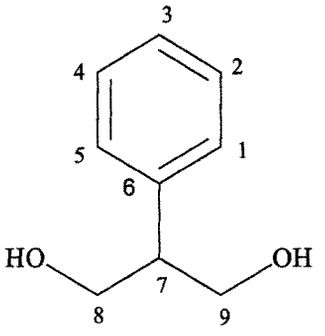


TABLE-[2]: Carbon (^{13}C) NMR:

Position	δ (ppm)
1 & 5	127.91
2 & 4	128.22
3	125.95
6	142.12
7	50.61
8 & 9	62.75

Conclusion: Proton and carbon NMR confirms the structure of 2-phenyl propane-1,3-diol.

7.0 CHARACTERIZATION BY DIFFERENTIAL SCANNING CALORIMETER [DSC]

The DSC of 2-phenyl propane-1,3-diol was performed and the DSC data was presented in **Exhibits** has the melting range 54.122°C. The observed peak is sharp and compound melts completely at the melting range of 2-phenyl propane-1,3-diol. Partial melting of the crystals was not observed before melting temperature is attained.

8.0 CHARACTERIZATION BY THERMO GRAVIMETRIC ANALYSIS [TGA]

The TGA of 2-phenyl propane-1,3-diol was performed and the TGA data presented in **Exhibits** depicts only residual moisture present in the sample. There is no hydrate form observed as there is no bounded moisture found in the sample. Also the 2-phenyl propane-1,3-diol sample does not exist as any solvate either. The compound does not lose any weight more than approximately 0.1% until the melting range temperature is attained.

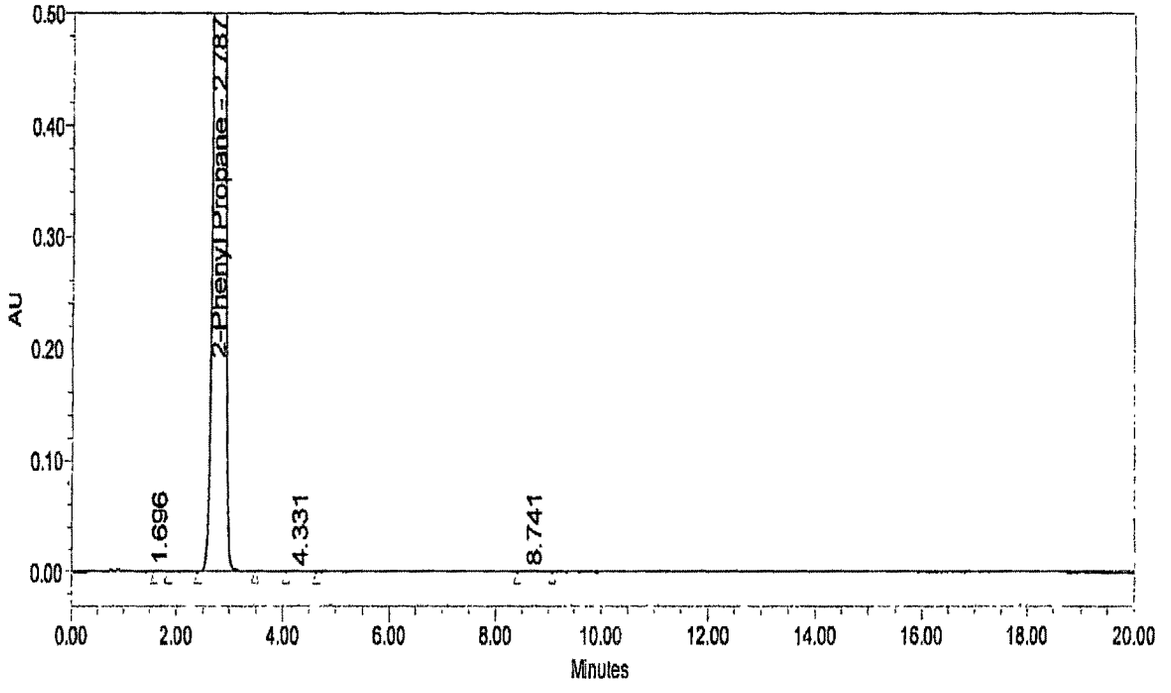
9.0 STORAGE CONDITION

Store in a well closed container at room temperature.

EXHIBITS

HPLC chromatogram of 2-phenyl propane-1,3-diol

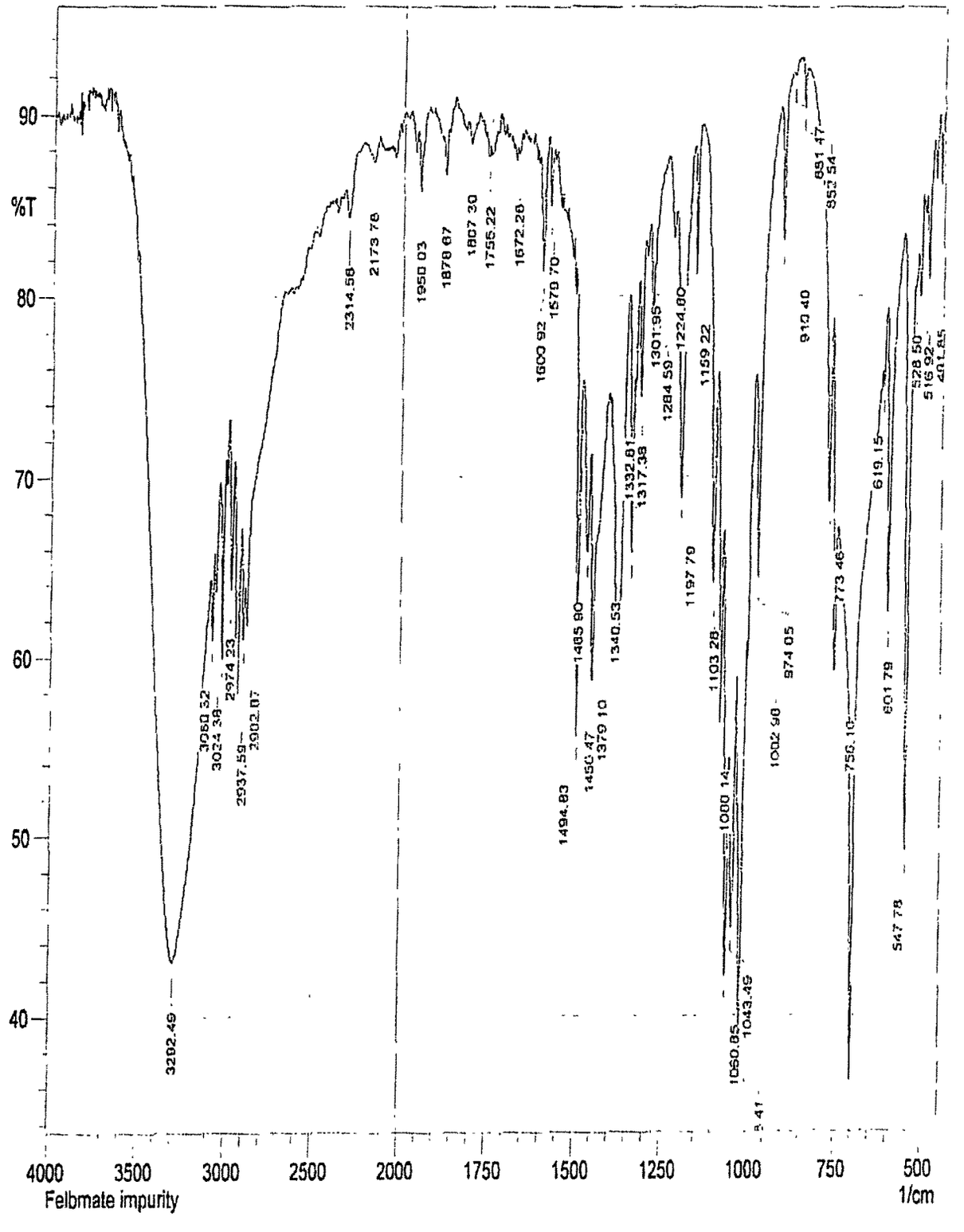
Chromatogram



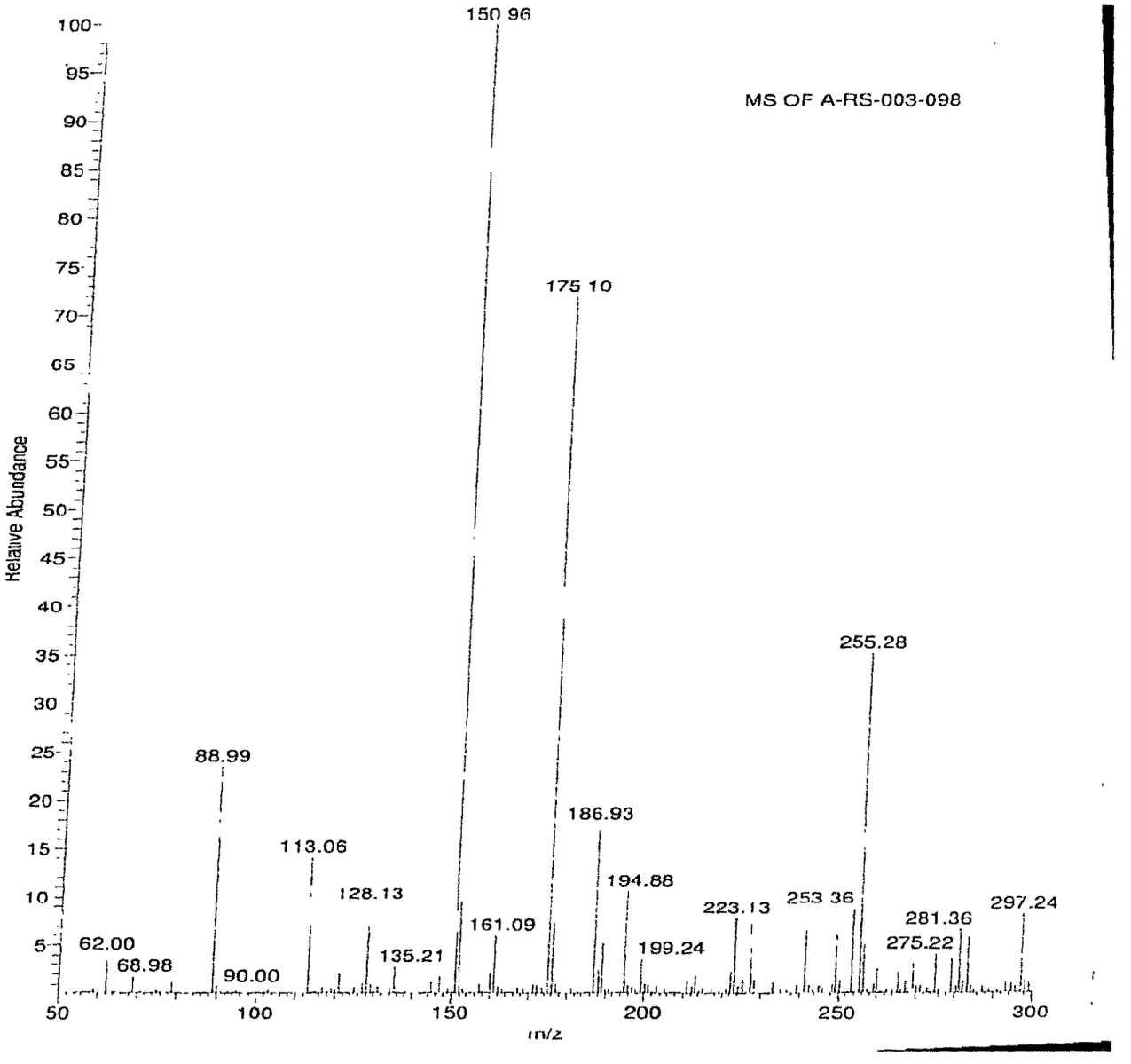
Peak Table

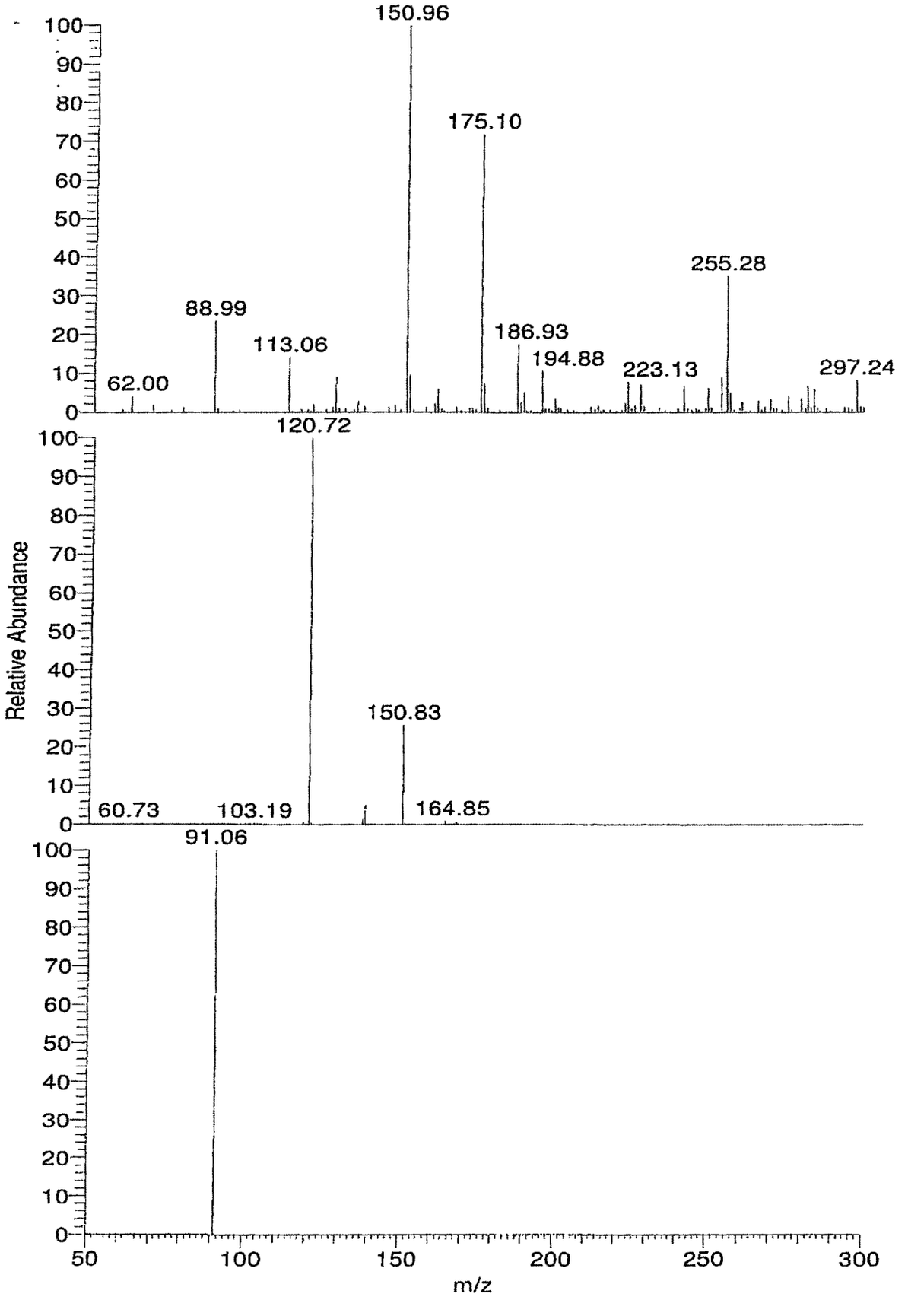
	Peak Name	RT	Area	% Area
1		1.70	2603	0.01
2	2-Phenyl Propane	2.79	23178960	99.96
3		4.33	1751	0.01
4		8.74	4565	0.02
5	Felbamate RCB	15.80		
Sum			23187880	100.00

Infrared Spectrum of 2-phenyl propane-1,3-diol

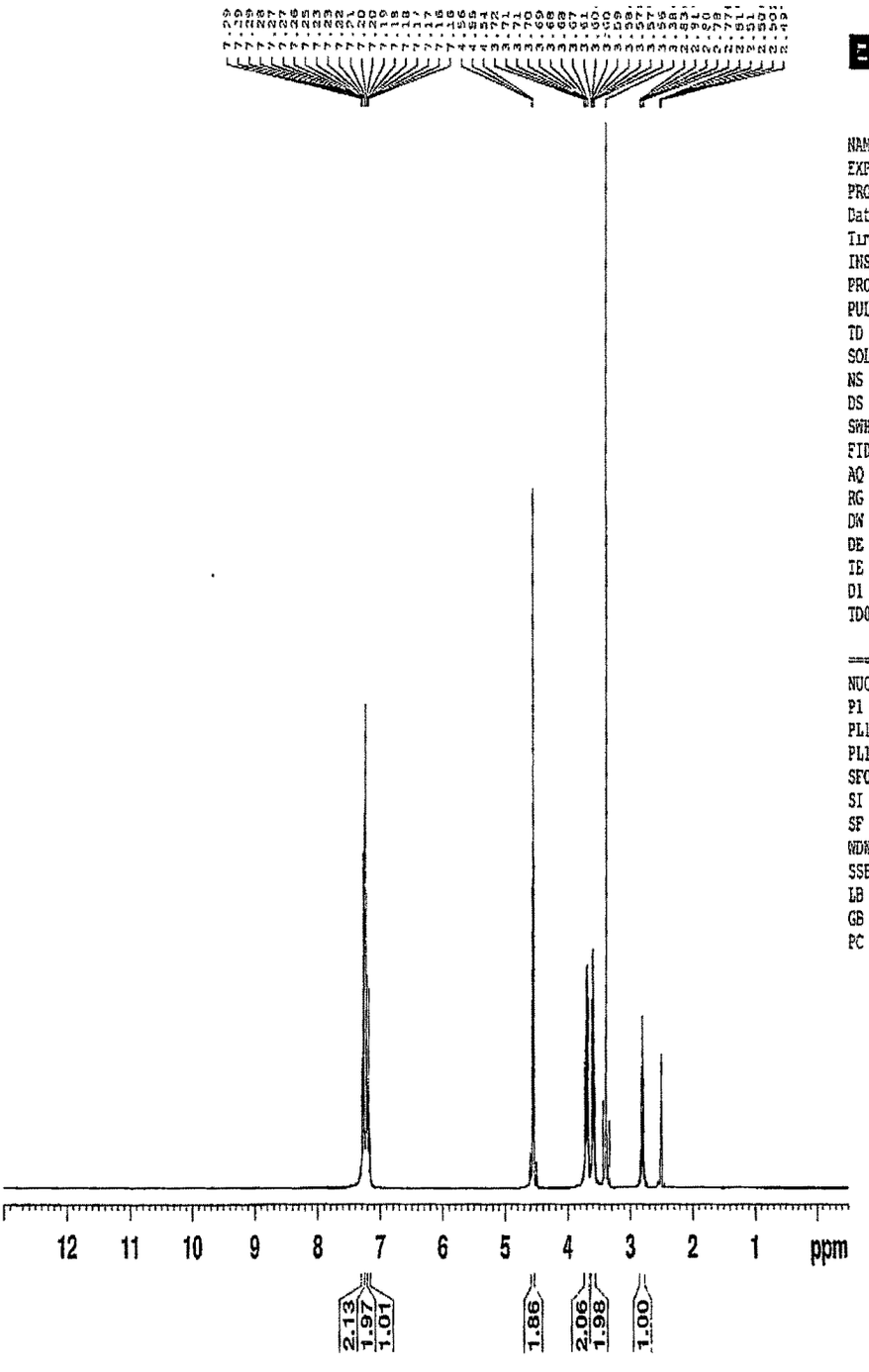


Mass Spectra of 2-phenyl propane-1,3-diol




150.96
175.10
255.28
88.99
186.93
113.06
194.88
223.13
297.24
62.00
120.72
150.83
60.73
103.19
164.85
91.06
Relative Abundance
m/z
100
90
80
70
60
50
40
30
20
10
0
50
100
150
200
250
300

Proton NMR Spectra of 2-phenyl propane-1,3-diol



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PROCNO	1
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NS	32
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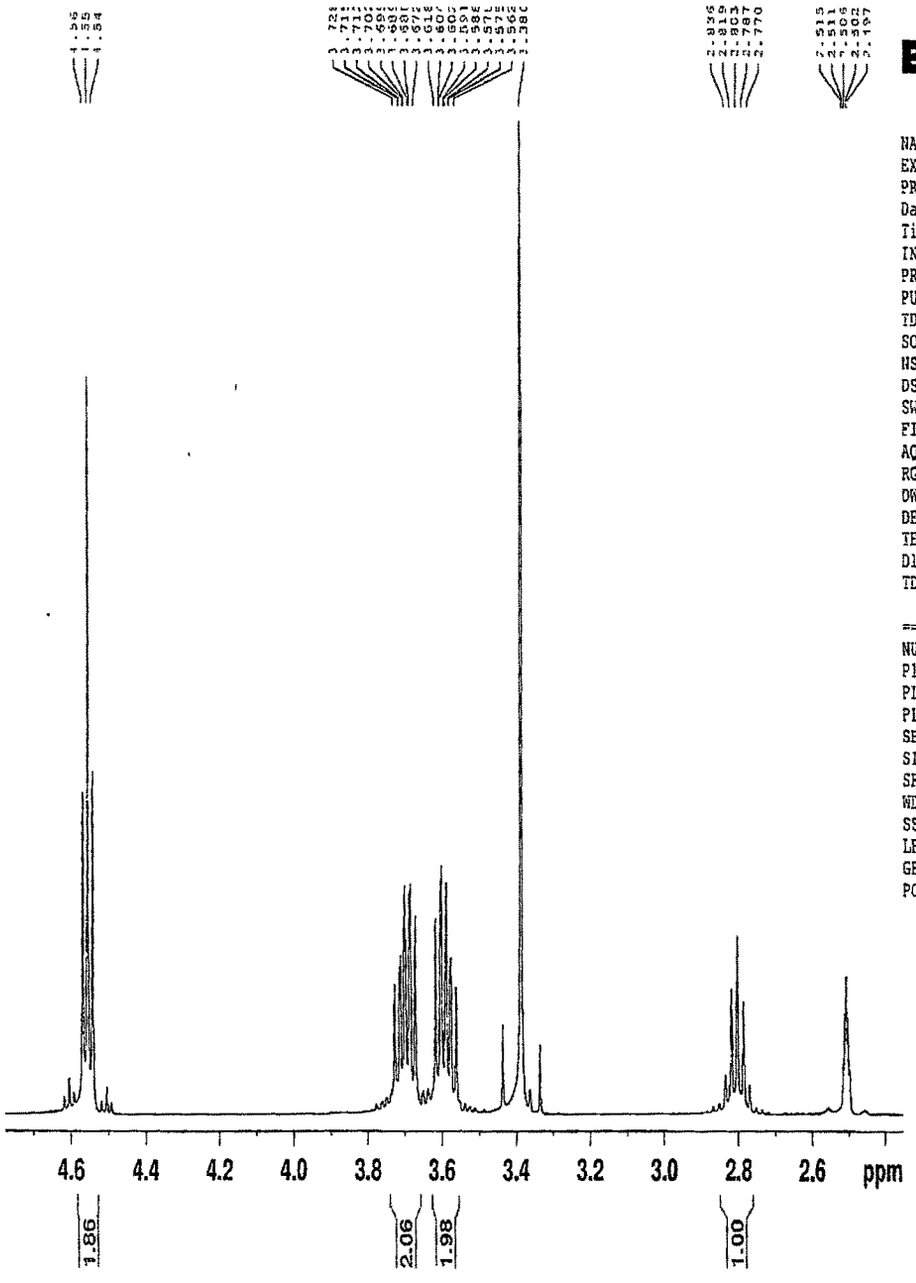
FIDRES	0.125483 Hz
AQ	3.9846387 sec
RG	57
DW	60.800 usec
DE	6.50 usec
TE	296.4 K
D1	1.00000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	1H
P1	11.20 usec
PL1	0.00 dB
PL1W	17.35713577 W
SFO1	400.1328009 MHz
SI	32768
SF	400.1300000 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00



BRUKER

NAME	060513 Apicore
EXPNO	1
PROCNO	1
Date_	20130508
Time	15.31
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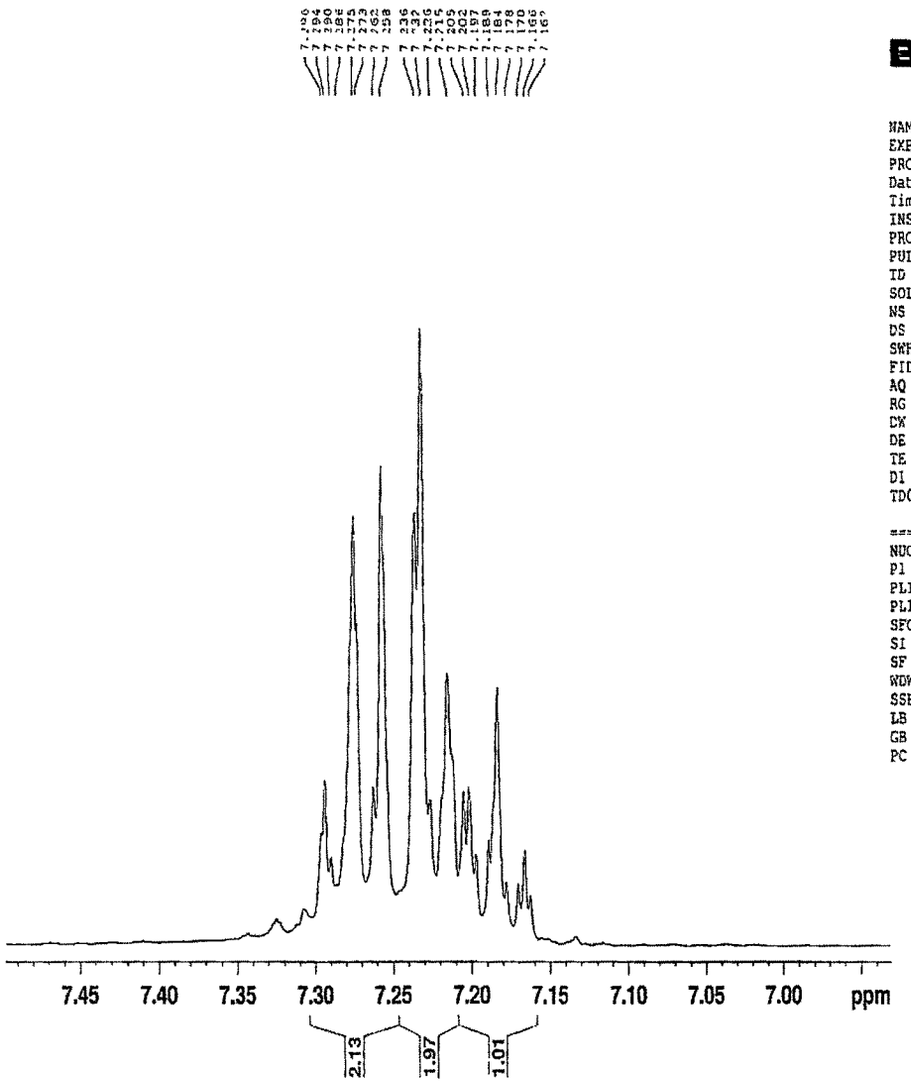
PULPROG	zg30
TD	65536
SOLVENT	DMSO
NS	32
DS	0
SWH	8223.685 Hz
FIDRES	0.125483 Hz
AQ	3.9846387 sec
RG	57
DW	60.800 usec
DE	6.50 usec
TE	296.4 K
D1	1.00000000 sec
TD0	1

======== CHANNEL f1 ========

NUC1	1H
P1	11.20 usec
PL1	0.00 dB
PL1W	17.35713577 W
SFO1	400.1328009 MHz
SI	32768
SF	400.1300000 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00



BRUKER

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PROCNO	1
Date_	20130508
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PULPROG	zg30
TD	65536
SOLVENT	DMSO
NS	32
DS	0
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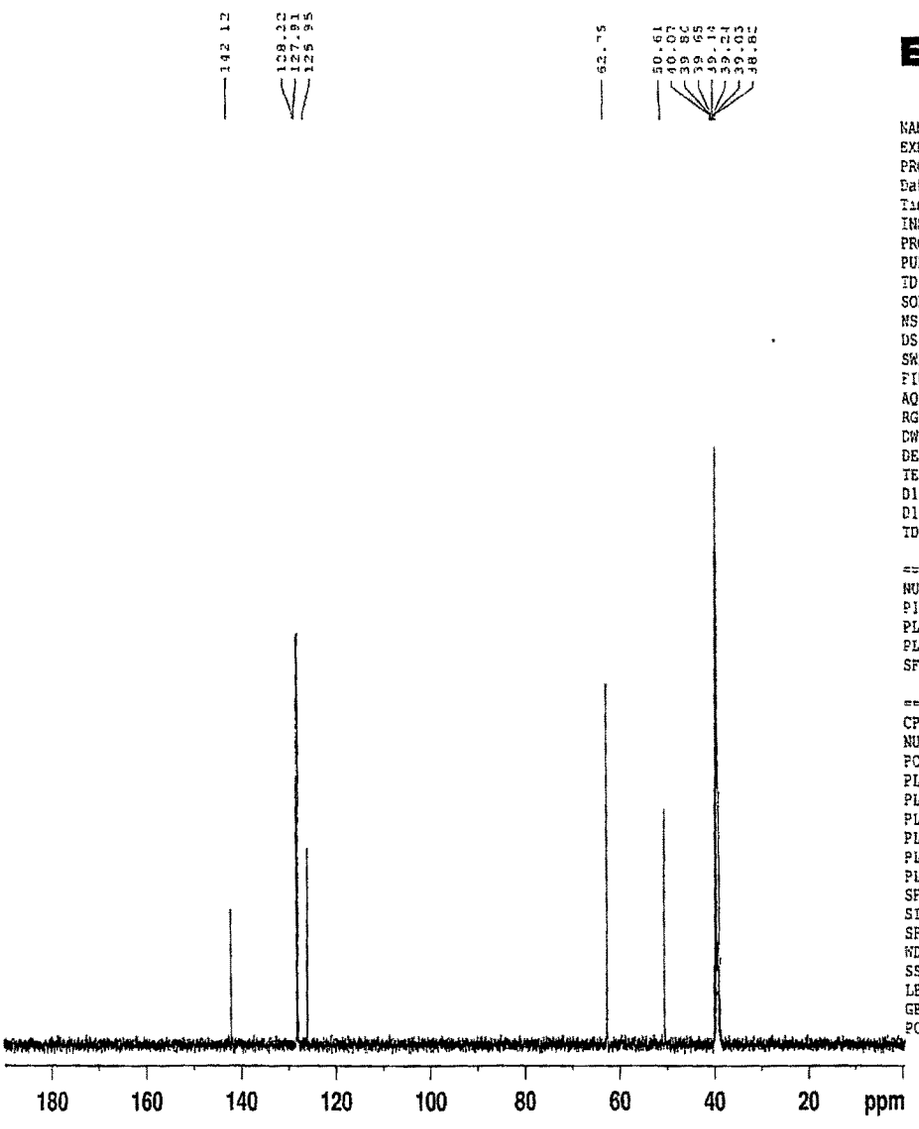
FIDRES	0.125483 Hz
AQ	3.9846357 sec
RG	57
DW	60.800 usec
DE	6.50 usec
TE	296.4 K
D1	1.00000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	1H
P1	11.20 usec
PL1	0.00 dB
PL1W	17.35713577 W
SFO1	400.1328009 MHz
SI	32768
SF	400.1300000 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

Carbon NMR Spectra of 2-phenyl propane-1,3-diol



BRUKER

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EXPNO	2
PROCNO	1
Date_	20130508
Time	15.35
INSTRUM	spect
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PULPROG	zgpg30
TD	65536
SOLVENT	DMSO
NS	50
DS	4
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FIDRES	0.397364 Hz
AQ	1.2583412 sec
RG	203
DW	19.200 usec
DE	6.50 usec
TE	297.1 K
D1	2.00000000 sec
D11	0.03000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	13C
P1	8.00 usec
PL1	1.60 dB
PL1W	63.25595093 W
SFO1	100.6228296 MHz
======== CHANNEL f2 ========	
CPDPRG2	waltz16
NUC2	1H
PCPD2	90.00 usec
PL2	0.00 dB
PL12	17.87 dB
PL13	20.87 dB
PL2W	17.35713577 W
PL12W	0.28345099 W
PL13W	0.14206201 W
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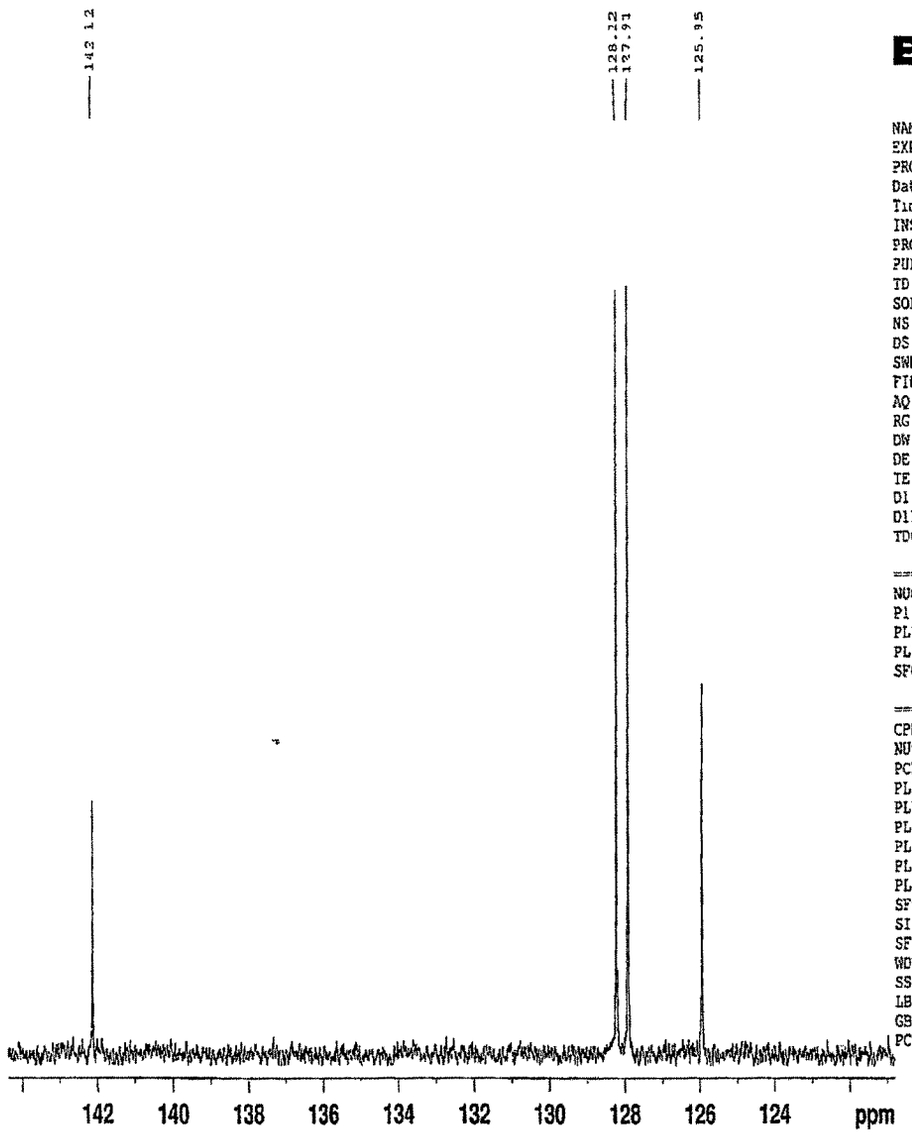
GB	0
PC	1.40



BRUKER

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EXPNO	2
PROCNO	1
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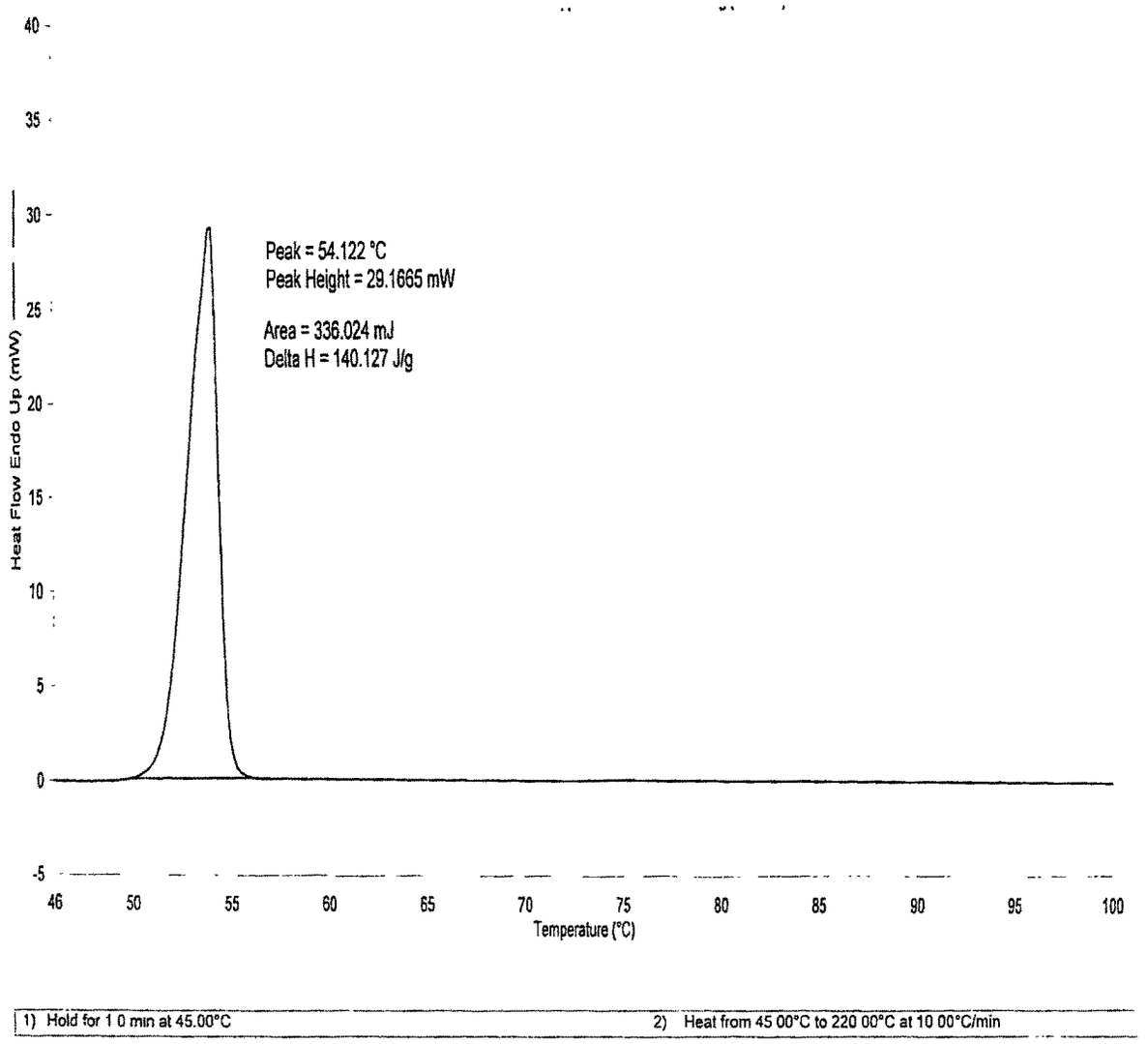
SWH	26041.666 Hz
FIDRES	0.397364 Hz
AQ	1.2583412 sec
RG	203
DW	19.200 usec
DE	6.50 usec
TE	297.1 K
D1	2.00000000 sec
D11	0.03000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	13C
P1	6.00 usec
PL1	1.60 dB
PL1W	63.25595093 W
SFO1	100.6228298 MHz
======== CHANNEL f2 ========	
CPDPRG2	waltz16
NUC2	1H
PCPD2	90.00 usec
PL2	0.00 dB
PL12	17.87 dB
PL13	20.87 dB
PL2W	17.35713577 W
PL12W	0.28345099 W
PL13W	0.14206201 W
SFO2	400.1316005 MHz
SI	32768
SF	100.6128193 MHz
WDW	EM
SSB	0
LB	1.00 Hz
GB	0
PC	1.40

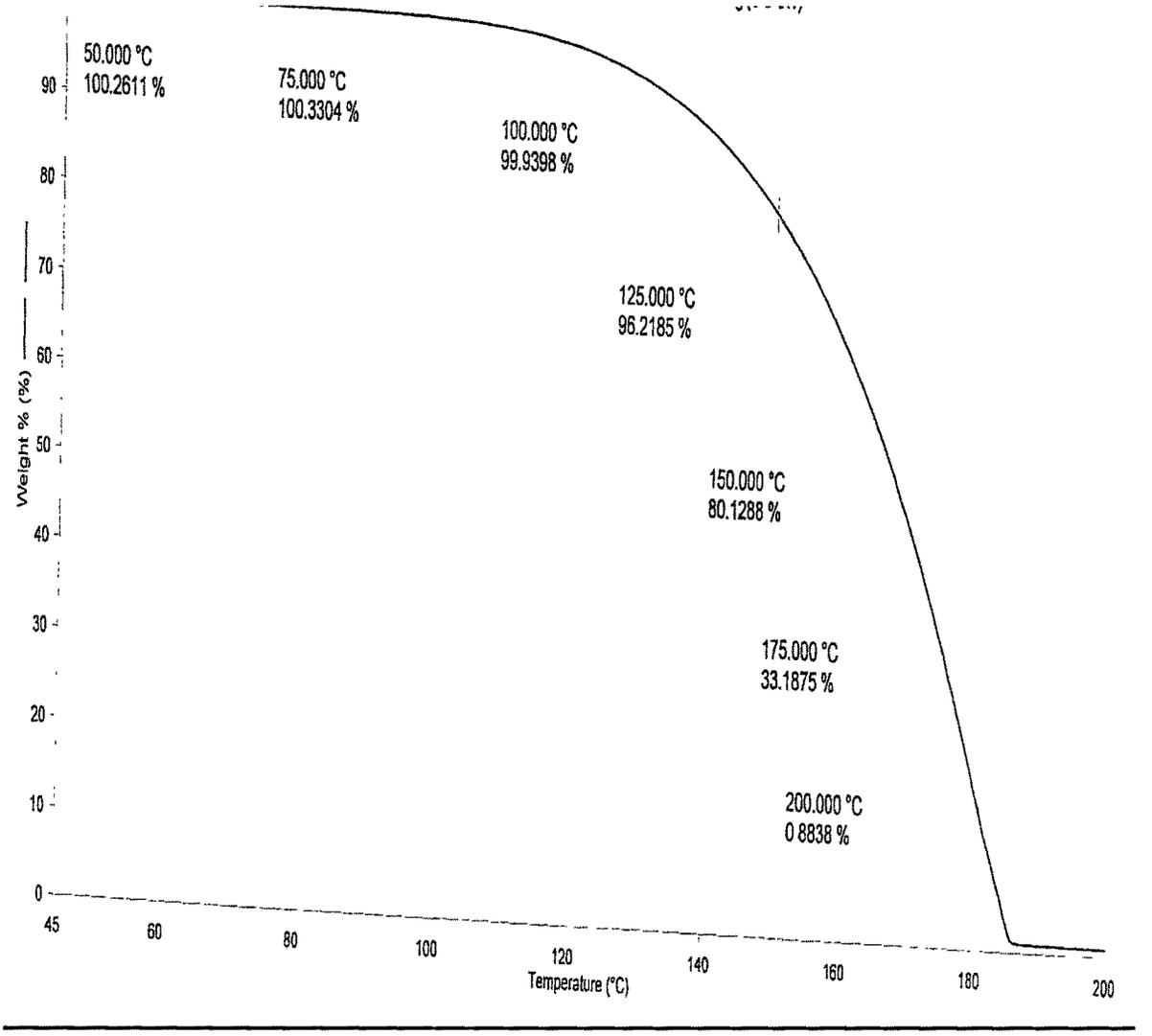
DSC report of 2-phenyl propane-1,3-diol



1) Hold for 1 0 min at 45.00°C

2) Heat from 45 00°C to 220 00°C at 10 00°C/min

TGA report of 2-phenyl propane-1,3-diol



PHYSICO-CHEMICAL CHARACTERIZATION OF FELBAMATE RELATED COMPOUND-A

The physico-chemical characterization of Felbamate Related Compound-A was established by analytical techniques such as HPLC, FT-IR, MASS, NMR, TGA and DSC analysis. The Chromatographic purity of Felbamate Related Compound-A Standard was determined by HPLC.

1.0 PHYSICAL PROPERTIES

1.1 APPEARANCE

TABLE-1 Appearance results for Felbamate Related Compound-A

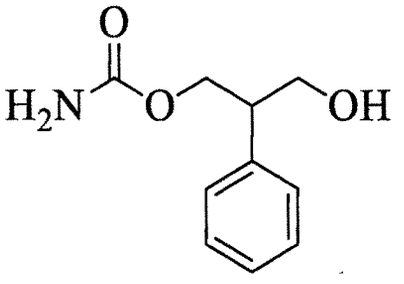
Name	Appearance
3-hydroxy-2-phenyl propyl carbamate	Off-white crystalline powder

2.0 CHEMICAL PROPERTIES:

2.1 Chemical Name: 3-hydroxy-2-phenyl propyl carbamate

2.2 Common Name: Felbamate Related Compound-A

2.3 Structural Formula:



2.4 Molecular Formula: $C_{10}H_{13}NO_3$

2.5 Molecular Weight: 195.22

3.0 CHROMATOGRAPHIC PURITY BY HPLC AND POTENCY :

The chromatographic purity of Felbamate Related Compound-A was determined by High performance liquid chromatograph (HPLC) using developed test procedure. The results are depicted in the **Table-2**. The chromatograms are depicted in **Exhibits**. The potency of Felbamate Related Compound-A was derived by subtracting all possible organic as well as inorganic impurities from 100.

TABLE-2 Results of Chromatographic purity by HPLC of Felbamate Related Compound-A

Name	Chromatographic purity by HPLC	Potency
3-hydroxy-2-phenyl propyl carbamate	98.39 %	97.66 %

4.0 CHARACTERIZATION BY INFRARED SPECTRUM

The Infrared spectra of Felbamate Related Compound-A was obtained by using FT-IR instrument and is depicted in **Exhibits below.**

TABLE-3 FT-IR Frequency table of Felbamate Related Compound A

Bond	Approx. Frequency (cm^{-1})	Intensity and range
$-NH_2$	3313.71, 3261.63	3300 to 3500 (Two bands)
-OH	3444.87	3550 -3200
-C=O	1693.5	1760 - 1670
$-CH_2-$	1408.04	1350 – 1470

Conclusion: The IR frequencies are in-line with the functional groups.

5.0 CHARACTERIZATION BY MASS SPECTROMETRY

The +ve Mass spectrum of Felbamate Related Compound A was performed for the parent ion scans using infusion-MS and infusion-MS/MS techniques on Quattro-LC mass spectrometer (Micromass-LC/MS/MS system). The result is depicted in **Table-4**. The mass spectrum is depicted in **Exhibits below.**

TABLE-4 Molecular ion data for Felbamate Related Compound-A by infusion-MS Technique

Name	m/z value + Na	m/z value	Molecular weight of Felbamate Related Compound A
3-hydroxy-2-phenyl propyl carbamate	218.13	196.10	195.22

Conclusion: Confirms the Mass of Felbamate Related Compound

6.0 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER (BRUKER 400 MHz, NMR SYSTEM)

The NMR of Felbamate Related Compound-A was performed by 400 MHz Bruker Nuclear Magnetic Resonance spectrometer by preparing sample in $CDCl_3$. The ^{1}H NMR & ^{13}C NMR spectra are depicted in **Exhibits** below and the interpretation is provided in **Table-5** and **Table-6**.

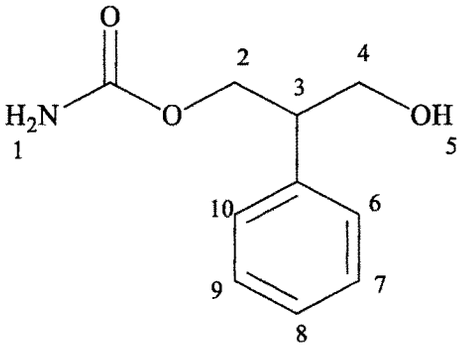


TABLE-5 Proton (^{1}H) NMR

Position	**1H**	3-hydroxy-2-phenyl propyl carbamate	
		δ (ppm)	**Multiplicity**
1	2H	6.47 (Broad peak)	Singlet
2	2H	4.159- 4.322	Doublet of doublet
3	1H	3.017 – 3.083	Multiplet
4	2H	3.633 – 3.673	Multiplet
5	1H	4.777 - 4.803	Triplet
6,7,8,9 &10	5H	7.249 – 7.352	Multiplet

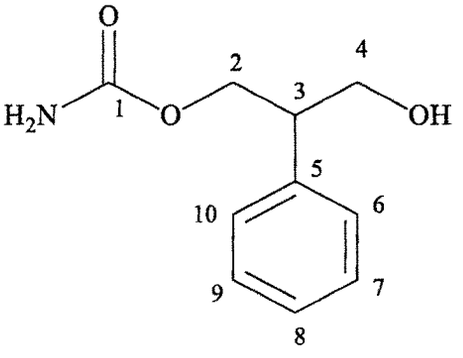


TABLE-6 Carbon (^{13}C) NMR

Position	δ (ppm)
1	156.76
2	62.63
3	47.41
4	64.52
5	140.79
6	128.12
7	126.38
8	126.38
9	126.38
10	128.12

Conclusion: Proton and carbon NMR confirms the structure of Felbamate Related Compound A.

7.0 CHARACTERIZATION BY DIFFERENTIAL SCANNING CALORIMETER [DSC]

The DSC of Felbamate Related Compound-A was performed and the DSC data presented in **Exhibits below** has the melting range 72.085°C. The observed peak is sharp and compound melts completely at the melting range of Felbamate Related Compound-A. Partial melting of the crystals was not observed before melting temperature is attained.

8.0 CHARACTERIZATION BY THERMO GRAVIMETRIC ANALYSIS [TGA]45

The TGA of Felbamate Related Compound was performed and the TGA data presented in **Exhibits below** depicts only residual moisture present in the sample. There is no hydrate form observed as there is no bounded moisture found in the sample. Also the Felbamate Related Compound-A sample does not exist as any solvate either. The compound does not lose any weight more than approximately 0.22% to 0.85% until the melting range temperature is attained.

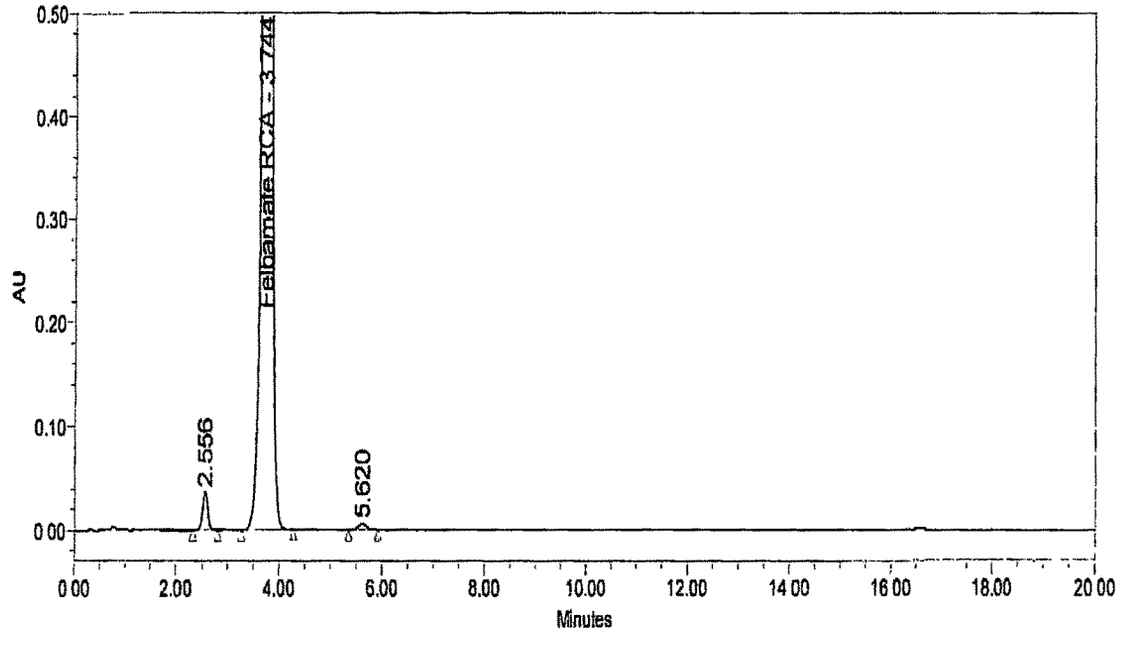
9.0 STORAGE CONDITION

Store in a well closed container at room temperature.

EXHIBITS

HPLC chromatogram of Felbamate Related Compound-A

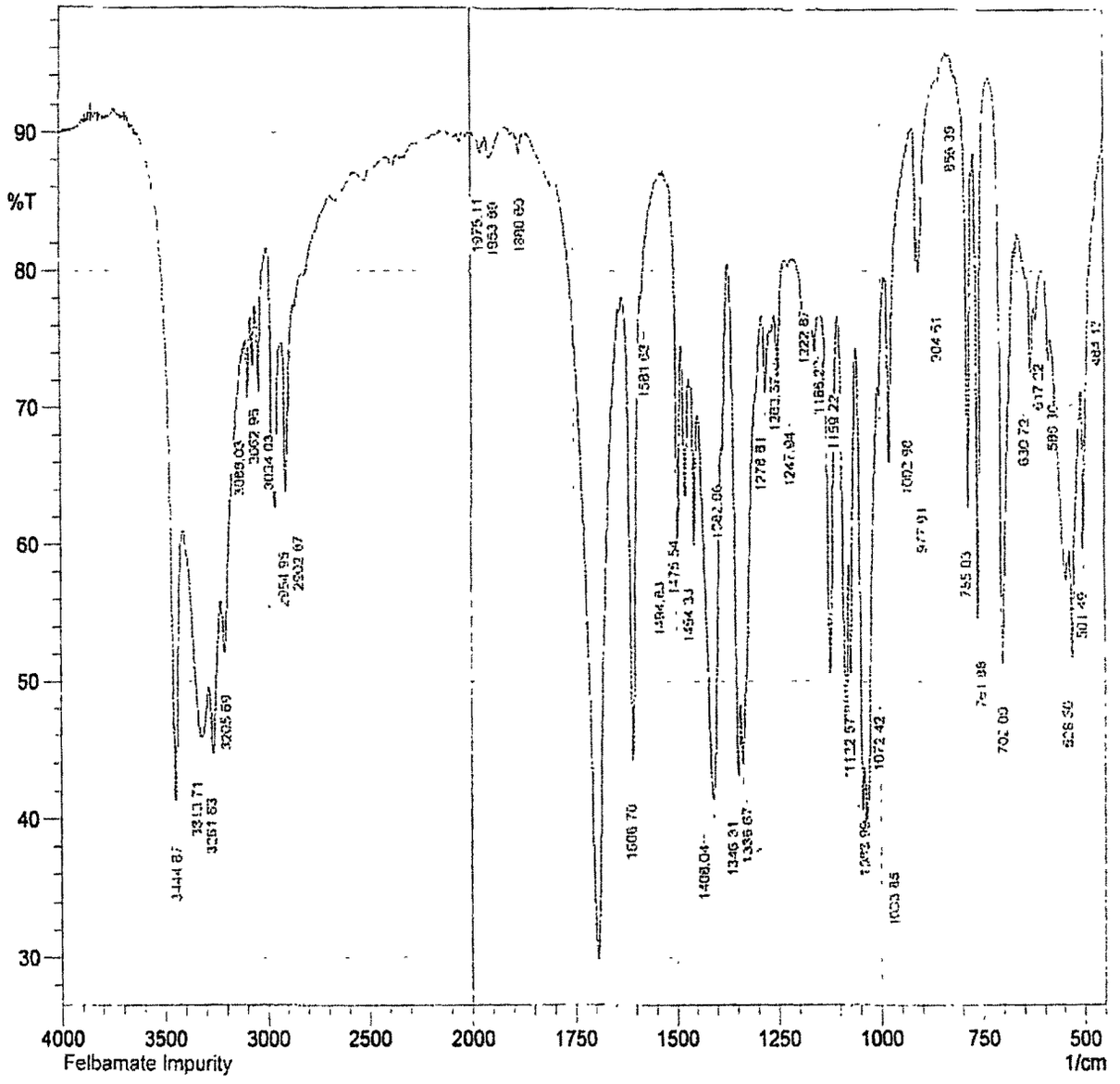
Chromatogram



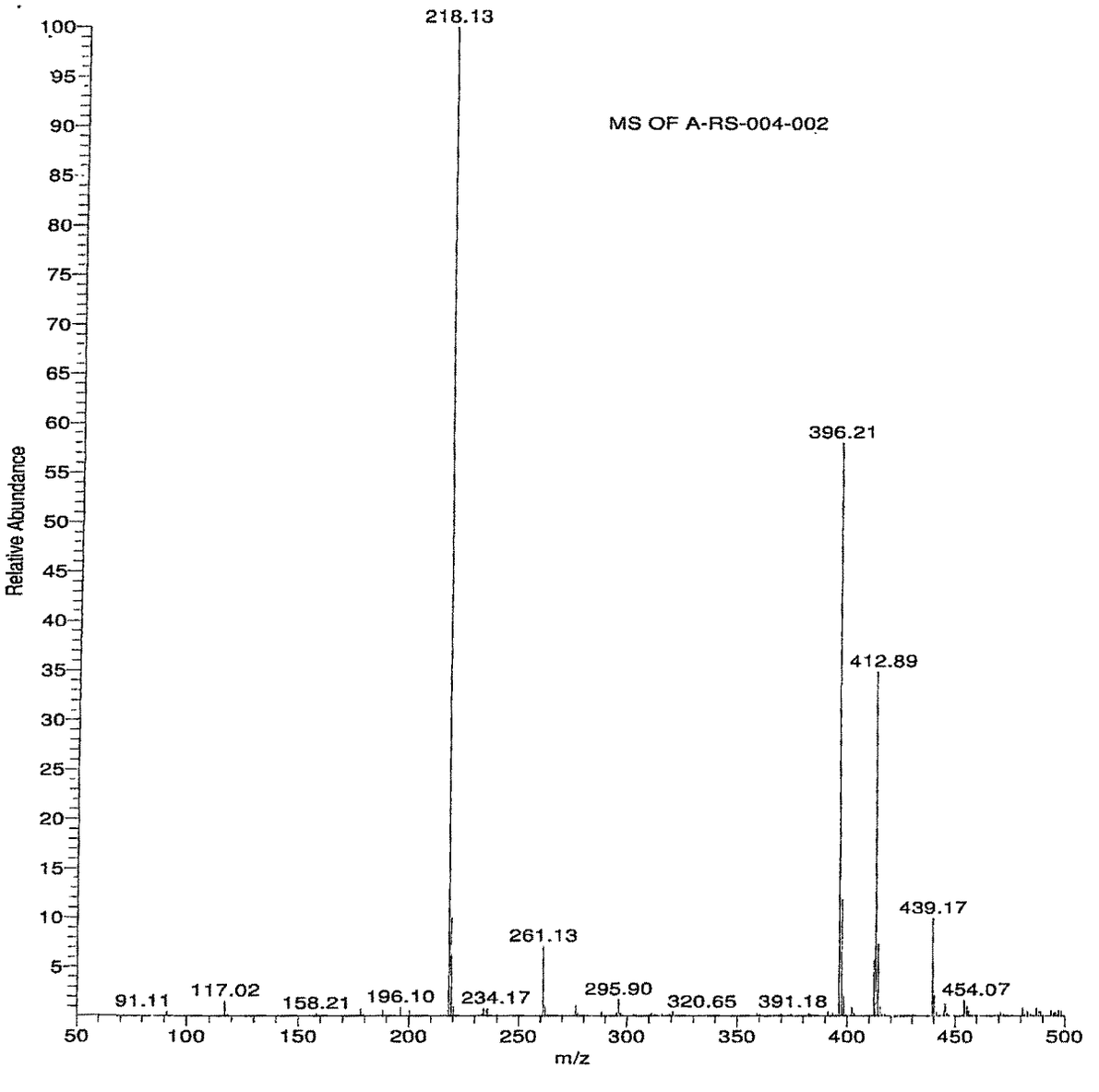
Peak Table

	Peak Name	RT	Area	% Area
1		2.56	257411	1.28
2	Felbamate RCA	3.74	19742144	98.39
3		5.62	66562	0.33
Sum			20066117	100.00

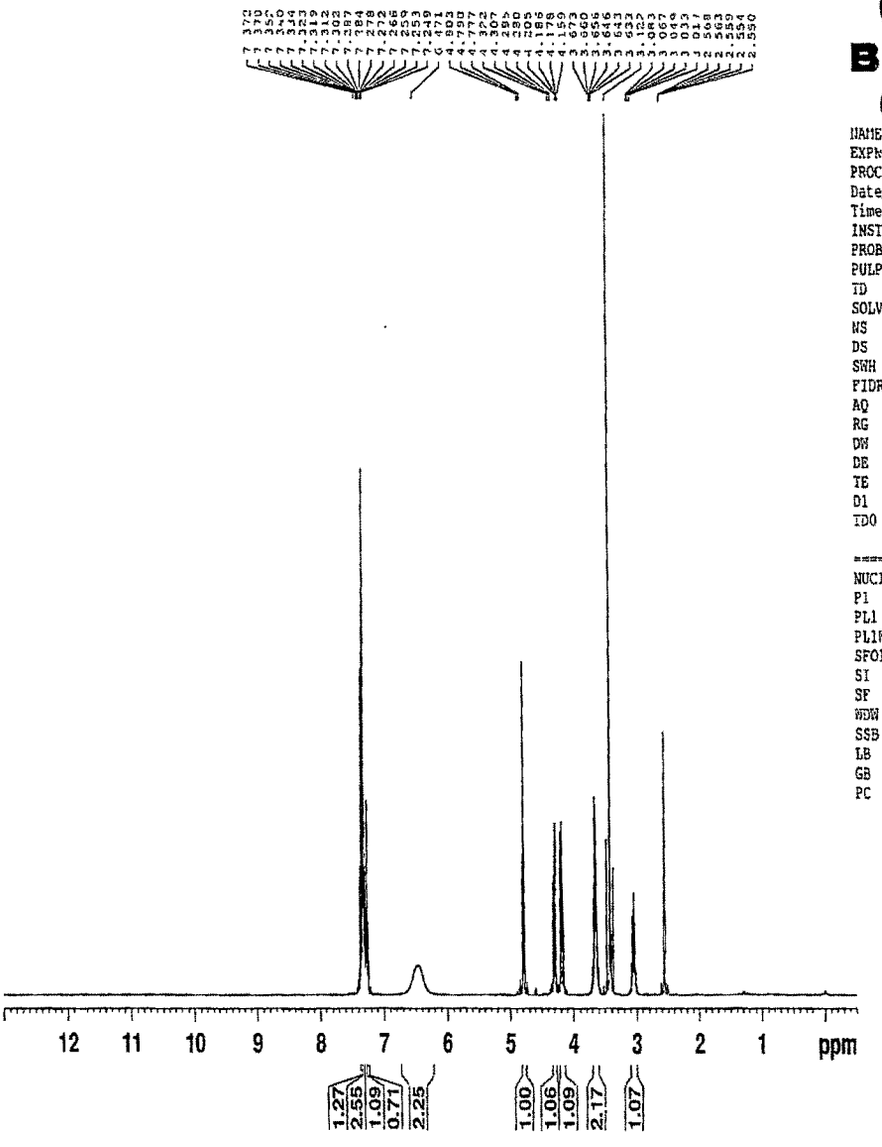
Infrared Spectrum of Felbamate Related Compound-A



Mass Spectra of Felbamate Related Compound-A



Proton NMR Spectra of Felbamate Related Compound-A



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NS	32
DS	0
SWH	8223.685 Hz
FIDRES	0.125483 Hz
AQ	3.9846387 sec
RG	114
DW	60.800 usec
DE	6.50 usec
TE	296.3 K
D1	1.00000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	1H
P1	11.20 usec
PL1	0.00 dB
PL1W	17.35713577 W
SFO1	400.1328009 MHz
SI	32768
SF	400.1299791 MHz
WDW	EM
SSB	0
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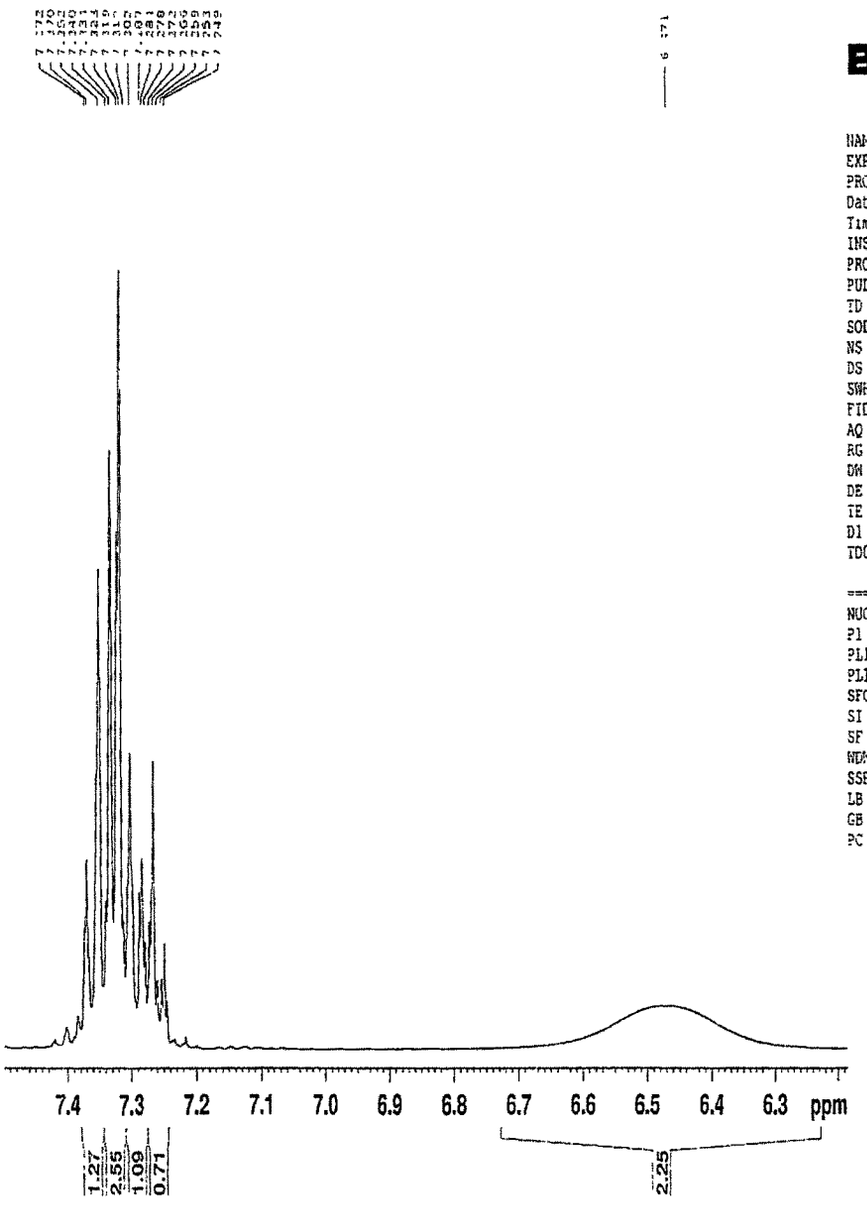
GB	0
PC	1.00

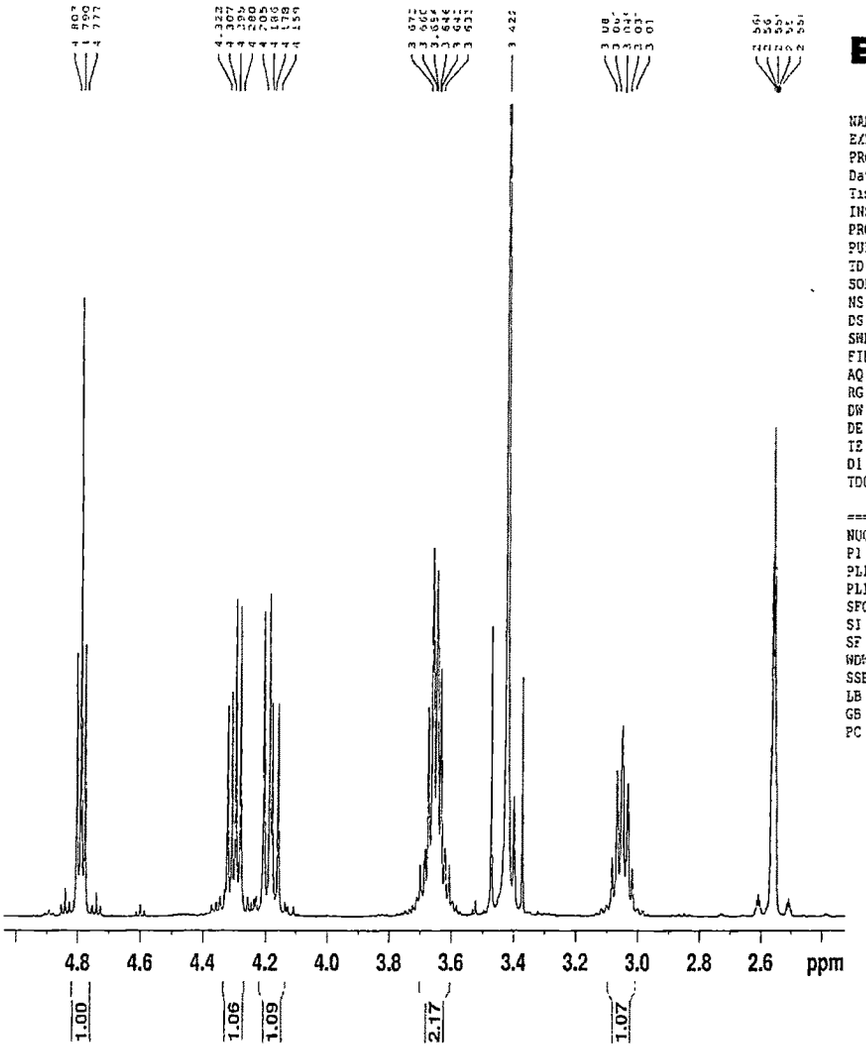


BRUKER

NAME	060513 Apicore
EXPNO	5
PROCNO	1
Date_	20130508
Time	15.55
INSTRUM	spect
PROBHD	5 mm PABBO BB-
PULPROG	zg30
TD	65536
SOLVENT	DMSO
NS	32
DS	0
SWH	8223.685 Hz
FIDRES	0.125483 Hz
AQ	3.9846387 sec
RG	114
DW	60.800 usec
DE	6.50 usec
TE	296.3 k
D1	1.00000000 sec
TD0	1

======== CHANNEL f1 ========

NUC1	1H
P1	11.20 usec
PL1	0.00 dB
PL1W	17.35713577 W
SFO1	400.1328009 MHz
SI	32768
SF	400.1299791 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00



BRUKER

NAME	060513 Apicore
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PROCNO	1
Date_	20130308
Time	15 55
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PROBHD	5 mm PABBO BB-
PULPROG	zg30
TD	65536
SOLVENT	DMSO
NS	32
DS	0
SWH	8223 685 Hz
FIDRES	0.125483 Hz
AQ	3.9846387 sec
RG	114
DW	60.800 usec
DE	6 50 usec
TE	295 3 K
D1	1.00000000 sec
TD0	1

======== CHANNEL f1 ========

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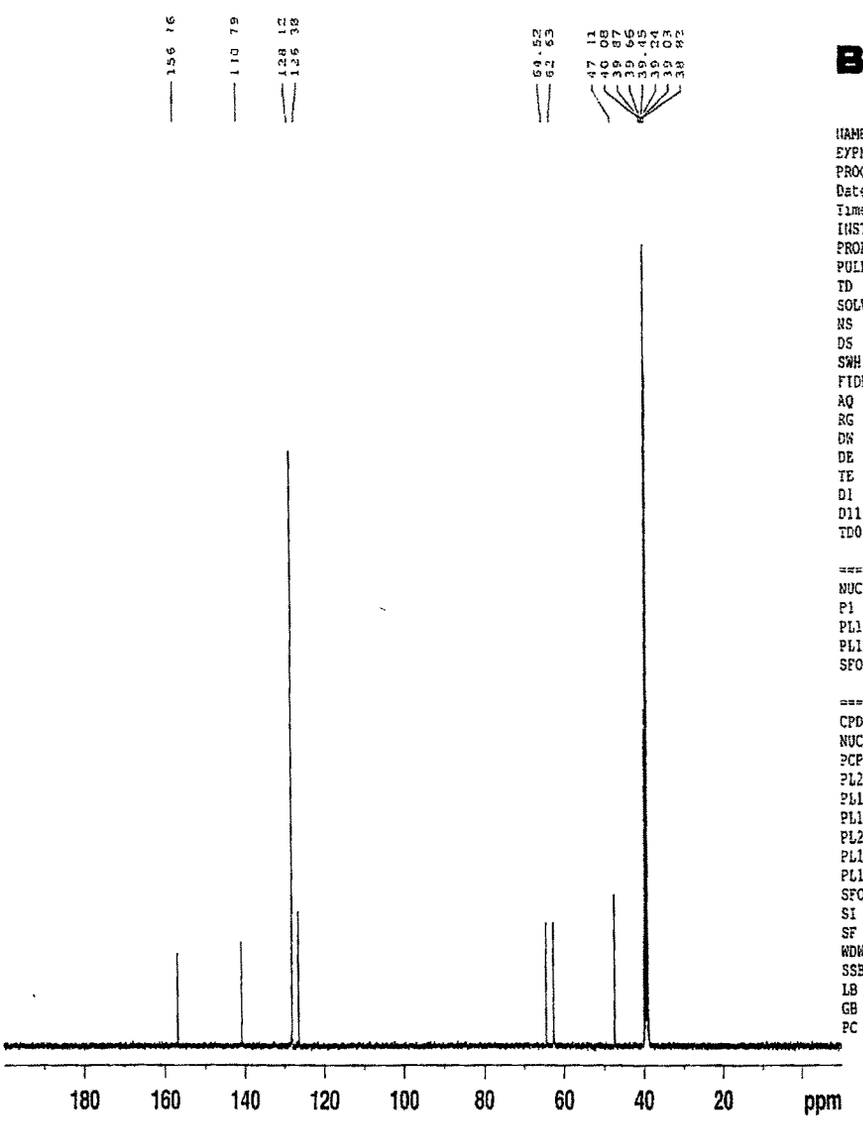
PL1	0 00 dB
PL1W	17.35713577 W
SFO1	400 1328009 MHz
SI	32768
SF	400 1299791 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

Carbon NMR Spectra of Felbamate Related Compound-A



BRUKER

NAME	060513 Apicore
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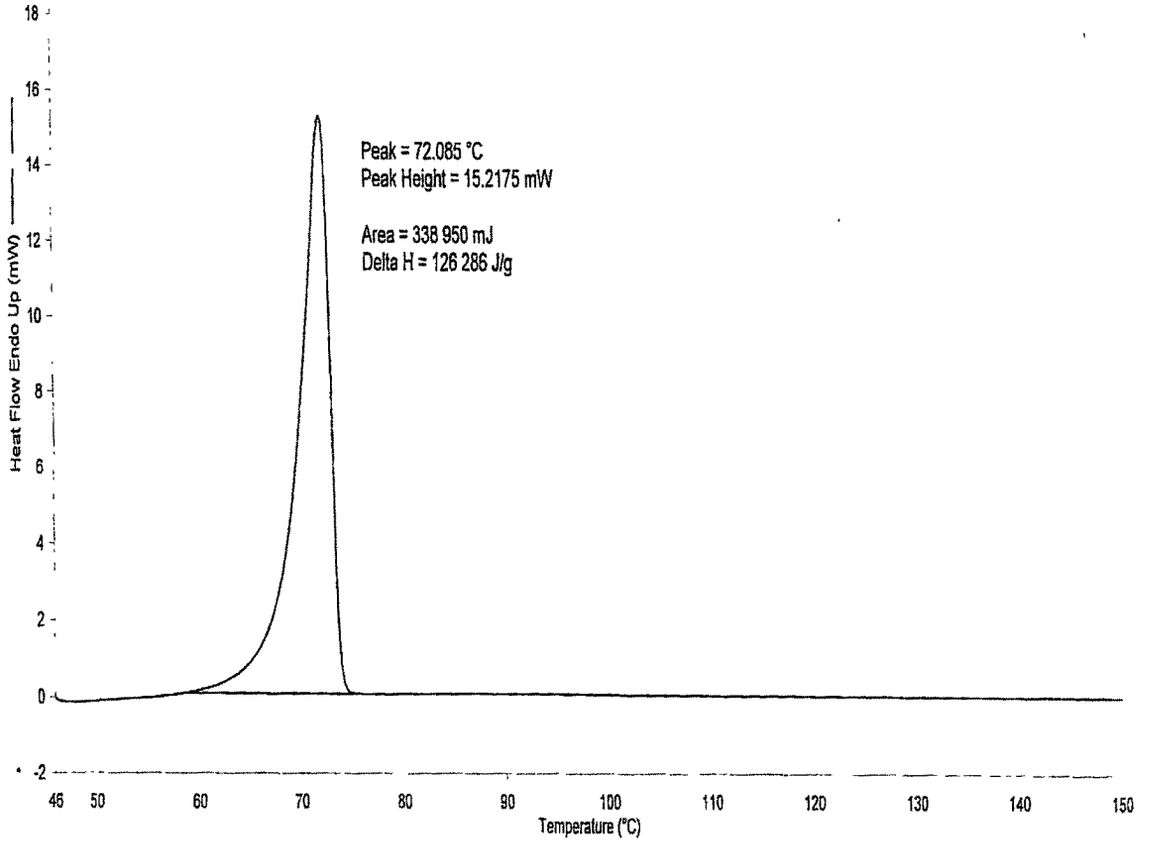
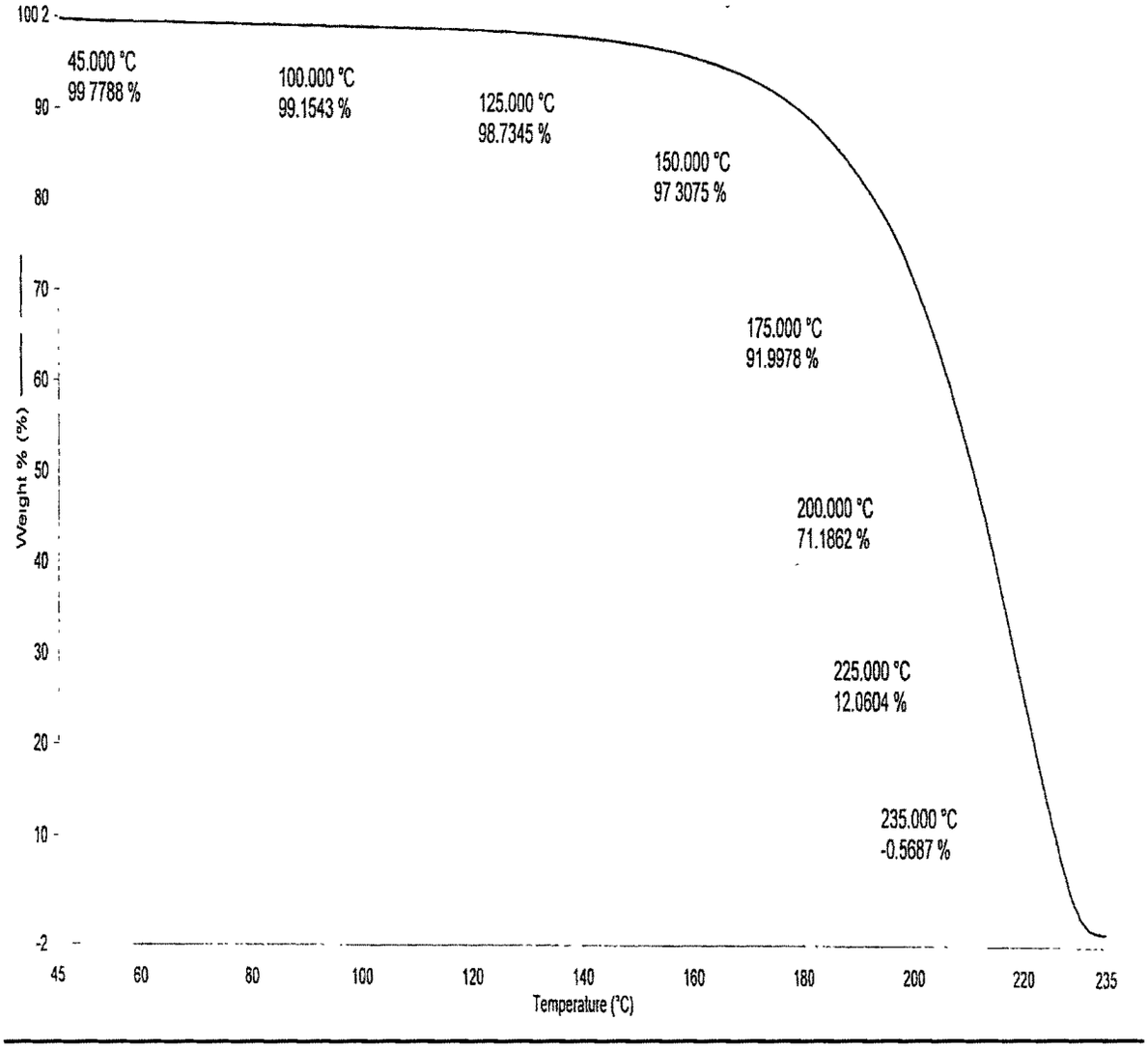
TD	65536
SOLVENT	DMSO
NS	464
DS	4
SWH	26041.666 Hz
FIDRES	0.397364 Hz
AQ	1.2583412 sec
RG	203
DW	19.200 usec
DE	6.50 usec
TE	297.2 K
D1	2.00000000 sec
D11	0.03000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	13C
P1	6.00 usec
PL1	1.60 dB
PL1W	63.25595093 W
SFO1	100.6228298 MHz
======== CHANNEL f2 ========	
CPDPRG2	waltz16
NUC2	1H
PCPD2	90.00 usec
PL2	0.00 dB
PL12	17.87 dB
PL13	20.87 dB
PL2W	17.35713577 W
PL12W	0.28345099 W
PL13W	0.14206201 W
SFO2	400.1316005 MHz
SI	32768
SF	100.6126193 MHz
WDW	EM
SSB	0
LB	1.00 Hz
GB	0
PC	1.40

DSC report of Felbamate Related Compound-A



1) Hold for 1.0 min at 45.00°C	2) Heat from 45.00°C to 200.00°C at 10.00°C/min

TGA report of Felbamate Related Compound-A



1). Heat from 45.00°C to 250.00°C at 10.00°C/min

PHYSICO-CHEMICAL CHARACTERIZATION OF FELBAMATE RELATED COMPOUND-B

The Physico-chemical characterization of Felbamate Related Compound-B was established by analytical techniques such as HPLC, FT-IR, MASS, NMR, TGA and DSC analysis. The Chromatographic purity of Felbamate Related Compound-B Standard was determined by HPLC.

1.0 PHYSICAL PROPERTIES

1.1 APPEARANCE

TABLE-1 Appearance results for Felbamate Related Compound-B

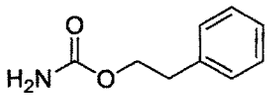
Name	Appearance
Phenethyl Carbamate	White powder

2.0 CHEMICAL PROPERTIES

2.1 Chemical Name: Phenethyl carbamate

2.2 Common Name: Felbamate Related Compound-B

2.3 Structural Formula:



phenethyl carbamate

2.4 Molecular Formula: $C_9H_{11}NO_2$

2.5 Molecular Weight: 165.19

3.0 CHROMATOGRAPHIC PURITY BY HPLC:

The chromatographic purity of Felbamate Related Compound-B was determined by High performance liquid chromatograph (HPLC) using developed test procedure. The results are depicted in the Table. The chromatograms are depicted in **Exhibits below**. The potency of Felbamate Related Compound-A was derived by subtracting all possible organic as well as inorganic impurities from 100.

TABLE-2 Results of Chromatographic purity by HPLC of Felbamate Related Compound-B

Name	Chromatographic purity by HPLC	Potency
Phenethyl carbamate	99.02%	98.68 %

4.0 CHARACTERIZATION BY INFRARED SPECTRUM

The Infrared spectra Felbamate Related Compound-B was obtained by using FT-IR instrument and is depicted in **Exhibits below.**

TABLE-3 FT-IR Frequency table of Felbamate Related Compound-B

Bond	Approx. Frequency (cm^{-1})	Intensity and range
-C=O	1680	1760 - 1670
$-NH_2$	3331.07, 3427.51	3450 - 3200
$-CH_2-$	1408.04	1350 - 1470

Conclusion: The IR frequencies are in-line with the functional groups.

5.0 CHARACTERIZATION BY MASS SPECTROMETRY

The –ve Mass spectrum of Felbamate Related Compound-B was performed for the parent ion scans using infusion-MS and infusion-MS/MS techniques on Quattro-LC mass spectrometer (Micromass-LC/MS/MS system). The result is depicted in Table. The mass spectrum is depicted in **Exhibits below.**

TABLE-4 Molecular ion data for Felbamate Related Compound-B by infusion-MS Technique

Name	m/z value + Na	Molecular weight of Felbamate Related Compound-B
Phenethyl carbamate (Felbamate Related compound B)	188.03	165.19

Conclusion: Confirms the Mass of Felbamate Related Compound-B

6.0 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER (BRUKER 400 MHz, NMR SYSTEM)

The NMR of Felbamate Related Compound-B was performed by 400 MHz Bruker Nuclear magnetic resonance spectrometer by preparing sample in DMSO. The ^{1}H NMR & ^{13}C NMR spectra are depicted in **Exhibits** and the interpretation is provided in below Tables.

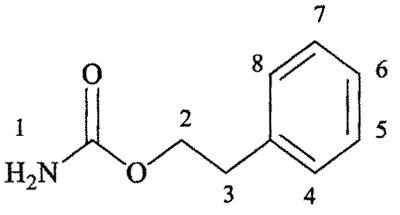


TABLE-5 Proton (^{1}H) NMR

Position	1H	Phenethyl Carbamate	
		δ (ppm)	Multiplicity
1	2H	6.475 – 6.538	singlet
2	1H	2.830 – 2.864	triplet
3	1H	4.091 – 4.125	triplet
4,5,6,7,8	5H	7.195 – 7.319	multiplet

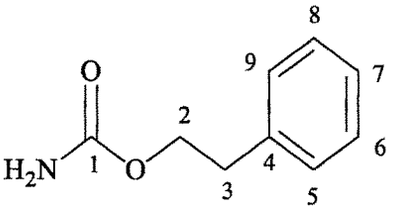


TABLE-6 Carbon (^{13}C) NMR

Position	δ (ppm)
1	156.660
2	63.907
3	34.077
4	138.276
5 , 9	128.79
6 , 8	128.37
7	126.29

Conclusion: Proton and carbon NMR confirms the structure of felbamate related compound-B

7.0 CHARACTERIZATION BY DIFFERENTIAL SCANNING CALORIMETER [DSC]

The DSC of Felbamate Related Compound-B was performed and the DSC data of batch, A-RS-004-008 presented in **Exhibits below** has the melting point 92.16°C. The observed peak is sharp and compound melts completely at the melting point of Felbamate Related Compound-B. Partial melting of the crystals was observed before melting temperature is attained.

8.0 CHARACTERIZATION BY THERMO GRAVIMETRIC ANALYSIS [TGA]

The TGA of Felbamate Related Compound-B was performed and the TGA data presented in **Exhibits** depicts only residual moisture present in the sample. There is no hydrate form observed as there is no bounded moisture found in the sample. Also, the Felbamate Related Compound-B sample does not exist as any solvate either. The compound does not lose any weight more than approximately 0.11 to 0.98% until the melting range temperature is attained.

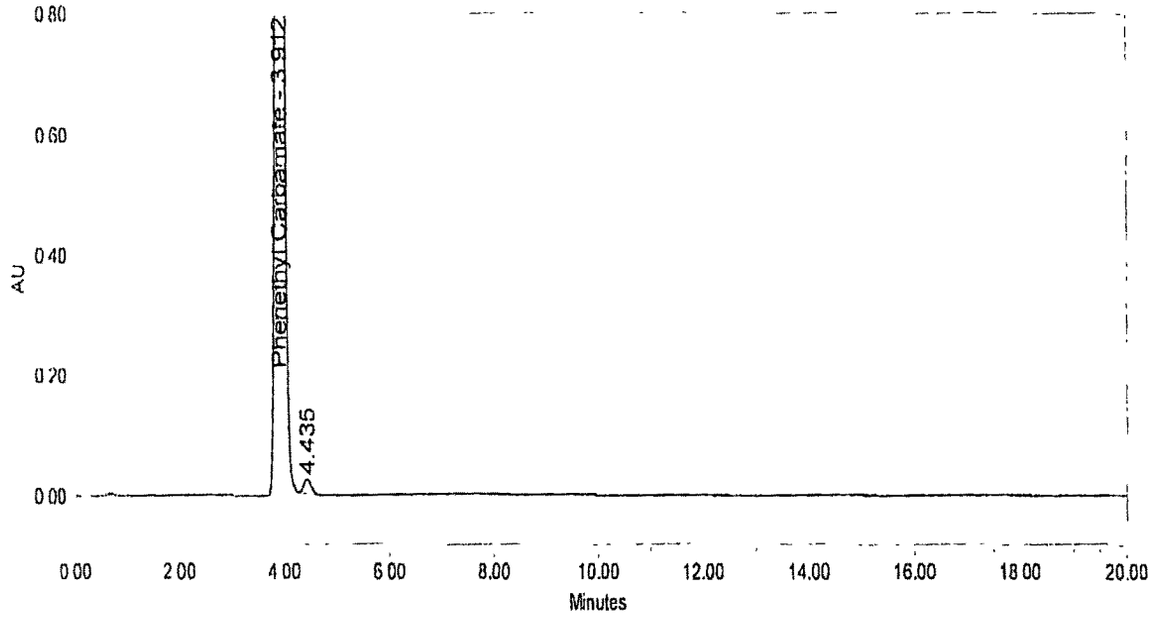
9.0 STORAGE CONDITION

Store in a well closed container at room temperature.

EXHIBITS

HPLC chromatogram of Felbamate Related Compound-B

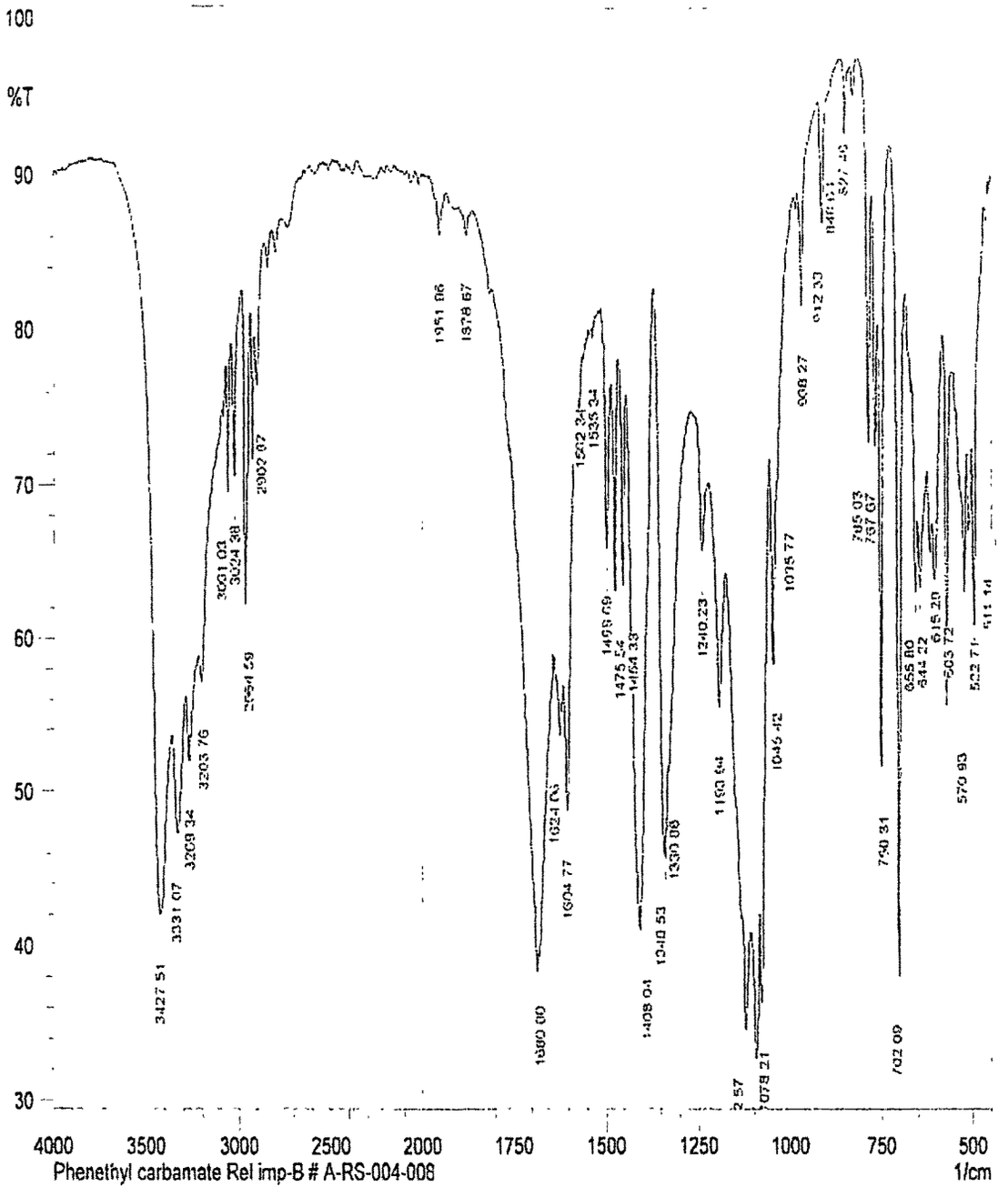
Chromatogram



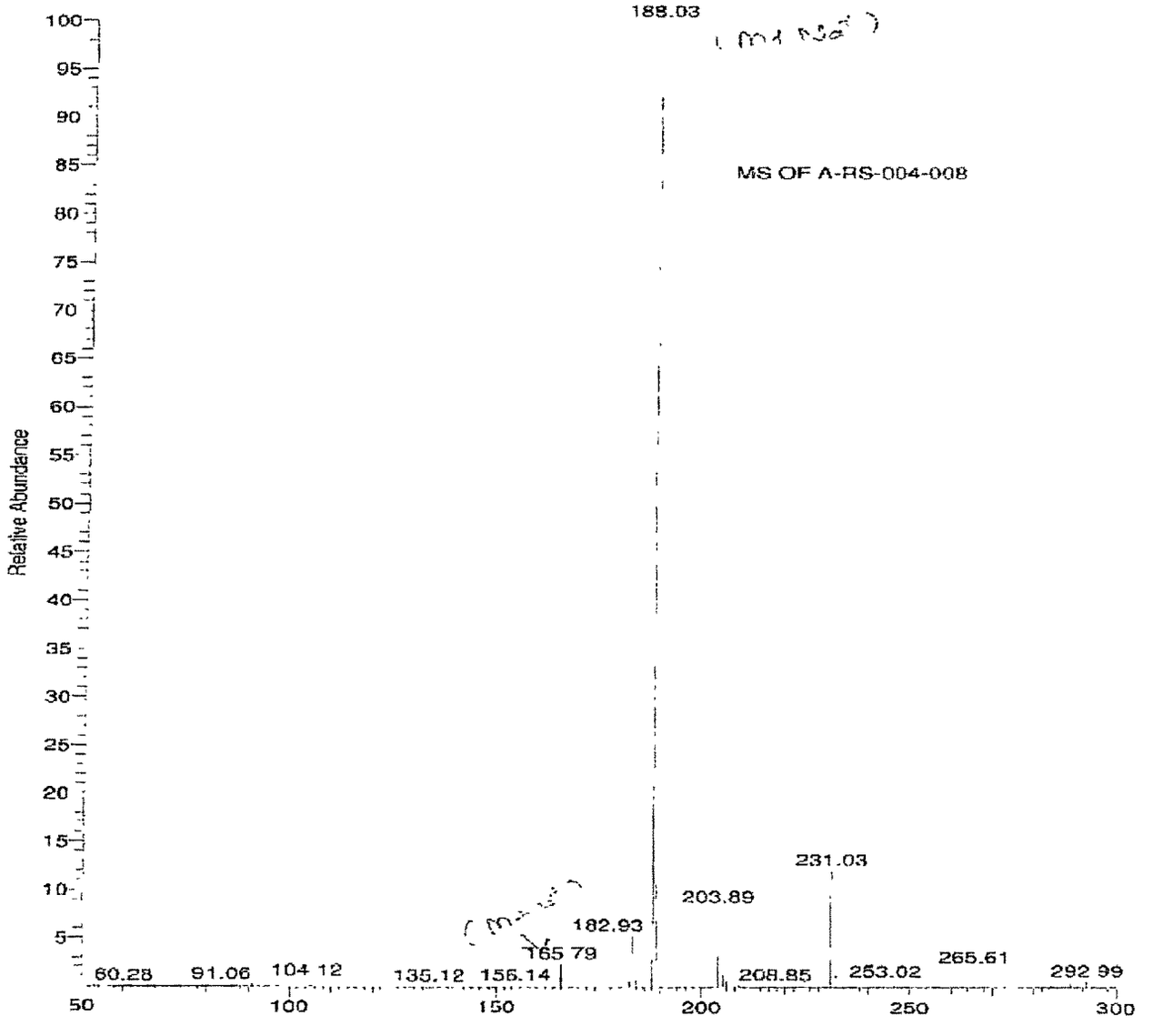
Peak Table

	Peak Name	RT	Area	% Area
1	Phenethyl Carbamate	3.91	21602936	99.02
2		4.44	214306	0.98
Sum			21817242	100.00

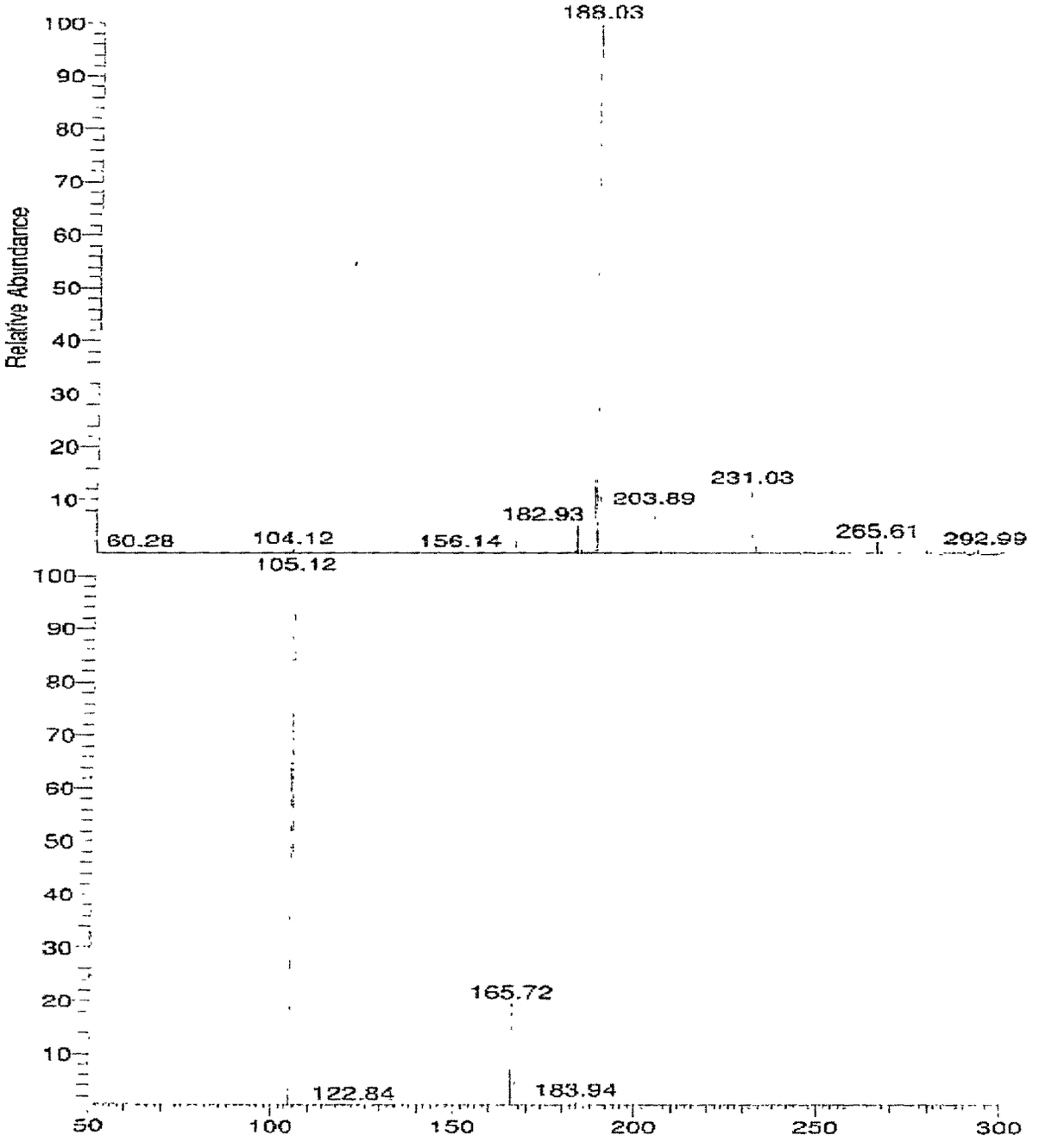
Infrared Spectrum of Felbamate Related Compound-B



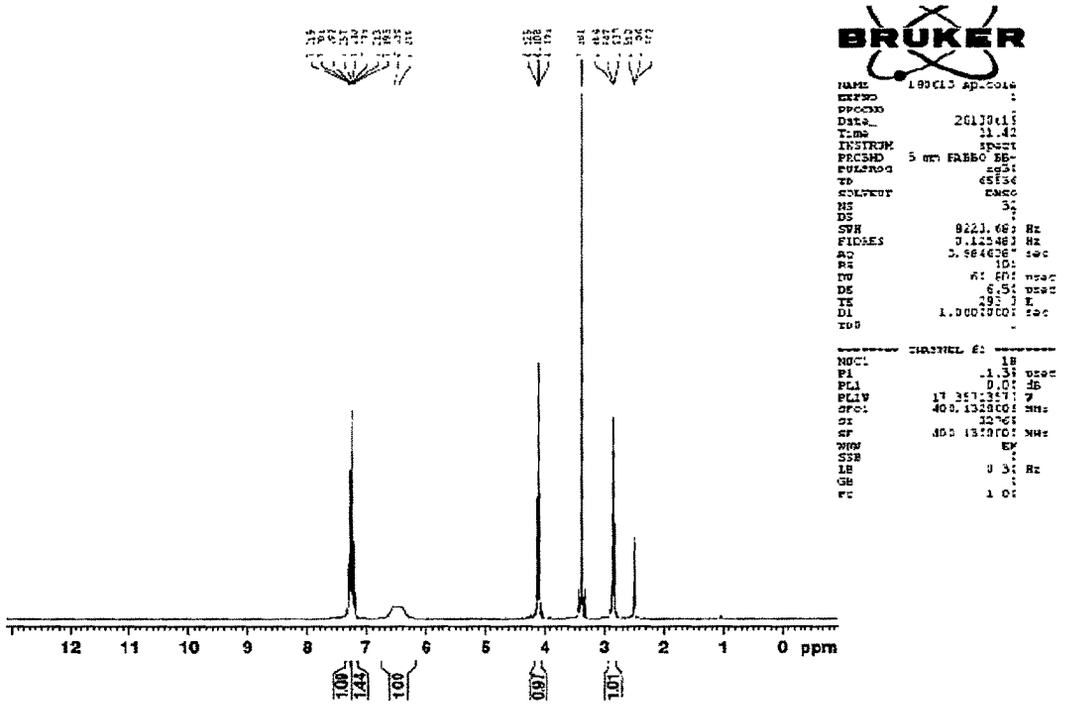
Mass Spectra of Felbamate Related Compound-B

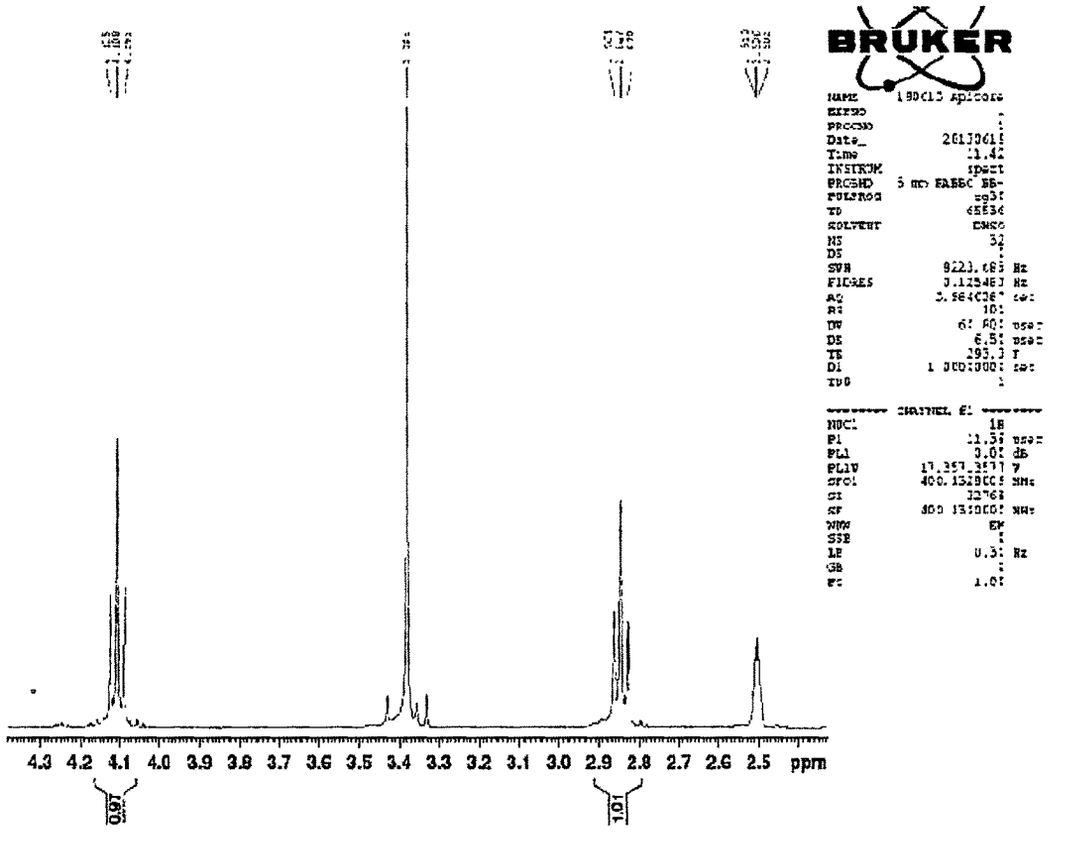


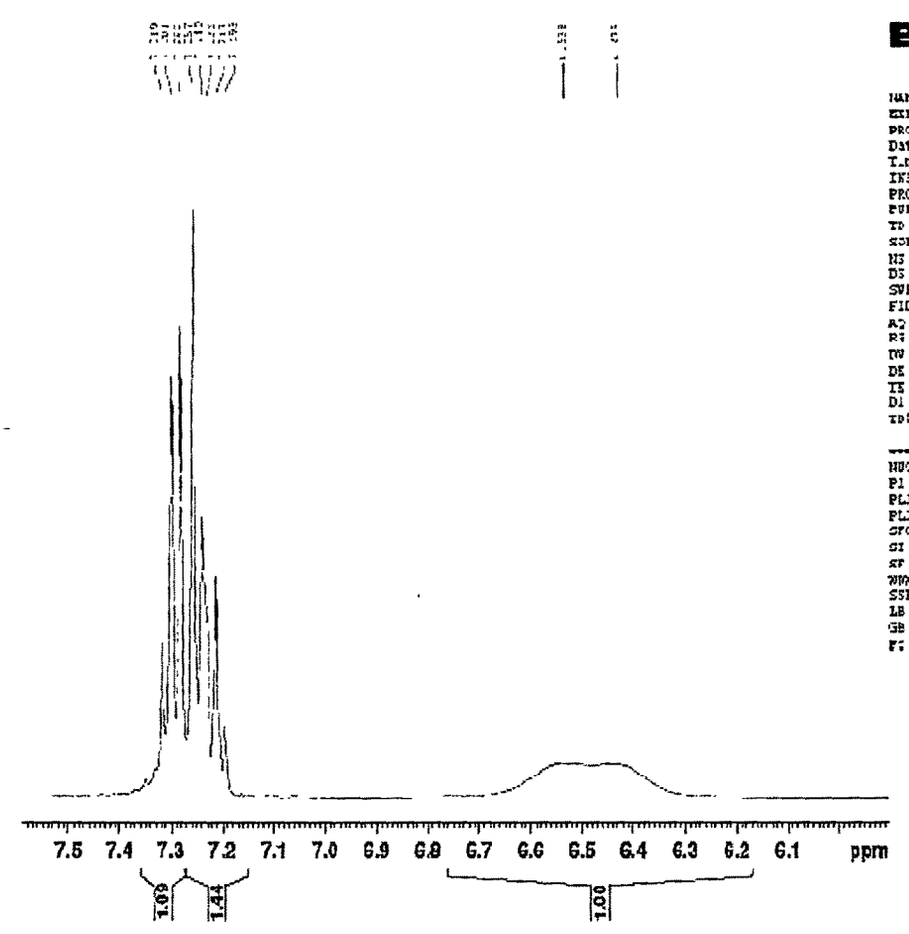
Mass Spectra (MS/MS) of Felbamate Related Compound-B



Proton NMR Spectra of Felbamate Related Compound-B







BRUKER

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PROCNO	1
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PULPROG	zg30
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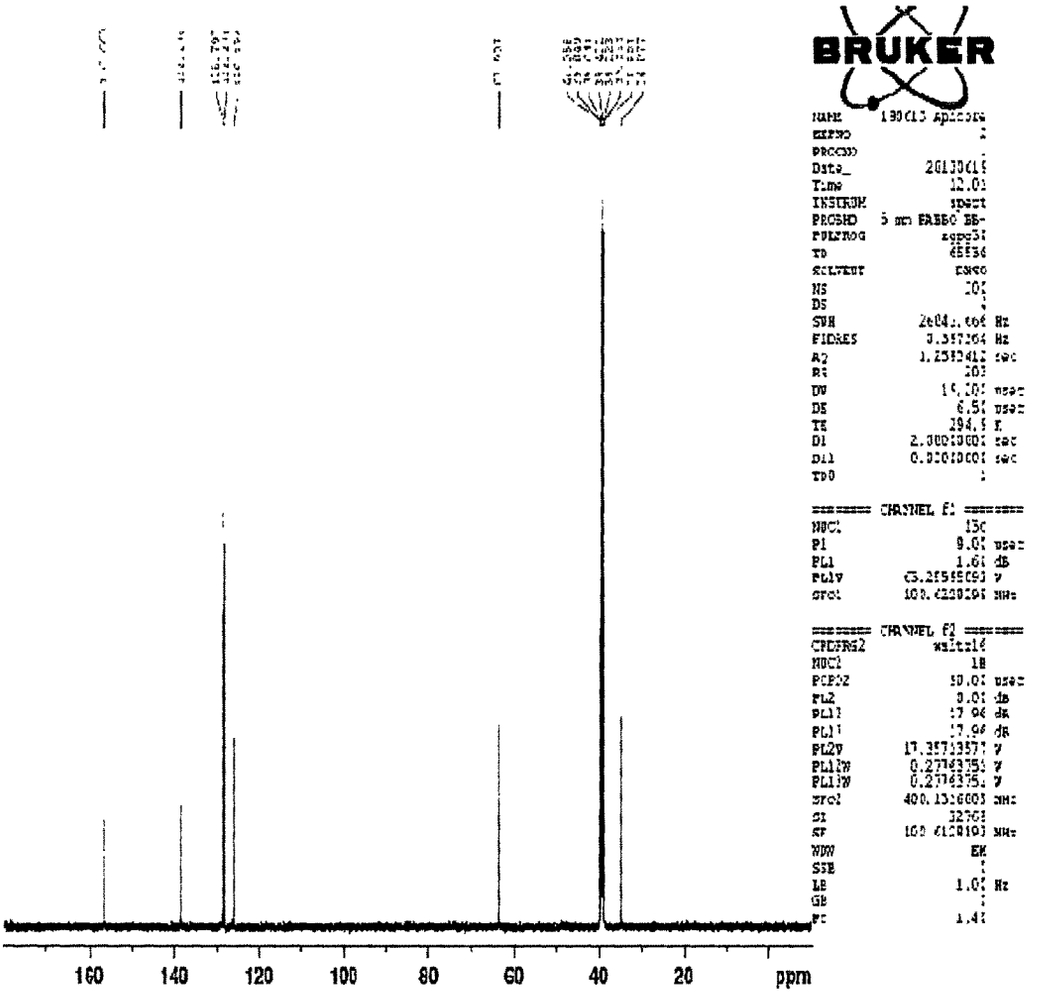
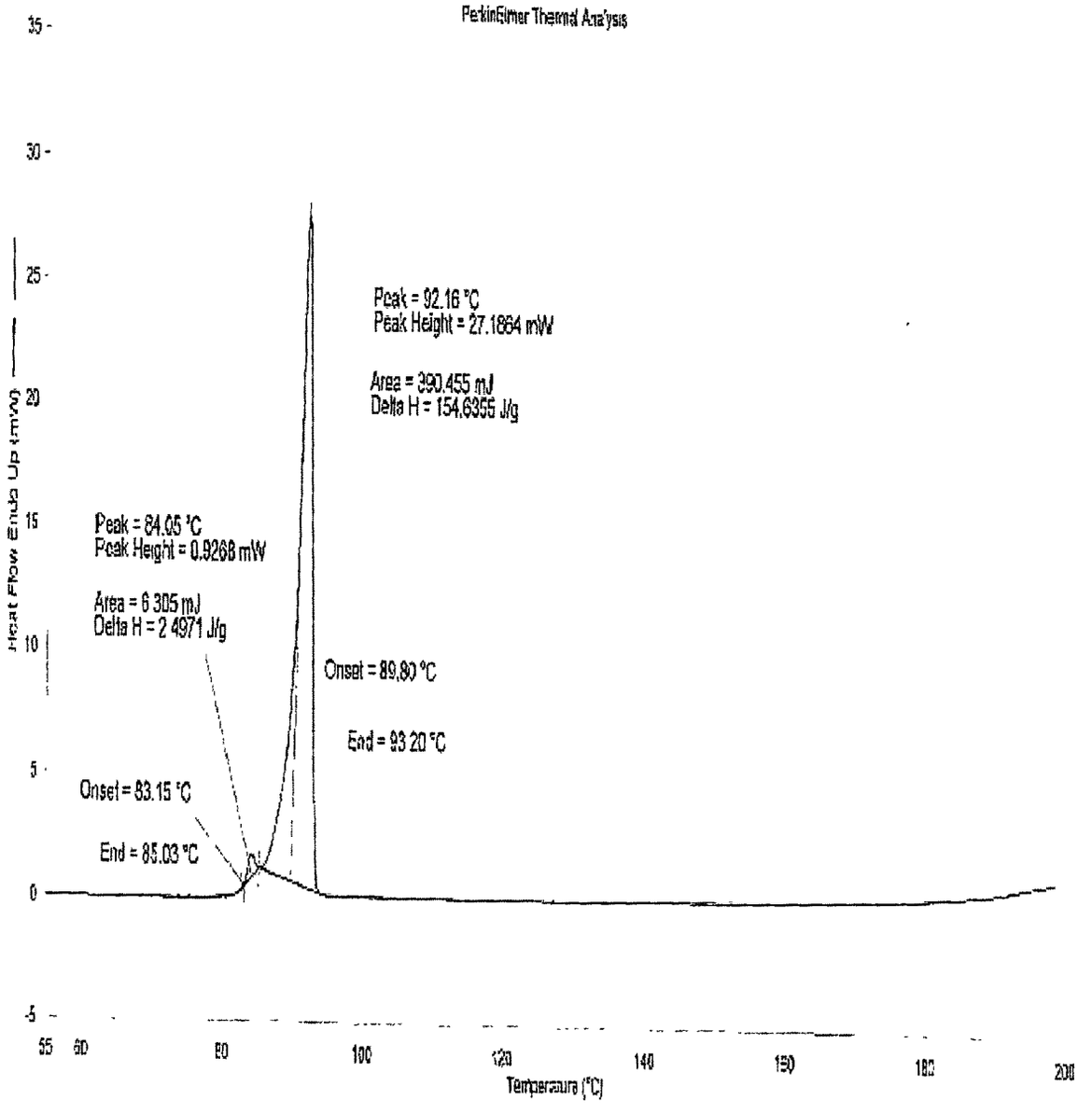
SOLVENT	CHCl3
NS	32
DS	4
SWH	9223.68[illegible] Hz
FIDRES	0.[illegible]2[illegible]48[illegible] Hz
AQ	3.984[illegible] sec
RG	101
DW	67.60[illegible] usec
DE	6.50[illegible] usec
TE	295.1[illegible] K
D1	1.000[illegible] sec
TD0	1

======== CHANNEL f1 ========	
NUC1	1H
P1	11.3[illegible] usec
PL1	0.00[illegible] dB
PL1W	17.357[illegible] W
SFO1	400.152[illegible] MHz
SI	32768
SF	400.13[illegible] MHz
WDW	EM
SSB	0
LB	0.30[illegible] Hz
GB	0
PC	1.00[illegible]

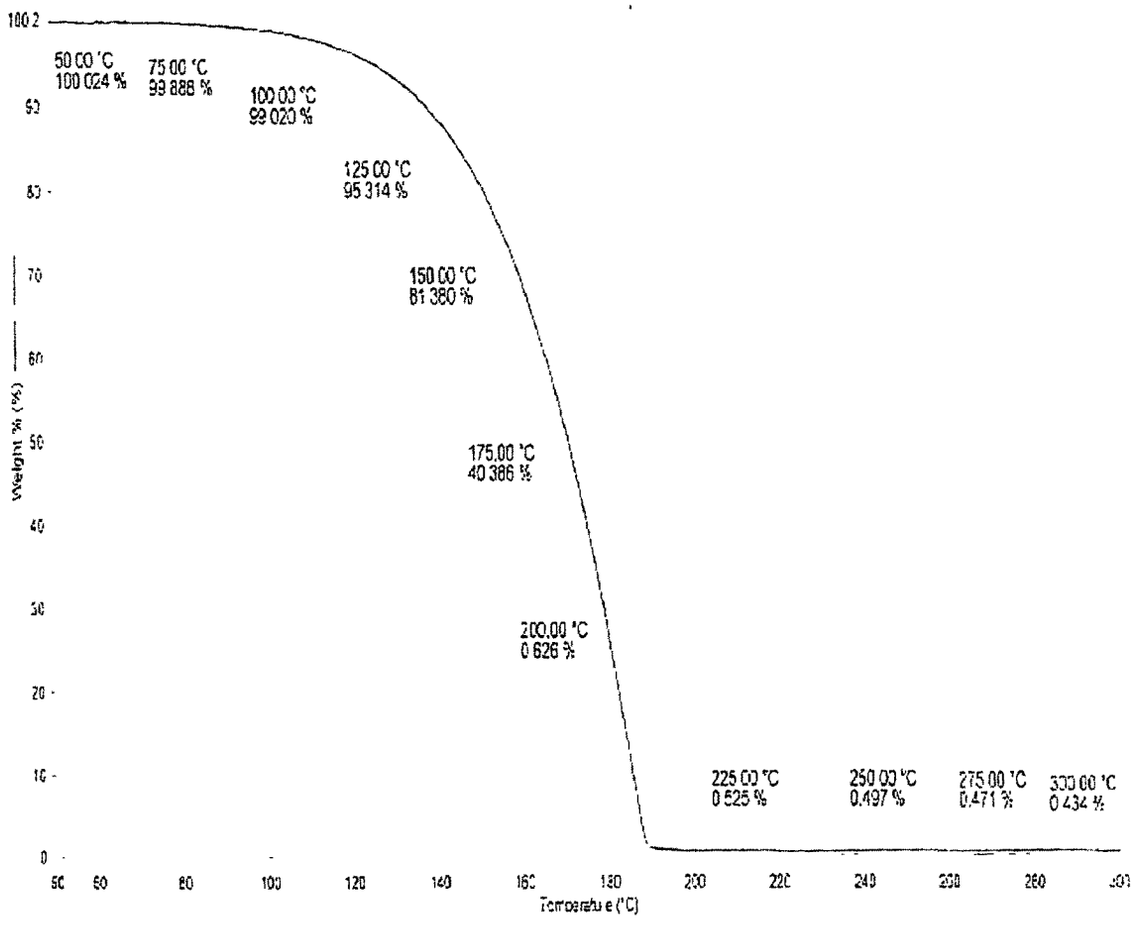
[13]Carbon NMR Spectra of Felbamate Related Compound-B



DSC report of Felbamate Related Compound-B



TGA report of Felbamate Related Compound-B



CONCLUSION

Based on the studies performed to study extensively the various degradation pathways we have observed that different impurities can be formed due to the stress effects on the felbamate drug substance. Not all stress conditions are generating the same impurities.

When the exercise was initiated on drug product also, we have observed the similar trend in the impurity profiles of the felbamate.

The impurity isolations and characterizations of the same, led to develop a better HPLC method which is a stability indicating method in order to monitor the related substances present in the drug substances and drug products. This method can be used at Quality Control laboratories which are required to test and release the felbamate drug substance and drug products for human use.

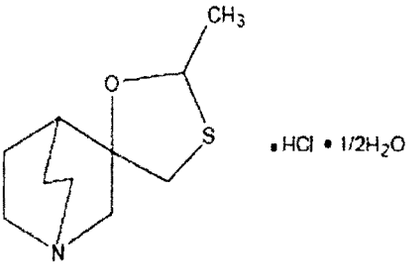
The knowledge of the possible degradation pathways achieved in this project prompted to understand the impurities behavior in the humans when administered either orally or systemically, and to get the toxicology information of the same. After thorough literature search, we have found that all the impurities found via forced degradation studies were similar to the metabolites found in animal studies and human patients performed on felbamate as a part of clinical studies. These metabolites are already studied for safety since felbamate dosed to human patients has seen extensive metabolism to give rise to the metabolites which are similar to our degradation impurities.

CEVIMELINE HCl

CEVIMELINE HYDROCHLORIDE

Cevimeline Hydrochloride is the active pharmaceutical ingredient (API) in Evoxac. EVOXAC® is available as white, hard gelatin capsules containing 30 mg of cevimeline hydrochloride. EVOXAC® capsules have a white opaque cap and a white opaque body. The capsules are imprinted with "EVOXAC" on the cap and "30 mg" on the body with a black bar above "30 mg".

Cevimeline is cis-2'-methylspiro {1-azabicyclo [2.2.2] octane-3, 5' - [1,3] oxathiolane} hydrochloride, hydrate (2:1). Its empirical formula is C10H17NOS.HCl.1/2 H2O, and its structural formula is:



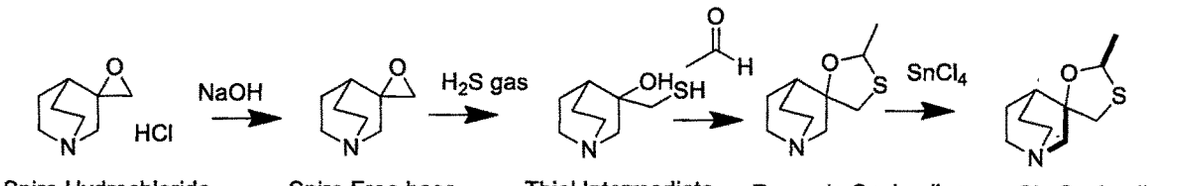
Cevimeline has a molecular weight of 244.79. It is a white to off white crystalline powder with a melting point range of 201 to 203°C. It is freely soluble in alcohol and chloroform, very soluble in water, and virtually insoluble in ether. The pH of a 1% solution ranges from 4.6 to 5.6. Inactive ingredients include lactose monohydrate, hydroxypropyl cellulose, and magnesium stearate.

Cevimeline is indicated for the treatment of symptoms of dry mouth in patients with Sjögren's syndrome

Storage condition on the label is store at 25°C (77°F) excursion permitted to 15°-30°C (59°-86°F)

Cevimeline Hydrochloride Synthesis

The synthetic scheme including the starting materials, reagents, catalysts and other solvents etc. is given below:



Cevimeline Hydrochloride can be synthesized by the similar synthetic pathways as given in the prior art literature.

Process related Impurities

The impurity profiling study has been conducted for the above synthetic process. The review of the manufacturing procedure reveals that elemental sulfur, boron and tin metals could be the likely inorganic impurities that could be formed and carried over to the final compound during the chemical transformations from the starting material to the finished API. BF_3 etherate was used as a reagent in the acetaldehyde reaction which could leave traces of boron metal ions.

The organic impurities include the starting material which is "Spiro [1-azabicyclo[2,2,2] octane-3,2'-oxirane] hydrochloride 1,3-cyclohexanedione" and the intermediate thiol compound "3-hydroxy-3-mercaptomethyl quinuclidine".

The enantiomeric impurities include the trans-isomer of Cevimeline hydrocloride which is formed as a by-product during the formation reaction of the Cevimeline hydrochloride (cis-isomer is the required form and is the therapeutically active form). The extent of trans impurity depends on the efficiency of the purification process and the conversion of the trans isomer to the required cis-isomer. Therefore the carryover of this enantiomeric impurity into the final compound is very much possible and therefore it is considered and monitored under the organic and enantiomeric impurity.

The residual solvents impurities are the solvents which are used in the process and also which are associated with the reagents like IPA.HCl, where in the isopropanol is the residual solvent. The solvents which could be potentially present in the final API of Cevimeline hydrochloride as per the manufacturing process given above are as follows:

1) Acetaldehyde
2) Methanol
3) Diethyl ether
4) Acetone
5) Isopropyl alcohol
6) Dichloromethane
7) Diisopropyl ether
8) Isopropyl chloride

Degradation Impurities

Since the drug substances and drug products will be stored for long durations in the pharmaceutical companies and the pharmacy retail outlets before they are consumed by the patient, it becomes imperative and prudent to study the degradation products which could be formed due to various stress factors on the drug substance either during shipment or storage. Hence, to establish the degradation pathways and the degradation products, we have performed several studies on Cevimeline hydrochloride by applying external stress factors like acid, base, heat, light etc. and characterized the structures of the same.

Stress studies (Induced degradation)

The following stress conditions were applied on the Cevimeline hydrochloride hemihydrate to understand the degradation profiles and identify the degradation impurities.

1) Hydrolytic degradation with acid
2) Hydrolytic degradation with alkali
3) Thermal degradation
4) Photolytic degradation
5) Sunlight degradation
6) Oxidative degradation

The forced degradation conditions and the sample preparations during the study are similar to the ones given under the Felbamate study.

Stability indicating Analytical method (By HPLC)

1.0 INTRODUCTION

The following stability indicating HPLC method has been developed to separate all the known and unknown impurities in Cevimeline hydrochloride hemihydrate API and drug product based on the degradation studies conducted.

2.0 RELATED SUBSTANCE (% w/w ON AS IS BASIS BY HPLC)

2.1 Instrumentation

A High Performance Liquid Chromatograph equipped with Gradient elution capability, Ultraviolet Spectrophotometer as detector and an auto sampler.

(Use Shimadzu LC 2010 system or equivalent).

Data handling system

LC Solution Software or equivalent chromatographic software

Column

A stainless steel column of length 250 mm, internal diameter 4.6 mm and filled with Octadecyl Silane chemically bonded to porous silica particles of 5µ diameter or Symmetry Shield RP 18 (250 x 4.6mm x 5µ) or equivalent.

2.2 Reagents

1) Process Water
2) Dipotassium hydrogen phosphate (K_2HPO_4)
3) Methanol
4) 50 % Hydrochloric Acid

2.3 Preparation of buffer

Dipotassium hydrogen Phosphate (3.7g) was weighed and dissolved in 2000 mL process water and mixed well to dissolve solids. The pH of solution was adjusted to 3.5±0.2 with diluent hydrochloric acid (1:1 water and concentrated hydrochloric acid) and mixed well. The solution was then filtered through 0.45µ or finer porosity membrane filter.

2.4 Preparation of Mobile [Mobile phase-A]

Transferred 1000 mL of buffer and 2 mL of methanol in a suitable container and mixed well. The solution was degassed by sonication.

2.5 Preparation of Mobile phase B

Transferred 300 mL of buffer and 700 mL of methanol in a suitable container and mixed well. The solution was degassed by sonication.

2.6 Elution Program

Time	Mobile phase A (% v/v)	Mobile phase B (% v/v)
0.00	100.0	0.0
7.00	100.0	0.0
40.00	70.0	30.0
50.00	100.0	0.0
65.00	100.0	0.0

2.7 Chromatographic parameters

Flow rate : 0.50 mL/minute

Detection : UV at 210 nm

Injection volume : 10 µL

Column oven temperature : 30°C

Run time : NLT 65 minutes.

2.8 Preparation of diluent

Used buffer as such prepared under section 2.3.

2.9 Preparation of Cevimeline standard solution

Cevimeline standard (25mg) was weighed and transferred into a 25 mL volumetric flask. About 20 mL of diluent was added and sonicated to dissolve the solid with occasional shaking. It was diluted to volume with diluent and mixed well.

5.0 mL of above stock solution was accurately transferred in to 50 mL volumetric flask, and diluted to volume with diluent and mixed.

2.10 Preparation of Sample solution for related substances (RS)

Cevimeline sample (50mg) was weighed accurately and transferred into 10 mL volumetric flask. 5 mL of diluent was added and sonicated to dissolve the solid with occasional shaking. It was diluted to volume with diluent and mixed well.

2.11 Procedure

Separately inject the diluent, standard preparation and sample preparation into the chromatograph. Record the chromatograms. Determine the peak responses of all eluting peaks in the chromatogram of the sample solution for related substances. Examine the diluent chromatogram for any extraneous peaks, and disregard the corresponding peaks observed in the chromatogram of the sample solution for related substances. Follow the injection sequence as mentioned below:

Sr. no.	Sample	No. of injections
1	Diluent	1
2	Diluted Standard S_1 (system suitability)	6
3	Sample preparation (RS)	1

2.12 System Suitability Parameter

Sr. No.	Parameter of system suitability	Specification
1	% Relative Standard deviation (RSD) for area of Cevimeline	Not more than 2.0 %
2	Theoretical plates (Column efficiency)	Not less than 3000
3	Tailing factor (Asymmetry)	Not more than 3.0

2.13 Calculation

2.13.1 Percentage individual Known / unknown impurity:

$$= \frac{At}{As} \times \frac{\text{Std. wt (mg)}}{25} \times \frac{5}{50} \times \frac{10}{\text{Sample wt (mg)}} \times P$$

Where,

At	:	Peak area of known/unknown impurity in sample preparation
As	:	Average peak area (six std) of Cevimeline in the chromatogram of standard preparation
Std. wt	:	Weight of Cevimeline standard in mg
Sample wt	:	Weight of Cevimeline sample preparation in mg
P	:	% Potency / Assay of Cevimeline standard (As is basis)

The retention time and relative retention time of known impurities and Cevimeline as under.

TABLE-1

Name of compound	**Retention time (RRT) in minutes**	**Relative retention time (RRT)**
Cevimeline	29.1	1.00
Cevimeline Sulfoxide	7.7	0.26
trans-isomer	33.1	1.14
Cevimeline N-Oxide	34.3	1.18

2.13.2 Maximum individual unknown impurity

Report maximum unknown impurity obtained from calculation.

2.13.3 Percentage total Impurities

Report summation of the all impurities

2.14 Specification

Cevimeline sulfoxide	: Not more than 0.15 %
Cevimeline N-oxide	: Not more than 0.15 %
trans-isomer	: Not more than 0.50 %
Maximum individual unknown impurity	: Not more than 0.10 %
Total impurities	: Not more than 1.00 %

Cevimeline Impurity Profile

Cevimeline hydrochloride subjected to the stress factors did undergo extensive degradation when exposed to oxidative degradation with hydrogen peroxide. It was found that the acid and base concentration has no effect on the Cevimeline degradation and the drug substance remained intact across the pH range. All the other conditions exposed also did not result in any degradation of the drug.

% Degradation of Cevimeline and peak purity data for Related Substances

Table-1 % Degradation in forced degradation studies for related substances

Mode of Degradation	**Condition**	**% Degradation of Cevimeline**
Hydrolytic degradation with acid	- Drug + 2 mL 1 N Hydrochloric acid, heated the solution for 1 hour at 80° C temperature on water bath	**12.38**
Hydrolytic degradation with base	- Drug + 2 mL 1 N Sodium hydroxide, heated the solution for 1 hour at 80° C temperature in water bath	**0.37**
Oxidative degradation with Hydrogen peroxide	- Drug + 2 mL 10 % Hydrogen peroxide, heated the solution for 1 hour at 80° C temperature in water bath	**14.33**
Photolytic degradation using UV light (254 nm)	- Exposed the drug for 12 hours under UV light	**1.23**
Photolytic degradation with Sun light	- Exposed the drug for 12 hours in sun light	**-1.40**
Thermal degradation (Sample)	- Heated the drug for 4 hour at 80° C temperature under oven.	**2.30**
Thermal degradation (Solution)	- Drug + 7 mL of diluent. Heated the solution for 4 hour at 80° C temperature under oven.	**3.3**

Table-2 Peak purity data of Cevimeline for Related Substances

Mode of Degradation	Condition	Peak purity data	
		* PA	** TH
Control Sample	- As such	0.239	0.293
Hydrolytic degradation with acid	- Drug + 2 mL 1 N Hydrochloric acid, heated the solution for 1 hour at 80° C temperature in water bath	0.239	0.316
Hydrolytic degradation with base	- Drug + 2 mL 1 N Sodium hydroxide, heated the solution for 1 hour at 80° C temperature in water bath	0.276	0.306
Oxidative degradation with Hydrogen peroxide	- Drug + 2 mL 10 % Hydrogen peroxide, heated the solution for 1 hour at 80° C temperature in water bath	0.227	0.306
Photolytic degradation using UV light (254 nm)	- Exposed the drug for 12 hours under UV light	0.287	0.305
Photolytic degradation with Sun light	- Exposed the drug for 12 hours in sun light	0.281	0.299
Thermal degradation (Sample)	- Heated the drug for 4 hour at 80° C temperature under oven.	0.240	0.309
Thermal degradation (Solution)	- Drug + 7 mL of diluent. Heated the solution for 4 hour at 80° C temperature under oven.	0.210	0.312

Note: * PA : Purity Angle, ** TH : Purity Threshold

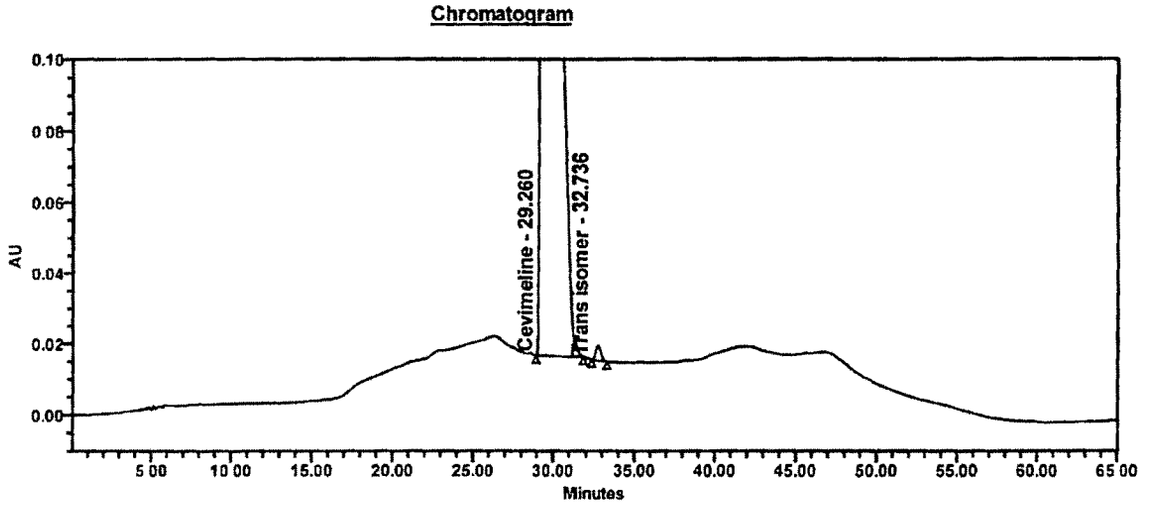
Table-2 Peak purity data of Cevimeline for Related Substances

Mode of Degradation	Condition	Peak purity data	
		* PA	** TH
Control Sample	- As such	0.239	0.293
Hydrolytic degradation with acid	- Drug + 2 mL 1 N Hydrochloric acid, heated the solution for 1 hour at 80° C temperature in water bath	0.239	0.316
Hydrolytic degradation with base	- Drug + 2 mL 1 N Sodium hydroxide, heated the solution for 1 hour at 80° C temperature in water bath	0.276	0.306
Oxidative degradation with Hydrogen peroxide	- Drug + 2 mL 10 % Hydrogen peroxide, heated the solution for 1 hour at 80° C temperature in water bath	0.227	0.306
Photolytic degradation using UV light (254 nm)	- Exposed the drug for 12 hours under UV light	0.287	0.305
Photolytic degradation with Sun light	- Exposed the drug for 12 hours in sun light	0.281	0.299
Thermal degradation (Sample)	- Heated the drug for 4 hour at 80° C temperature under oven.	0.240	0.309
Thermal degradation (Solution)	- Drug + 7 mL of diluent. Heated the solution for 4 hour at 80° C temperature under oven.	0.210	0.312

Note: * PA : Purity Angle, ** TH : Purity Threshold

The HPLC chromatograms are depicted below:

Control sample Chromatogram

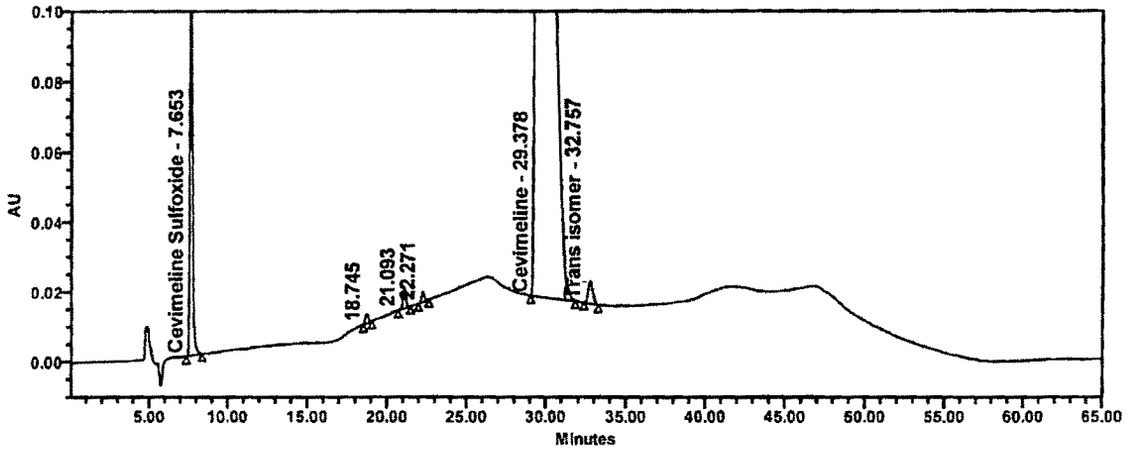


Peak Table

	Peak Name	RT	Area	% Area	RT Ratio
1	Cevimeline Sulfoxide	7.80			
2	Cevimeline	29.26	20614782	99.56	
3	Trans isomer	32.74	90615	0.44	1.12
4	Cevimeline N-Oxide	34.20			
Sum			20705397	100.00	

Acid degradation sample

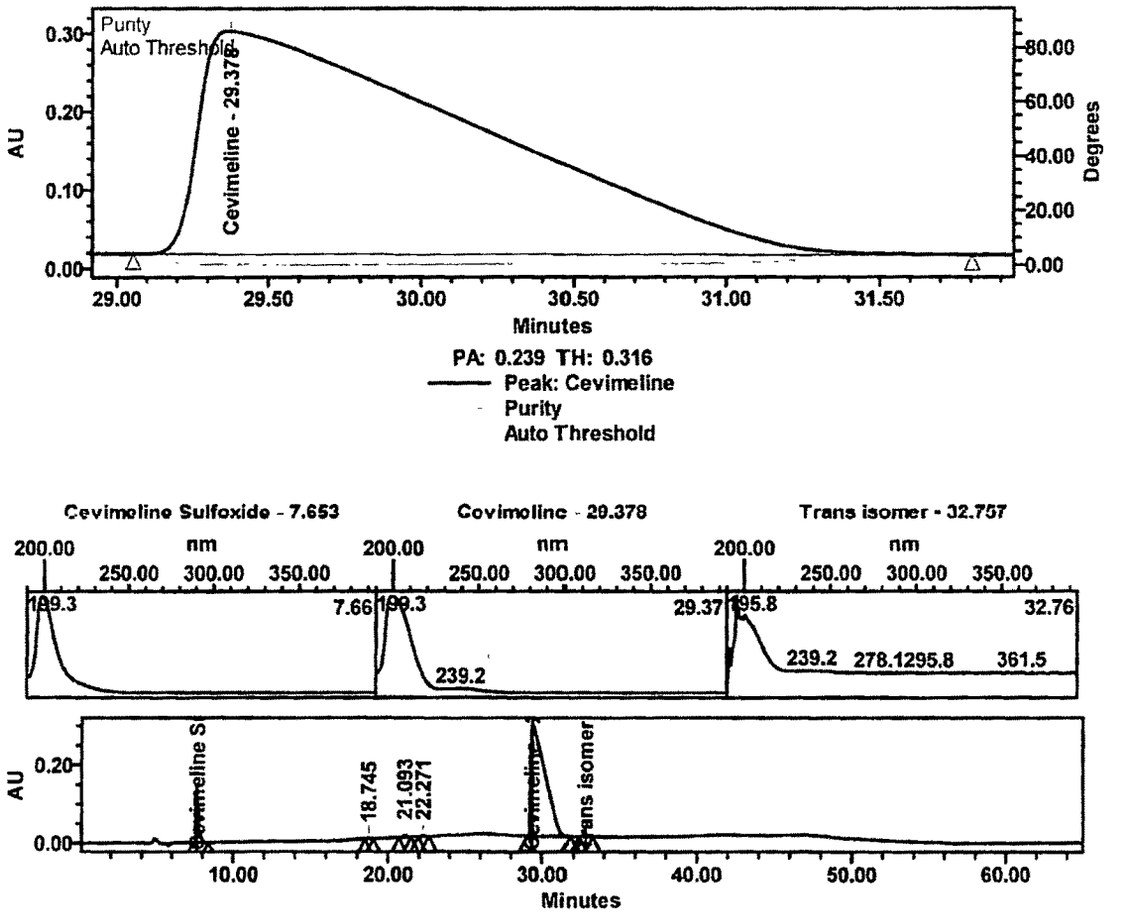
Chromatogram



Peak Table

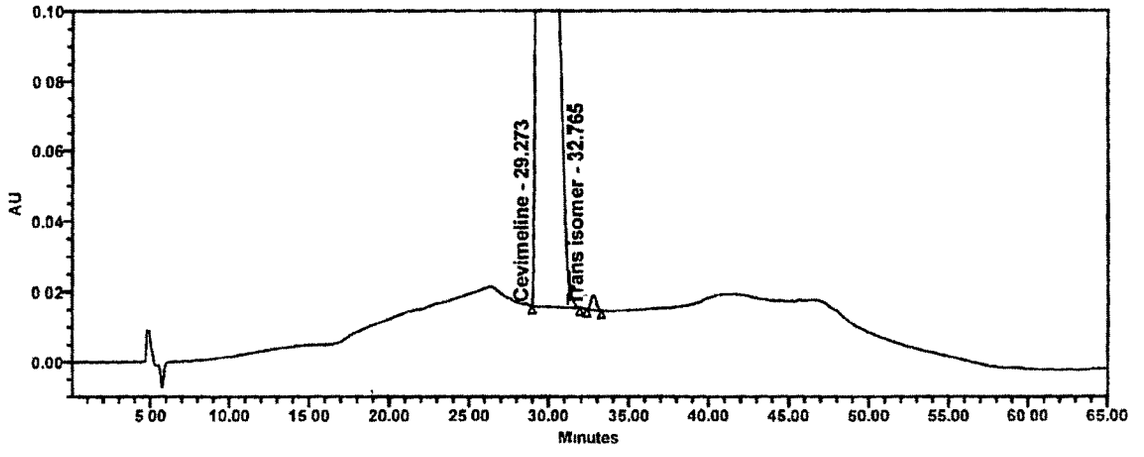
	Peak Name	RT	Area	% Area	RT Ratio
1	Cevimeline Sulfoxide	7.65	1425799	7.21	0.26
2		18.75	33182	0.17	
3		21.09	72895	0.37	
4		22.27	33917	0.17	
5	Cevimeline	29.38	18080869	91.41	
6	Trans isomer	32.76	132502	0.67	1.11
7	Cevimeline N-Oxide	34.34			
Sum			19779163	100.00	

Acid degradation peak purity data



Base degradation sample

Chromatogram

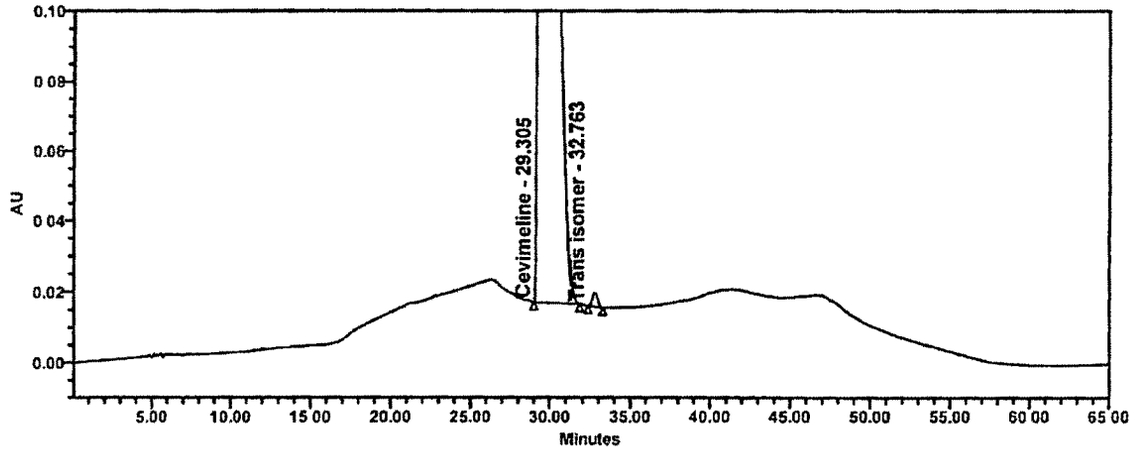


Peak Table

	Peak Name	RT	Area	% Area	RT Ratio
1	Cevimeline Sulfoxide	7.80			
2	Cevimeline	29.27	20608751	99.55	
3	Trans isomer	32.77	93931	0.45	1.12
4	Cevimeline N-Oxide	34.22			
Sum			20702682	100.00	

Thermal Degradation

Chromatogram

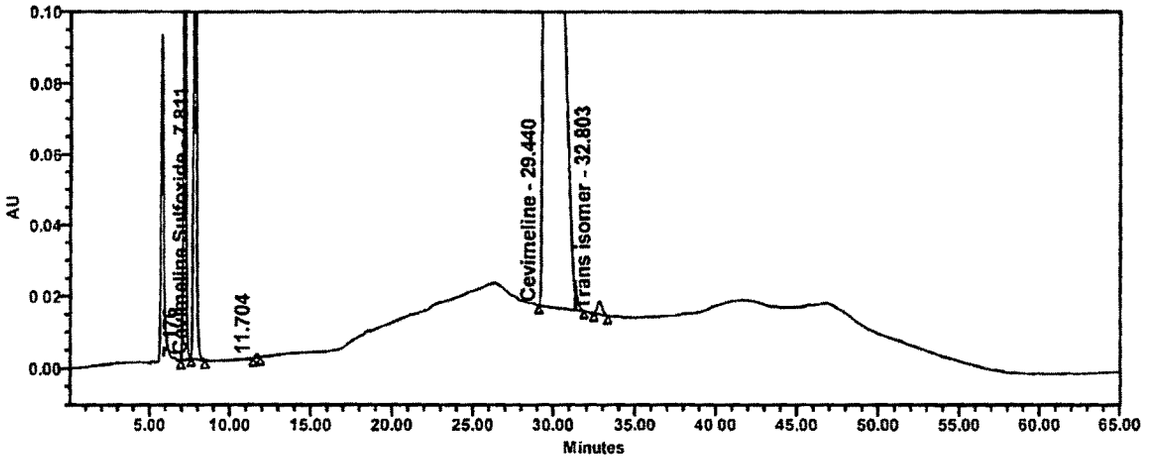


Peak Table

	Peak Name	RT	Area	% Area	RT Ratio
1	Cevimeline Sulfoxide	7.81			
2	Cevimeline	29.30	20233114	99.56	
3	Trans isomer	32.76	89674	0.44	1.12
4	Cevimeline N-Oxide	34.25			
Sum			20322788	100.00	

Oxidative degradation

Chromatogram

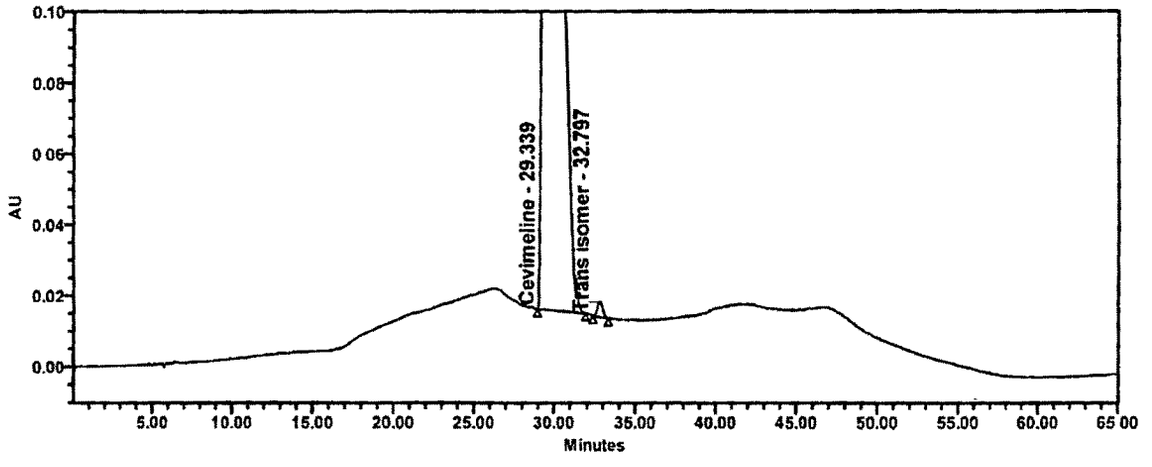


Peak Table

	Peak Name	RT	Area	% Area	RT Ratio
1		7.18	1490769	7.01	
2	Cevimeline Sulfoxide	7.81	1933226	9.09	0.27
3		11.70	10189	0.05	
4	Cevimeline	29.44	17754910	83.49	
5	Trans isomer	32.80	77059	0.36	1.11
6	Cevimeline N-Oxide	34.41			
Sum			21266154	100.00	

UV light degradation

Chromatogram

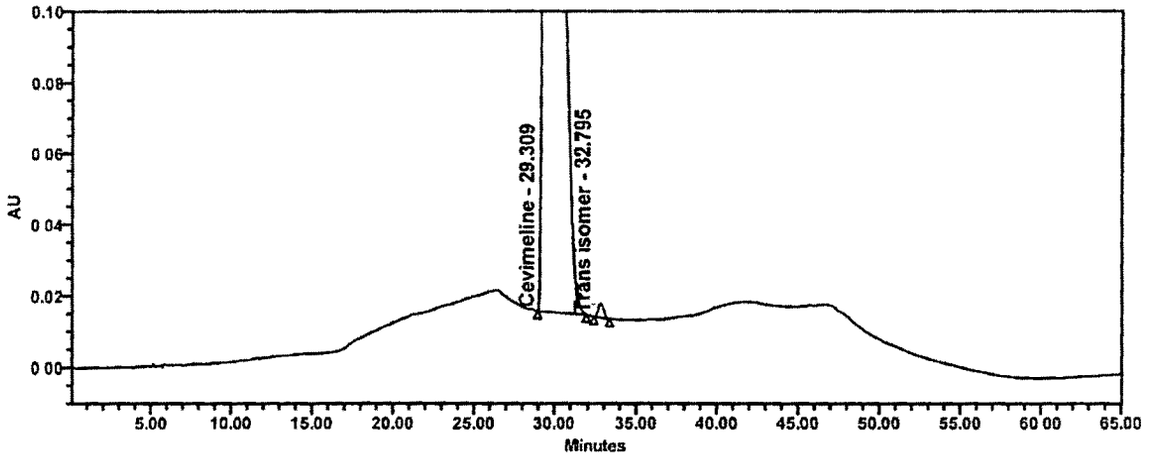


Peak Table

	Peak Name	RT	Area	% Area	RT Ratio
1	Cevimeline Sulfoxide	7.82			
2	Cevimeline	29.34	20333528	99.51	
3	Trans isomer	32.80	99247	0.49	1.12
4	Cevimeline N-Oxide	34.29			
Sum			20432775	100.00	

Sun light degradation

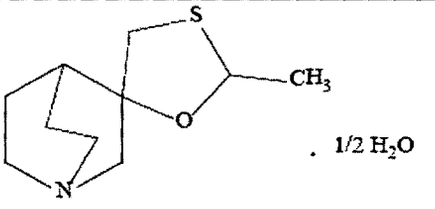
Chromatogram

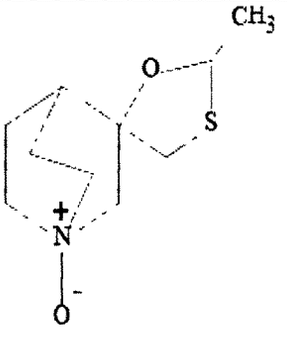


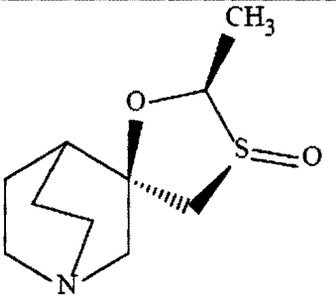
Peak Table

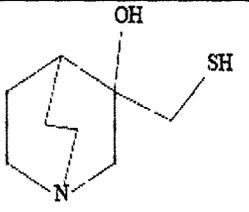
	Peak Name	RT	Area	% Area	RT Ratio
1	Cevimeline Sulfoxide	7.81			
2	Cevimeline	29.31	20881718	99.57	
3	Trans isomer	32.80	89745	0.43	1.12
4	Cevimeline N-Oxide	34.26			
Sum			20971463	100.00	

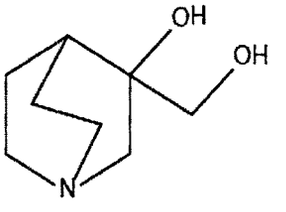
The route of synthesis for Cevimeline Hydrochloride was evaluated for potential impurities. The impurities, which have been identified based on the route of synthesis and forced degradation studies of Cevimeline Hydrochloride, are listed below.

Name	Chemical Name, Structure, Molecular Formula and Molecular Weight	Source	Limits
Trans Cevimeline Hydrochloride (Trans Isomer)	 . 1/2 H_2O Trans-2-methylspiro (1,3-oxathiolane-5,3-quinuclidine) hydrochloride hemihydrate $C_{10}H_{17}NOS.HCl.1/2\ H_2O$ 244.77	Process Impurity	Not more than 0.50%
Origin: This is a process impurity formed during synthesis of racemic Cevimeline hydrochloride. During resolution of the racemic mixture there is a chance of leftover of this impurity.			

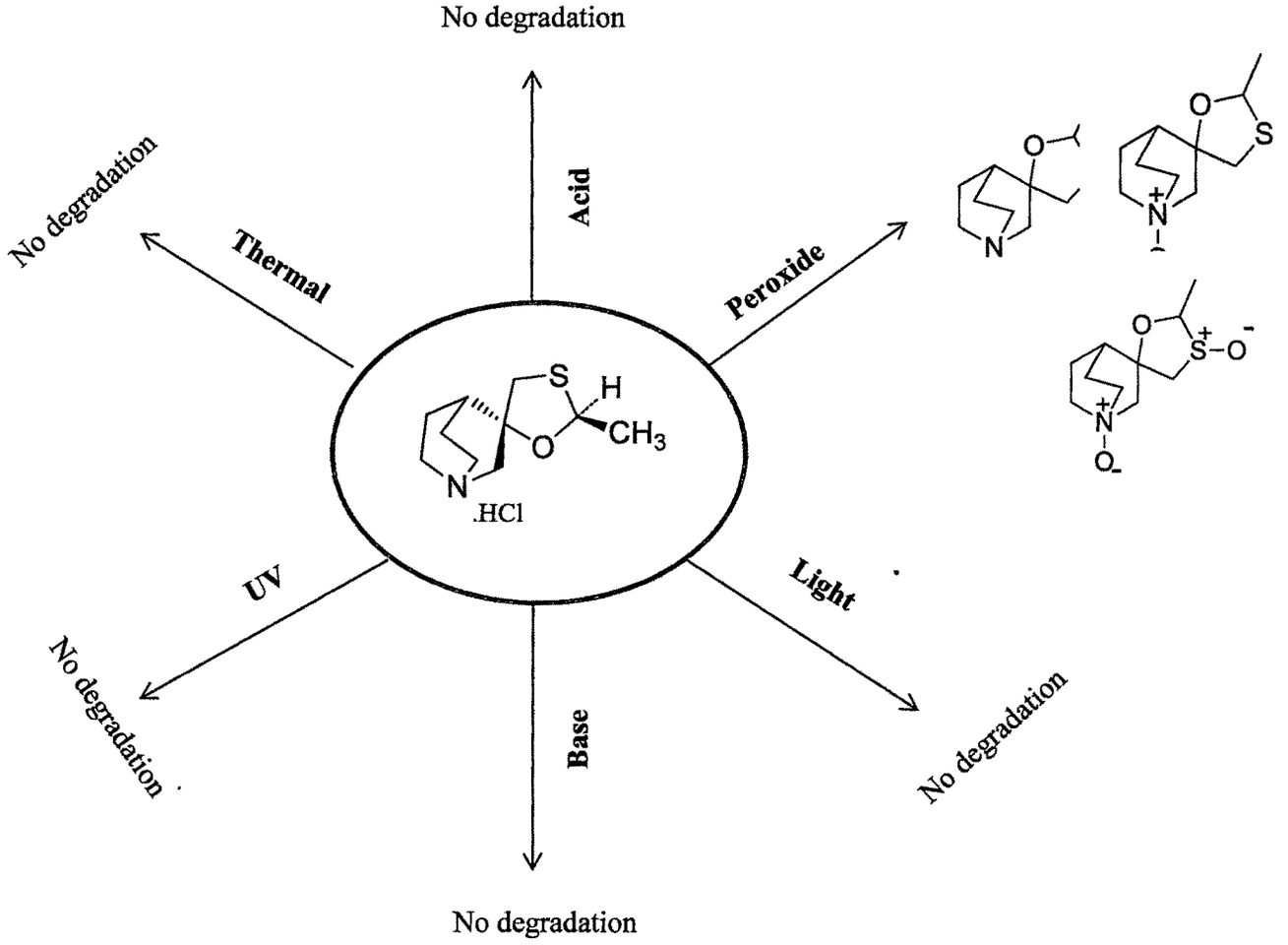
Cevimeline N-Oxide	 2-methyl-1'-azaspiro[[1,3]oxathiolane-5,3'-bicyclo[2.2.2]octane] 1-Oxide $C_{10}H_{17}NO_2S$ 215.31	Degradation Impurity	Not more than 0.15%
Origin: This is a degradation impurity formed due to oxidation of Cevimeline Hydrochloride. As the manufacturing process doesn't involve any oxidation steps, the formation of this impurity in the process is remote.			

Name	Chemical Name, Structure, Molecular Formula and Molecular Weight	Source	Limits
Cevimeline sulfoxide	 (2'R,3R,3'S)-rel- 2'-Methylspiro [1-azabicyclo [2.2.2] octane-3,5'-oxathiolane] 3'-oxide $C_{10}H_{17}NO_2S$ 215.31	Degradation Impurity	Not more than 0.15%
Origin: This is a degradation impurity formed due to oxidation of Cevimeline Hydrochloride. As the manufacturing process doesn't involve any oxidation steps, the formation of this impurity in the process is remote.			

Thiol Impurity	 $C_8H_{15}SNO$ 173.28	Process Impurity
Origin: This is a process impurity formed during synthesis of racemic Cevimeline hydrochloride as an in-situ intermediate which is further converted to Cevimeline hydrochloride.		

Diol Impurity	 $C_8H_{15}NO_2$ 157.21	Degradation impurity
Origin: This is a degradation impurity formed due to reaction of epoxide starting material (Spiro Compound) with Sodium hydroxide at high temperatures.		

Cevimeline


No degradation
Acid
Thermal
No degradation
Peroxide
S
H
CH3
O
N
.HCl
UV
No degradation
Light
No degradation
Base
No degradation

CHARACTERIZATION AND STRUCTURE ELUCIDATION

All the possible process and degradation individual impurities are either synthesized or isolated by enriching the impurity in the mother liquors and characterized by various analytical techniques to confirm the structure of the impurities. Later these impurities were spiked to confirm the retention times (RT's) and relative retention times (RRT's). The full physico-chemical characterization of each individual impurity is provided below:

PHYSICO-CHEMICAL CHARACTERIZATION OF DIOL REFERENCE STANDARD

The physico-chemical characterization of diol was established by analytical techniques such as FT-IR, HPLC, Mass and NMR.

PHYSICO-CHEMICAL CHARACTERIZATION OF DIOL

1.0 INTRODUCTION

The physico-chemical characterization of diol was established by analytical techniques such as High performance liquid chromatograph (HPLC), FT-IR, Mass and NMR.

2.0 PHYSICAL PROPERTIES

2.1 APPEARANCE

Table-1 Appearance results for diol

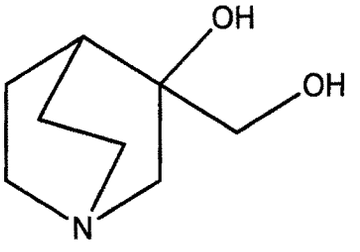
Name	Appearance
3-(hydroxymethyl) quinuclidin-3-ol (Diol)	Light yellow semi solid

3.0 CHEMICAL PROPERTIES

3.1 Chemical Name : 3-(hydroxymethyl) quinuclidin-3-ol

3.2 Common Name : Diol

3.3 Structural Formula



3.4 Molecular Formula : $C_8H_{15}NO_2$

3.5 Molecular Weight : 157.21

4.0 CHARACTERIZATION BY INFRARED SPECTROPHOTOMETER (FT-IR)

The Infrared spectra of diol was obtained by IR-Prestige-21 instrument.

The FT-IR spectra for diol are depicted in **Exhibits below** and interpretation of functional groups are presented in **table-2.**

Table-2 FT-IR Frequency of diol

Bond	Approx. Frequency (cm-1)	Intensity and range
-OH	1321.24	Med (1330-1430)
-CH2	2873.94 and 2945.3	Strong (2850-3000)

Conclusion: The IR frequencies of functional groups are in line with functional groups of diol.

5.0 CHROMATOGRAPHIC PURITY BY HPLC

The chromatographic purity of diol was determined by high performance liquid chromatograph (HPLC) using a developed test procedure. The result is depicted in the following **Table-3**. The chromatograms are depicted in **Exhibits.**

Table-3 Chromatographic Purity of diol

Name	Average % Chromatographic Purity By HPLC of diol
3-(hydroxymethyl) quinuclidin-3-ol	94.53 %

6.0 CHARACTERIZATION BY MASS SPECTROMETRY

The diol sample was analyzed for the parent ion scan by using infusion-MS on Quattro-LC mass spectrometer. The result data is presented in the following **Table-4**. The mass spectrum is depicted in **Exhibits**.

Table-4 Molecular ion data of diol by infusion-MS Technique

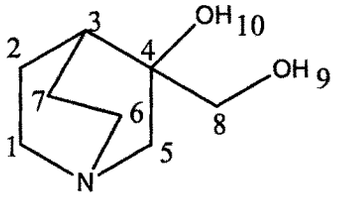
Name	m/z value	(M+H)+	Molecular weight of Diol
3-(hydroxymethyl) quinuclidin-3-ol	158.09	158.09	157.21

Conclusion: Confirms the Mass of diol

7.0 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER

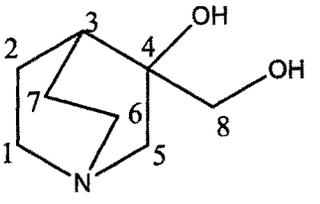
The diol sample was analyzed on 400 MHz Nuclear magnetic resonance spectrometer (NMR) by preparing sample in $CDCl_3$. The same was analyzed for the proton and carbon NMR. The NMR spectra for proton scan and carbon scans are depicted in **Exhibits.**

Proton (^{1}H) NMR:



Position	1H	δ (ppm)	Multiplicity
1,5,6	6H	2.964-2.628	multiplet
2,7	4H	1.385-1.251	multiplet
3	1H	1.379-1.364	Multiplet
8	2H	3.568-3.437	Dd
9,10	2H	4.827	singlet

Carbon (^{13}C) NMR:



Carbon Position	δ (ppm)
C8	71.35
C7	28.07
C6	63.94
C5	66.95
C4	77.40-76.76
C3	46.77-46.12
C2	25.33
C1	59.30

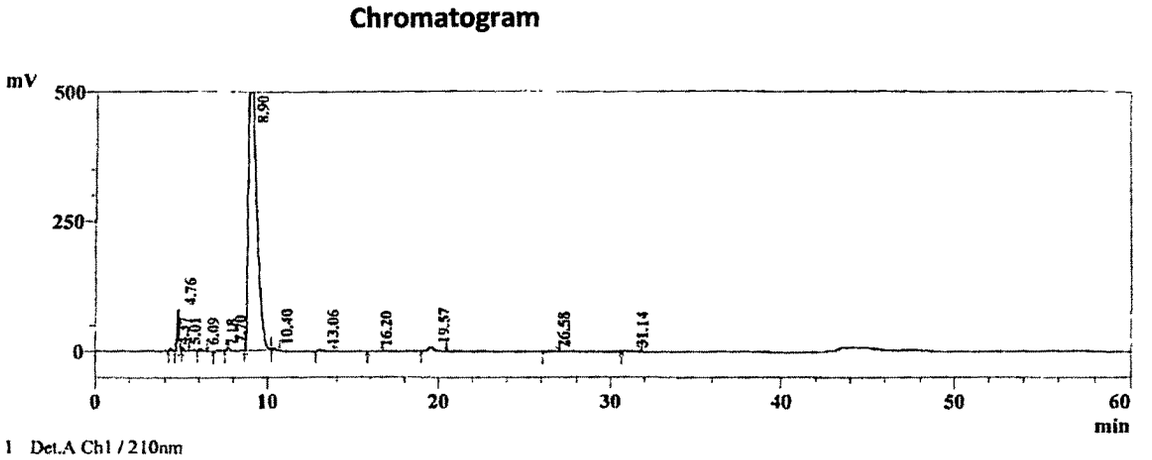
Conclusion: Proton (^{1}H) and Carbon (^{13}C) NMR confirms the structure of diol.

8.0 STORAGE CONDITION

Store in refrigerator

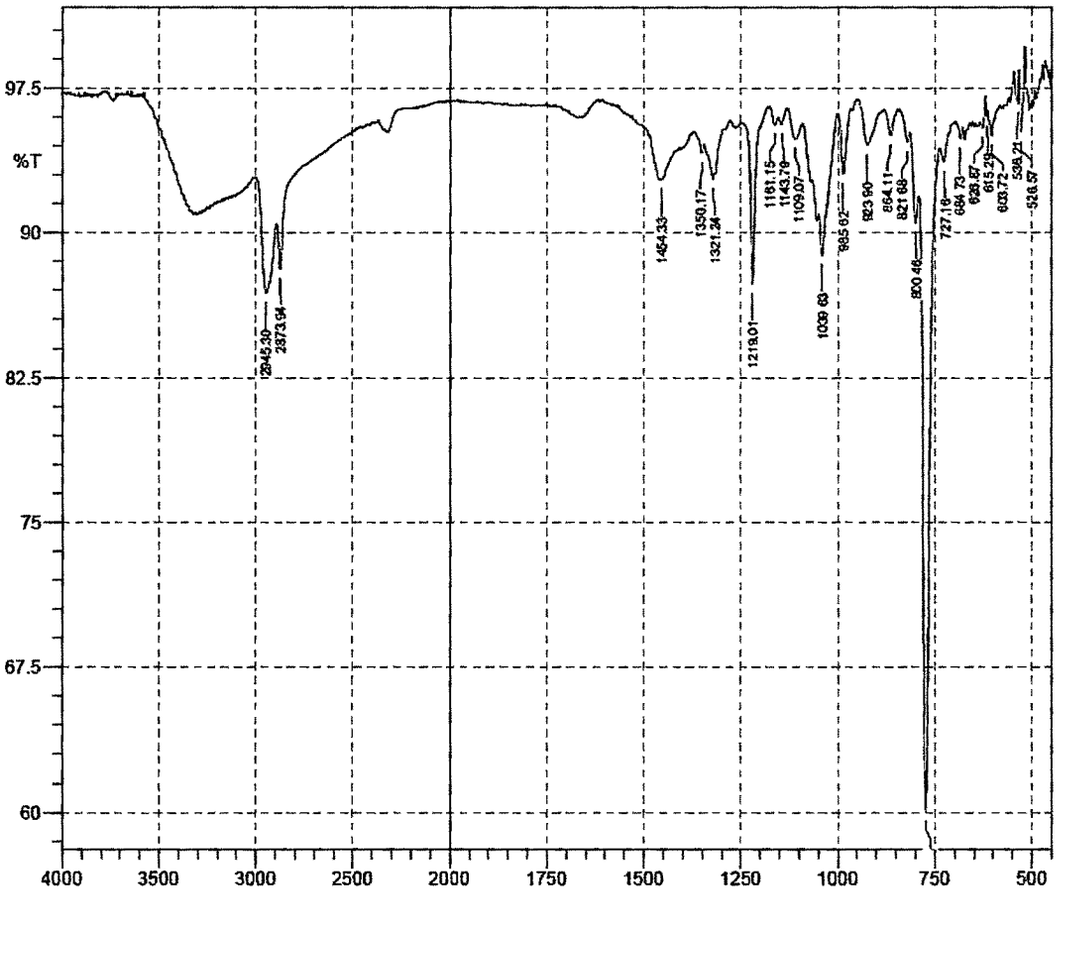
EXHIBITS

HPLC Chromatogram of diol

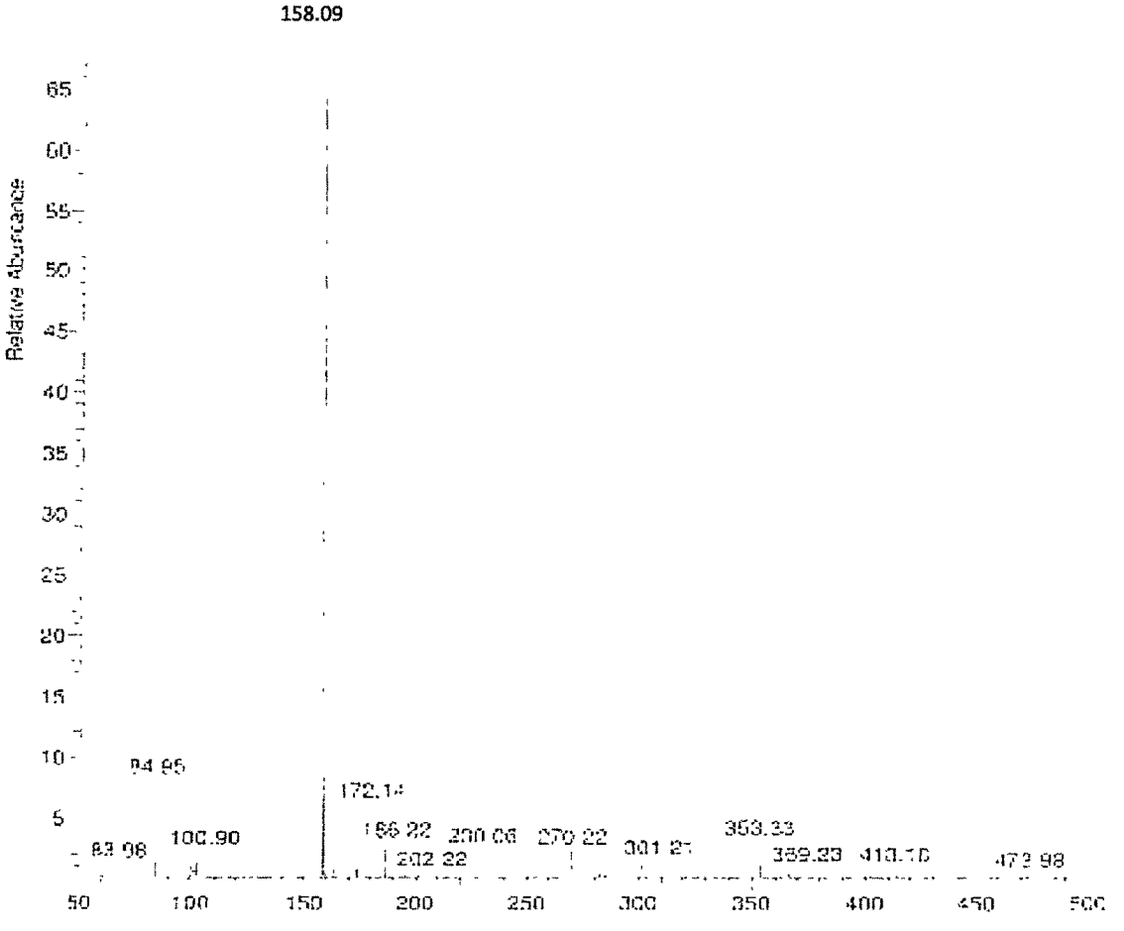


Peak#	Ret. Time	Area	Area %	Name
1	4.37	43779	0.22	Unknown impurity
2	4.76	483365	2.47	Unknown impurity
3	5.01	59785	0.31	Unknown impurity
4	6.09	45339	0.23	Unknown impurity
5	7.18	29794	0.15	Unknown impurity
6	7.70	81547	0.42	Unknown impurity
7	8.90	18501688	94.53	DIOL
8	10.40	28640	0.15	Unknown impurity
9	13.06	83424	0.43	Unknown impurity
10	16.20	22129	0.11	Unknown impurity
11	19.57	172538	0.88	Unknown impurity
12	26.58	6427	0.03	Unknown impurity
13	31.14	14665	0.07	Unknown impurity
Total		19573120	100.00	

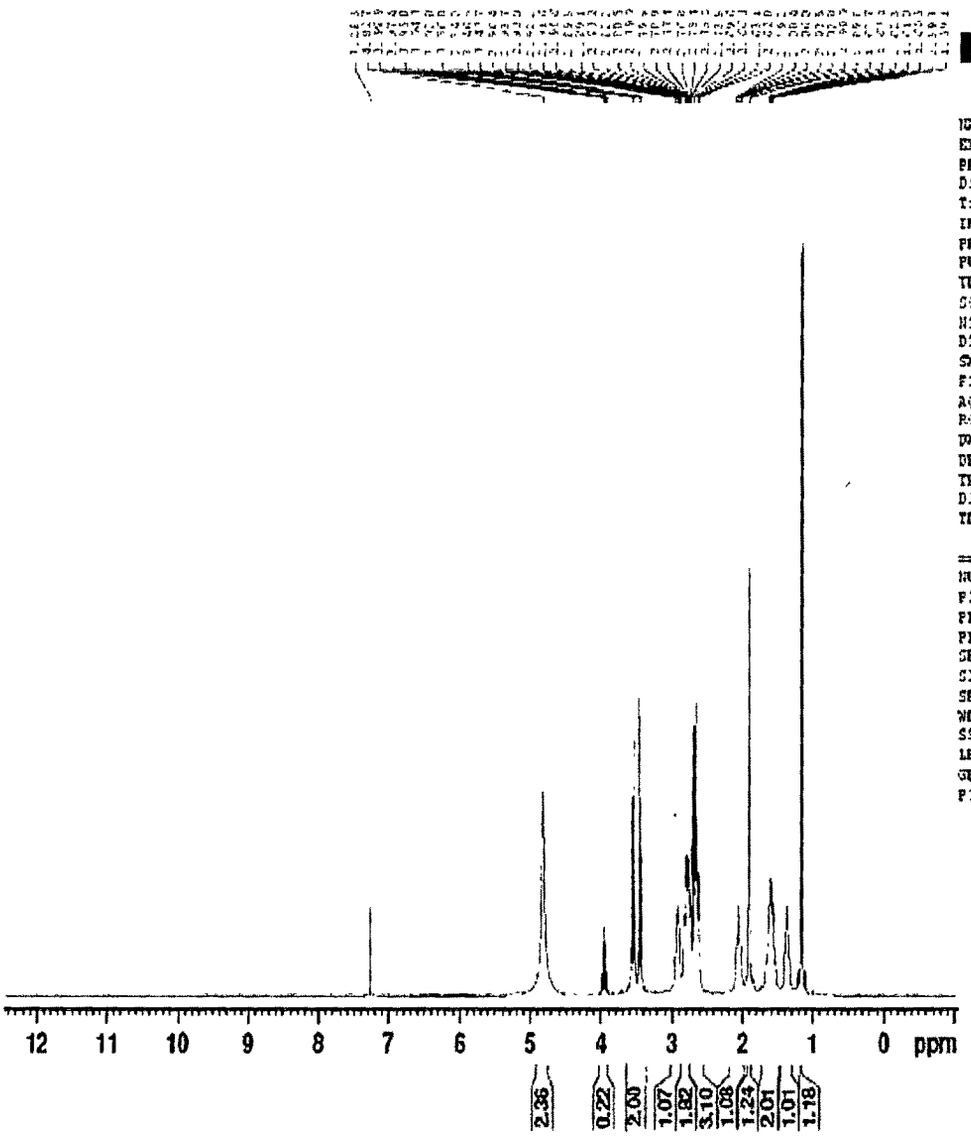
IR Spectra of diol

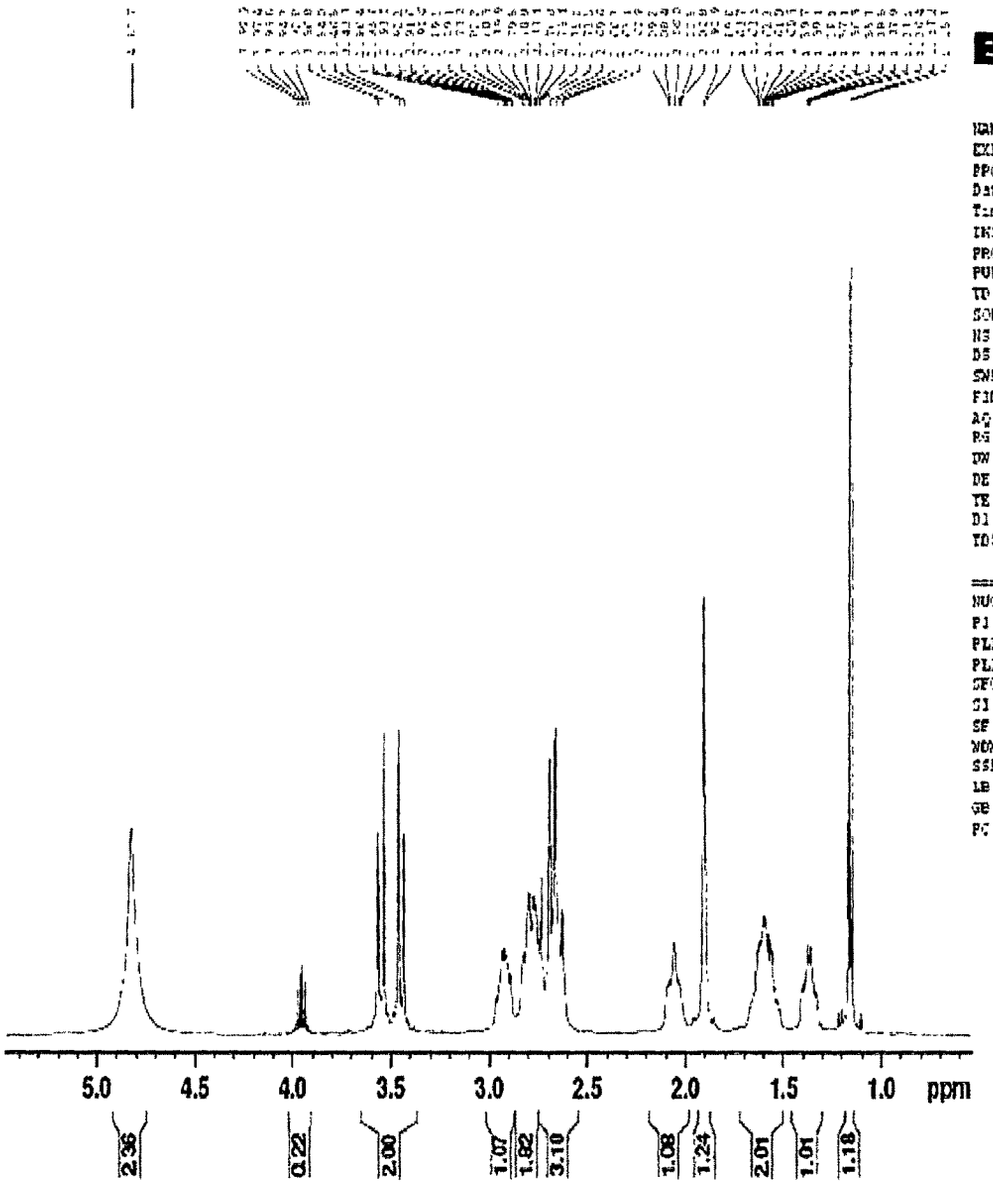


Mass Spectra of diol

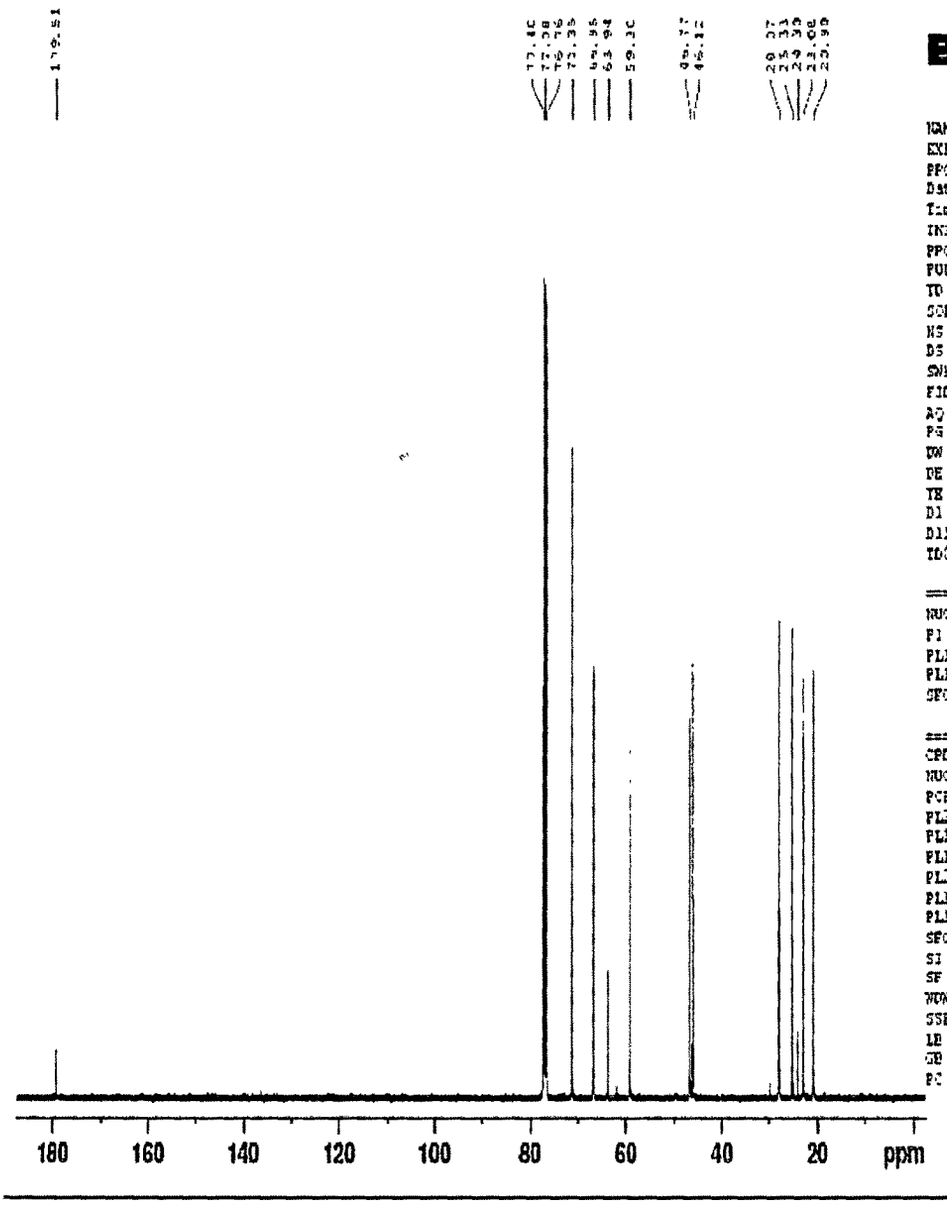


^{1}H NMR Spectra of diol




BRUKER
NAME 130313 Apixaba
EXPNO 5
PROCNO 1
Date_ 20130317
Time 17.09
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 32
DS 3
SWH 8223.685 Hz
FIDRES 0.125483 Hz
AQ 3.9846387 sec
RG 36
DW 60.800 usec
DE 6.50 usec
TE 295.7 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 11.20 usec
PL1 0.00 dB
PL1W 17.35713577 W
SFO1 400.1329009 MHz
SI 32768
SF 400.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
2.36
0.22
2.00
1.07
1.82
3.10
1.08
1.24
2.01
1.01
1.18

[13] Carbon



BRUKER

NAME	150313 Apicore
EXPNO	6
PROCNO	1
Date_	20130317
Time	19.03
INSTRUM	spect
PROBHD	5 mm PABBO BB-
PULPROG	zgpg30
TD	65536
SOLVENT	CDCl3
NS	2048
DS	4
SWH	26041.666 Hz
FIDRES	0.397364 Hz
AQ	1.2583412 sec
RG	203
DW	19.200 usec
DE	6.50 usec
TE	294.7 K
D1	2.00000000 sec
D11	0.03000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	13C
P1	9.00 usec
PL1	1.00 dB
PL1W	63.25595093 W
SFO1	100.6228099 MHz
======== CHANNEL f2 ========	
CPDPRG2	waltz16
NUC2	1H
PCPD2	90.00 usec
PL2	1.00 dB
PL12	17.67 dB
PL13	20.67 dB
PL2W	17.15713577 W
PL12W	0.28348099 W
PL13W	0.14206201 W
SFO2	400.1316005 MHz
SI	32768
SF	100.6127690 MHz
WDW	EM
SSB	0
LB	1.00 Hz
GB	0
PC	1.40

PHYSICO-CHEMICAL CHARACTERIZATION OF CEVIMELINE N-OXIDE

The Physico-chemical characterization of Cevimeline N-oxide sourced from Toronto Research chemicals Inc. was established by analytical techniques such as Mass and proton NMR.

1.0 PHYSICAL PROPERTIES

2.0 APPEARANCE

Table-1: (Appearance results for Cevimeline N-oxide)

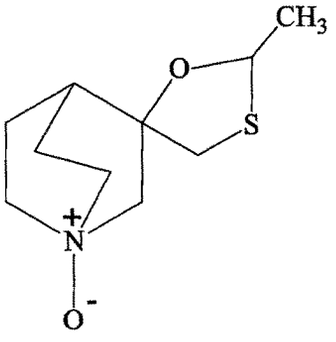
Name	Appearance
Cevimeline N-Oxide	White solid

3.0 CHEMICAL PROPERTIES

3.1 Chemical Name: 2-methyl-1'-azaspiro [[1,3] oxathiolane-5,3'-bicyclo[2.2.2]octane] 1-Oxide **(or)** (2'R, 3R)-rel-2'-Methylspiro[1-azabicyclo[2.2.2]octane-3,5'-oxathiolane]-1-Oxide

3.2 Common Name: Cevimeline N-oxide

3.3 Structural Formula



3.4 Molecular Formula: $C_{10}H_{17}NO_2S$

3.5 Molecular Weight: 215.31

3.6 CAS number: [469890-14-0]

3.7 CHARACTERIZATION BY MASS SPECTROMETRY

The MASS spectroscopy of Cevimeline N-oxide was performed for the parent ion scans using infusion-MS LC mass spectrometer. The result is depicted in **Table-2.** The mass spectrum is depicted in **Exhibits below.**

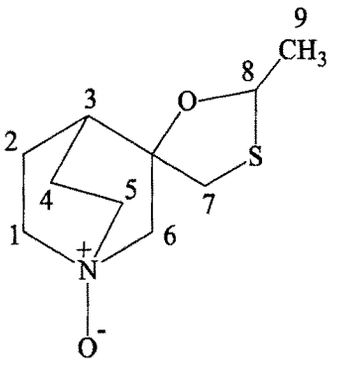
Table-2 Molecular ion data of Cevimeline N-oxide by infusion-MS Technique

Name	m/z value	Molecular weight of Cevimeline N-oxide
Cevimeline N-Oxide	216.52	215.31

Conclusion: Confirms the Mass of Cevimeline N-oxide

3.8 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER

The proton NMR of Cevimeline N-oxide was performed by 400 MHz Nuclear magnetic resonance spectrometer by preparing sample in Deuterated chloroform ($CDCl_3$). The NMR spectra for Proton scans is depicted in **Exhibits**.



Proton (^{1}H) NMR

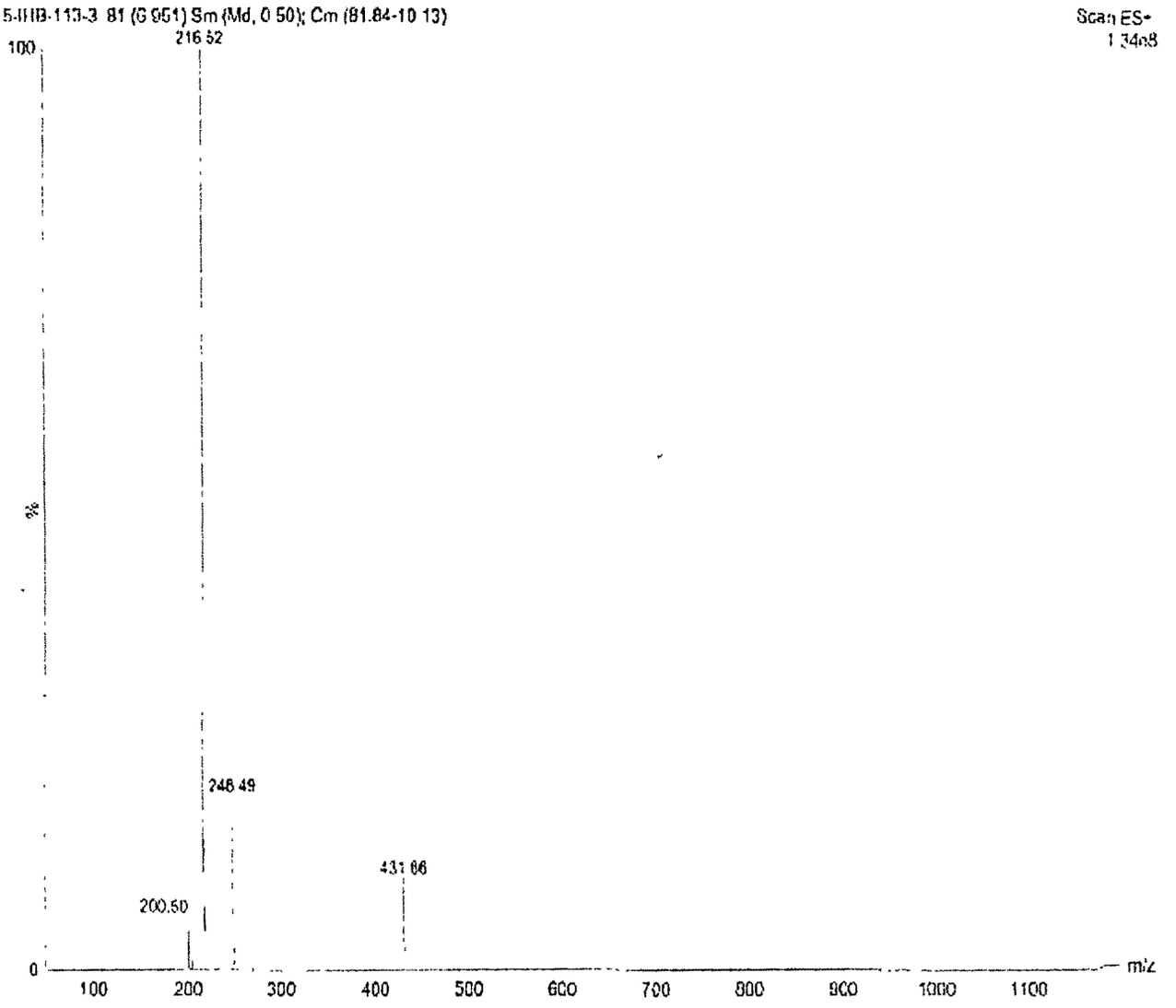
Position	^{1}H	δ (ppm)	Multiplicity
1 & 5	4H	2.293-2.318	multiplet
2 & 4	4H	1.713-2.144	multiplet
3	1H	1.216-1.232	multiplet
6 & 7	4H	2.989-3.256	multiplet
8	1H	5.169-5.212	quartet
9	3H	1.582-1.614	doublet

Conclusion: Proton (1H) NMR confirms the structure of Cevimeline N-oxide.

4.0 STORAGE CONDITION

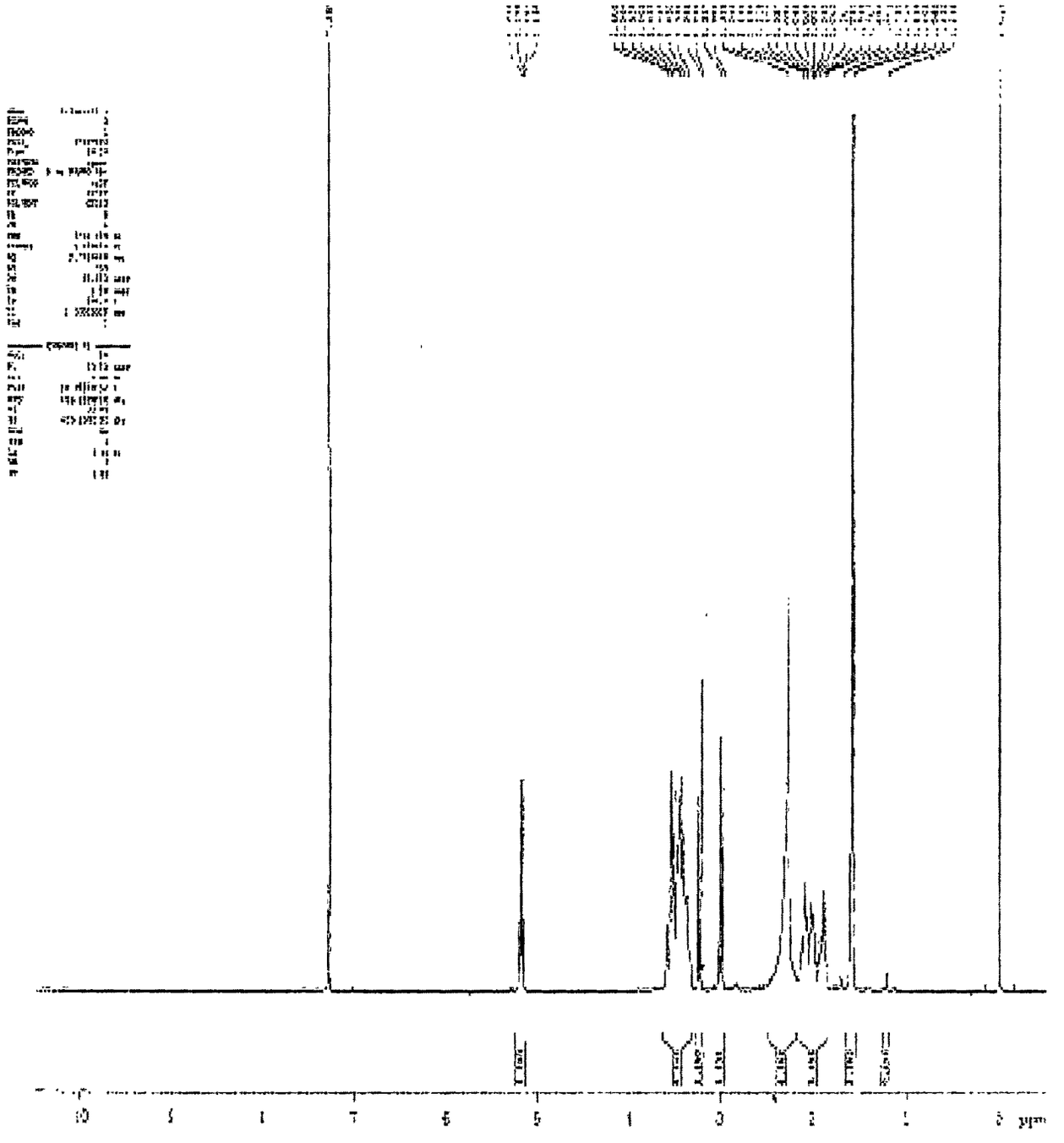
Store at -20°C Freezer

Mass Spectra of Cevimeline N-oxide



^{1}H NMR Spectra of Cevimeline N-oxide

Proton



PHYSICO-CHEMICAL CHARACTERIZATION OF CEVIMELINE SULFOXIDE

1.0 INTRODUCTION

The physico-chemical characterization of Cevimeline sulfoxide was established by analytical techniques such as FT-IR, HPLC, Mass and NMR analysis.

2.0 PHYSICAL PROPERTIES

2.2 APPEARANCE

Table-1 Appearance results for Cevimeline sulfoxide

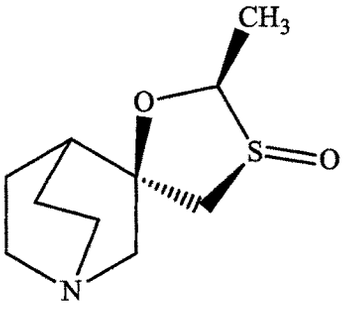
Name	Appearance
Cevimeline sulfoxide	Brown color semi solid

3.0 CHEMICAL PROPERTIES

3.1 Chemical Name: (2'R,3R,3'S)-rel- 2'-Methylspiro [1-azabicyclo[2.2.2]octane-3,5'-oxathiolane] 3'-Oxide

3.2 Common Name: Cevimeline sulfoxide

3.4 Structural Formula



3.4 Molecular Formula: $C_{10}H_{17}NO_2S$

3.5 Molecular Weight: 215.31

3.6 CAS number: [124751-36-6]

3.7 INFRARED SPECTRUM

The Infrared spectra of Cevimeline sulfoxide was obtained by using FT-IR instrument and is depicted in **Exhibits below.**

Table-2 FT-IR Frequency table of Cevimeline sulfoxide

Bond	Approx. Frequency (cm^{-1})	Intensity and range
$-CH_3$ (Methyl)	2875.86	2865–2845 (stretch)
-C-O-C- (Cyclic ether)	1224.8	1200-1300
S=O	1053.13	1030-1060 (stretch)

Conclusion: The IR frequencies are in-line with the functional groups present in the Cevimeline sulfoxide. Therefore the FT-IR conforms to the structure of Cevimeline sulfoxide.

3.8 CHROMATOGRAPHIC PURITY BY HPLC

The purity of Cevimeline sulfoxide was determined by Liquid Chromatography using the developed method. The results are depicted in **Table-3.**

Table-3 Chromatographic purity by HPLC

Name	Chromatographic purity
Cevimeline sulfoxide	98.14%

3.9 CHARACTERIZATION BY MASS SPECTROMETRY

The MASS of Cevimeline sulfoxide was performed for the parent ion scans using infusion-MS and infusion-MS/MS techniques on Quattro-LC mass spectrometer (Micromass-LC/MS/MS system). The result is depicted in **Table-4**. The mass spectrum is depicted in **Exhibits.**

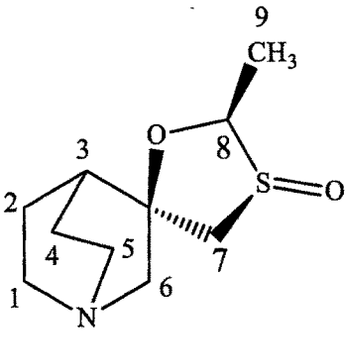
Table-4 Molecular ion data for Cevimeline sulfoxide by infusion-MS Technique

Name	m/z value	Molecular weight of Cevimeline sulfoxide
Cevimeline sulfoxide	216.14	215.31

Conclusion: Confirms the Mass of Cevimeline sulfoxide.

3.10 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER

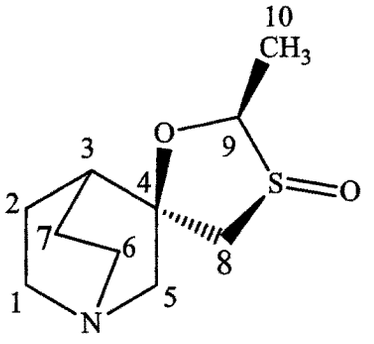
The NMR of Cevimeline sulfoxide was performed by 400 MHz Bruker Nuclear magnetic resonance spectrometer by preparing sample in Deuterated chloroform ($CDCl_3$). The NMR spectra for proton scans and carbon scans are depicted in **Exhibits** and interpretation is provided below.



Proton (^{1}H) NMR

Position	^{1}H	δ (ppm)	Multiplicity
1, 5 & 7	6H	2.657-2.973	doublet
2 & 4	4H	1.625-1.716	multiplet
3	1H	1.827-1.897	multiplet
6	2H	2.471-2.551	multiplet
7	2H	3.253-3.288	multiplet
8	1H	4.603-4.650	quartet
9	3H	1.186-1.573	multiplet

Carbon (^{13}C) NMR



Position	δ (ppm)
C10	15.66
C9	101.74
C8	57.11
C7	21.81
C6	45.58
C5	59.05
C4	86.85
C3	25.79
C2	19.20
C1	45.82

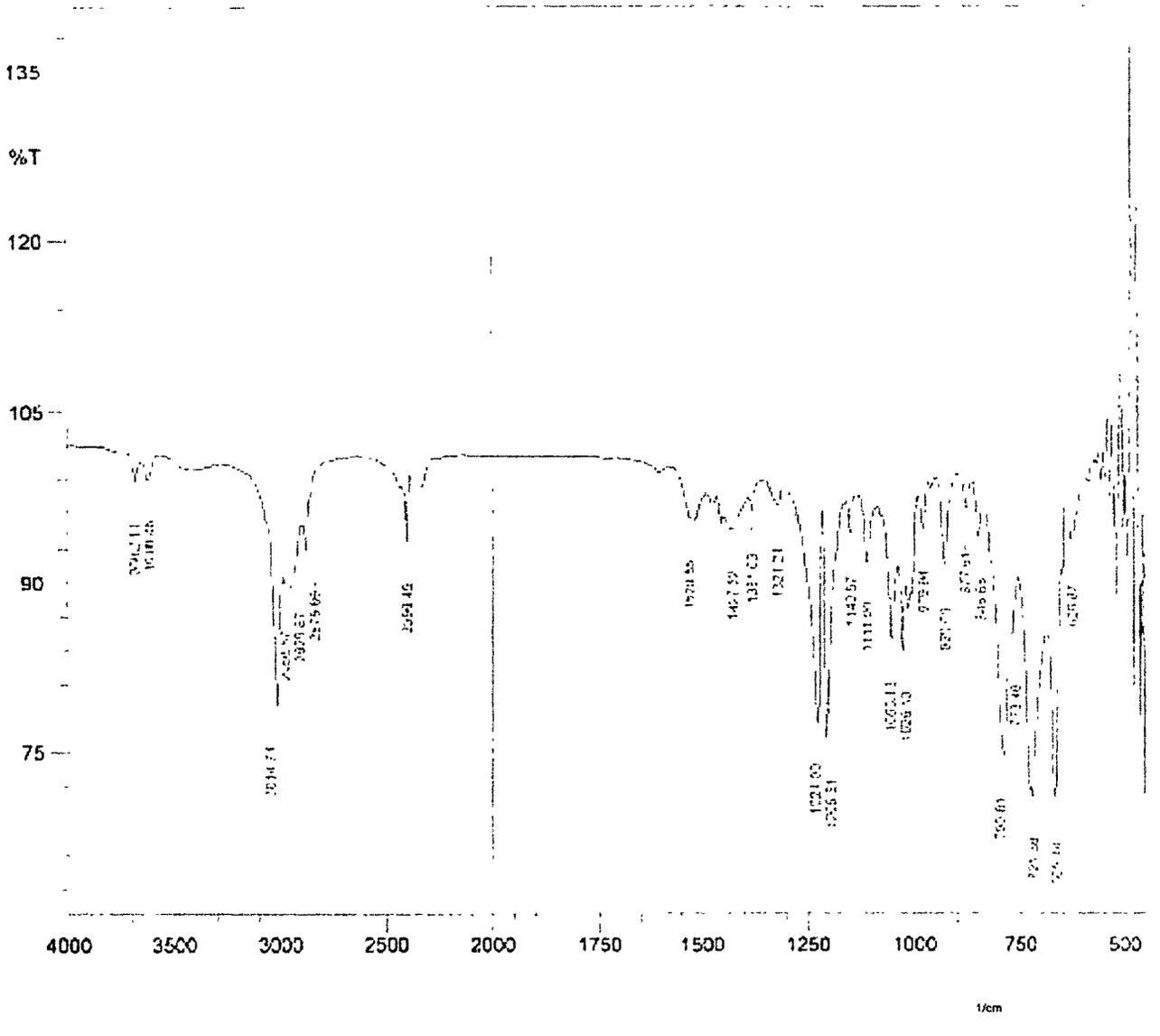
Conclusion: Proton (^{1}H) and carbon (^{13}C) NMR confirms the structure of Cevimeline Sulfoxide.

4.0 STORAGE CONDITION

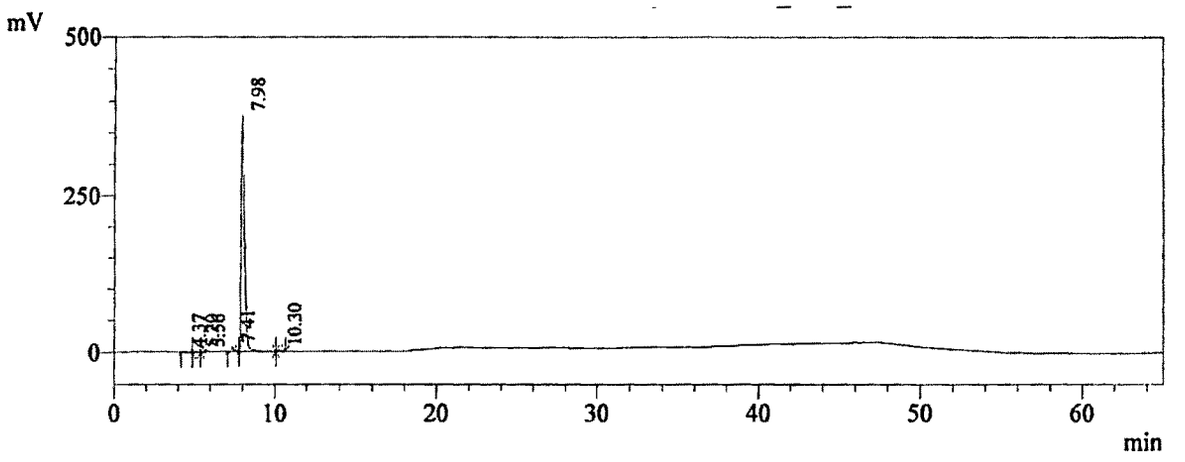
Store in refrigerator

EXHIBITS

Infrared Spectrum of Cevimeline Sulfoxide for identification



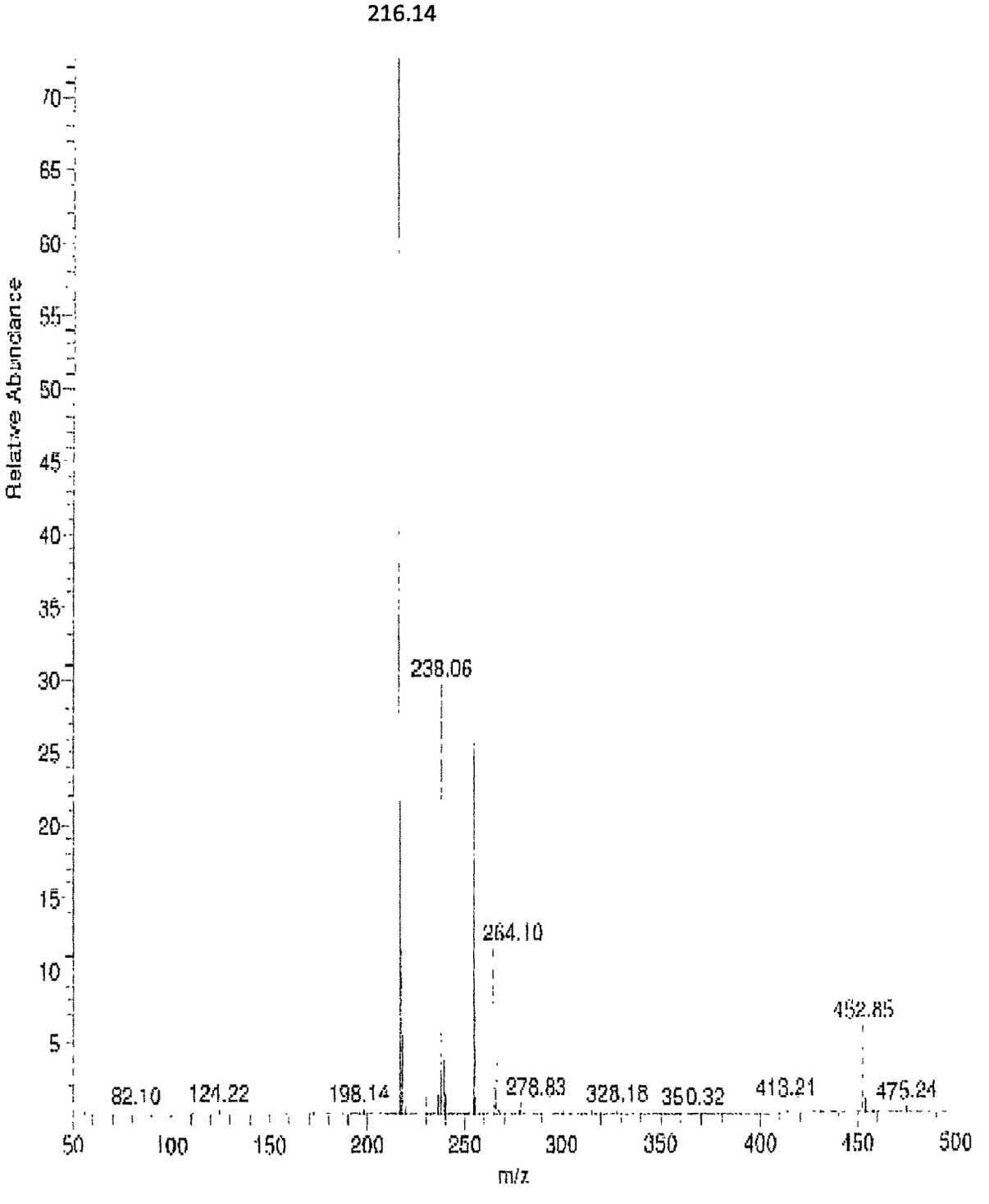
Chromatograms of Chromatographic purity by HPLC



Detector A Ch1 210nm

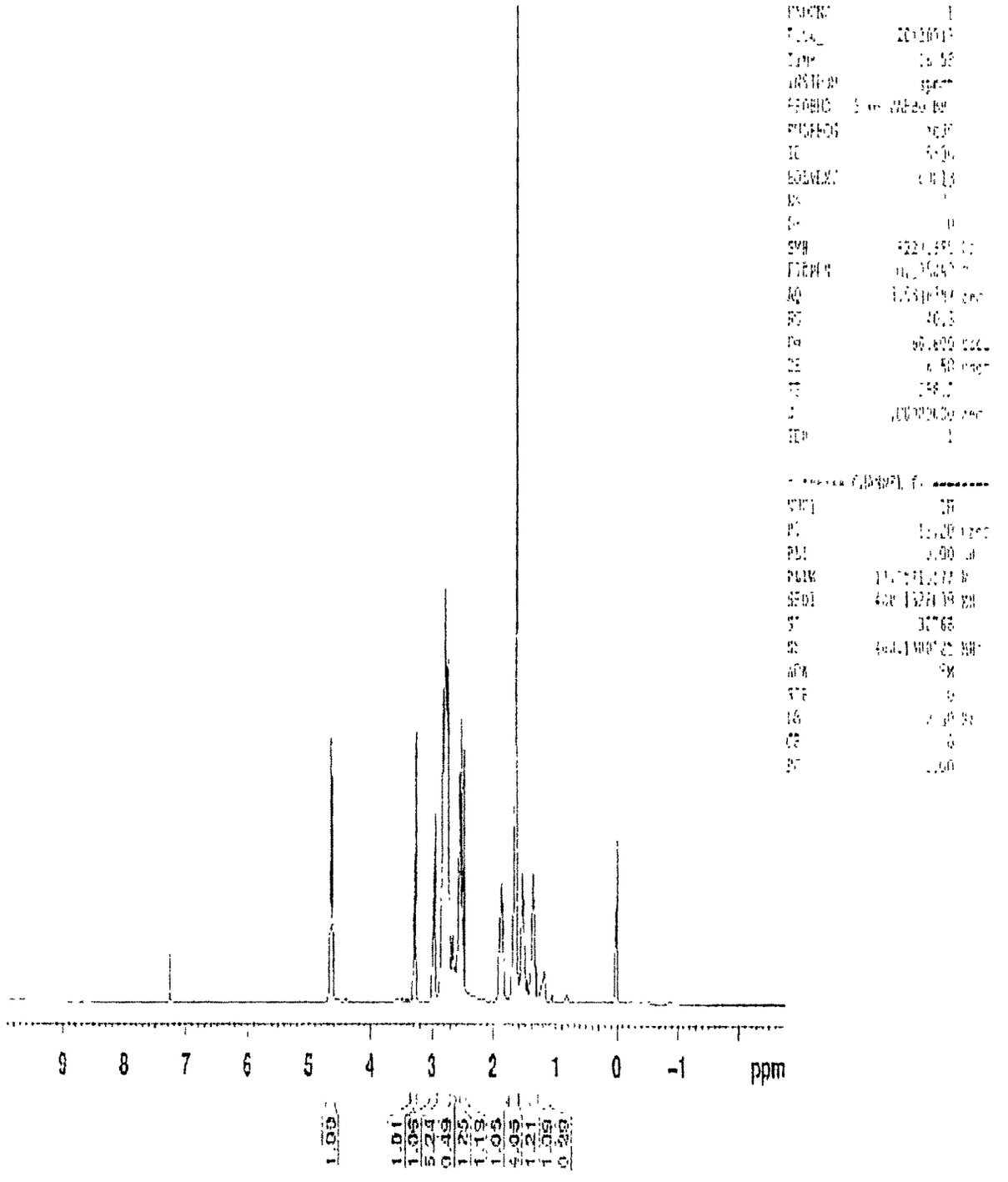
Peak#	Ret. Time	Area	Area %	Name
1	4.37	9785	0.18	Unknown impurity
2	5.20	5500	0.10	Unknown impurity
3	5.56	5112	0.09	Unknown impurity
4	7.41	64305	1.16	Unknown impurity
5	7.98	5448050	98.14	cevimeline sulfoxide
6	10.30	18636	0.34	Unknown impurity
Total		5551388	100.00	

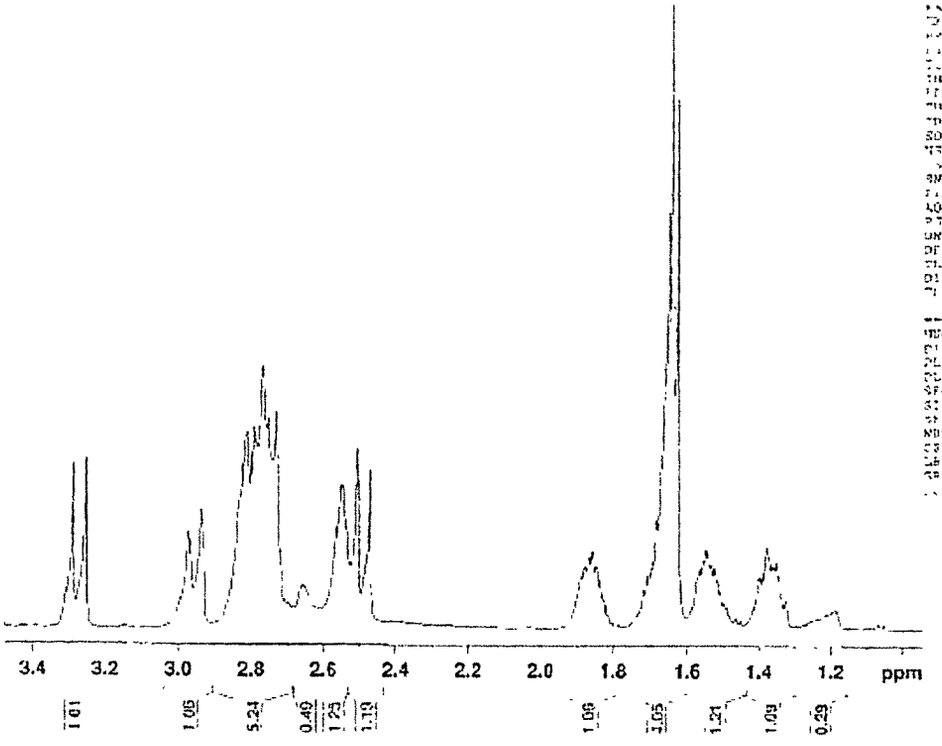
Mass Spectra of Cevimeline Sulfoxide



^{1}H NMR of Cevimeline sulfoxide

Proton

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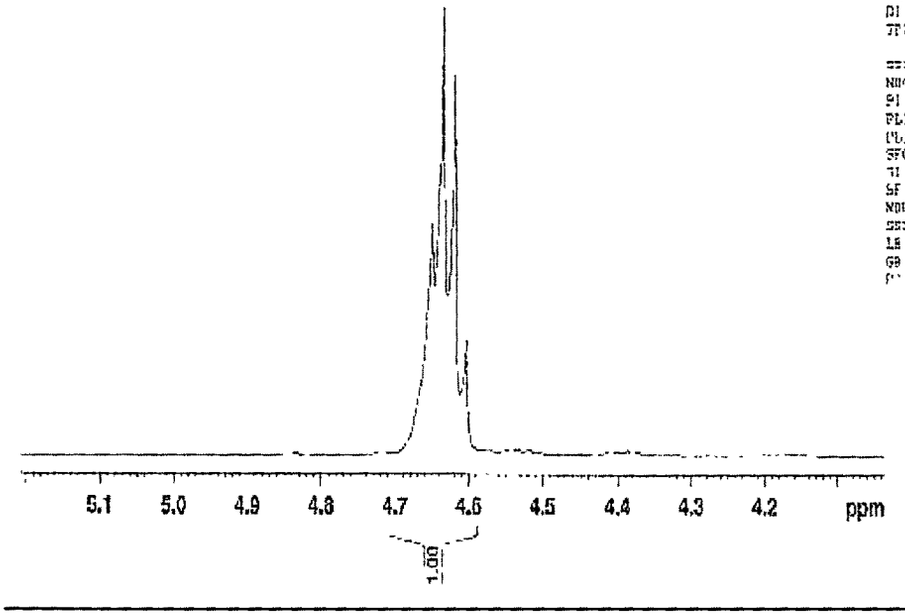
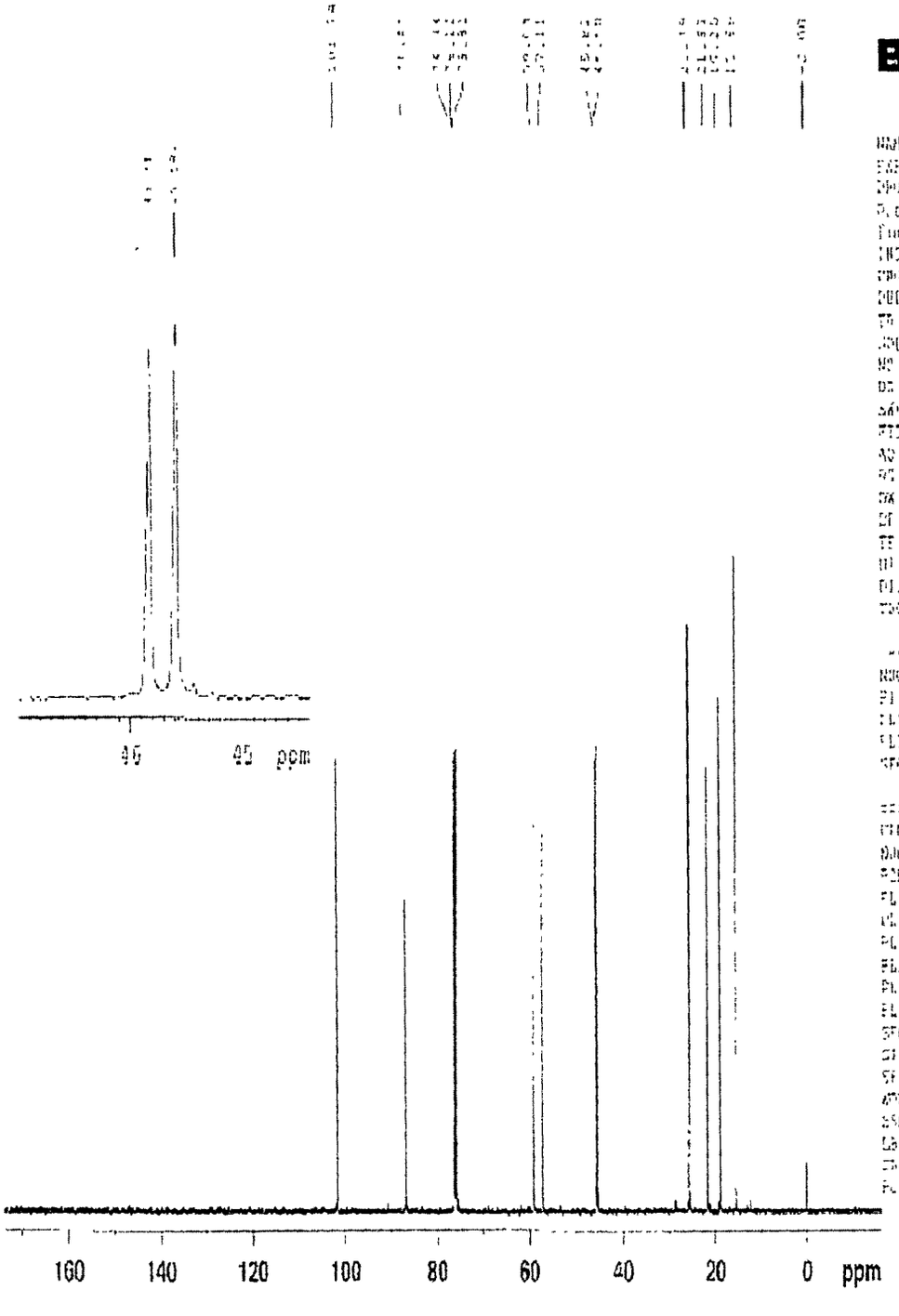
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
ppm
1.01
1.05
5.24
0.49
1.23
1.19
1.09
1.05
1.21
1.09
0.29


5.1
5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
ppm
1.00

Carbon



PHYSICO-CHEMICAL CHARACTERIZATION OF THIOL IMPURITY

The Physico-chemical characterization of Thiol was established by analytical techniques such as FT-IR, HPLC, Mass and ^{13}C NMR.

1.0 INTRODUCTION

This report describes the Physico-chemical characterization of Thiol standard manufactured at Apicore Pharmaceuticals Pvt. Ltd., India

The Physico-chemical characterization of Thiol for batch number A-ST-007-072 was established by analytical techniques such as High performance liquid chromatograph (HPLC), FT-IR, Mass and ^{13}C NMR.

2.0 PHYSICAL PROPERTIES

2.1 APPEARANCE

Table-1 Appearance results for Thiol

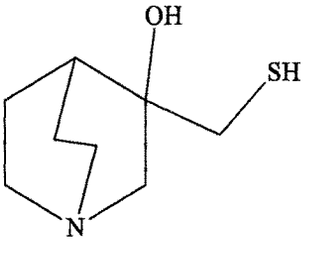
Name	Appearance
Thiol Impurity	A white solid powder

3.0 CHEMICAL PROPERTIES

3.1 Chemical Name : 3-Hydroxy-3-Mercaptomethyl Quinuclidine OR 1-Azabicyclo [2.2.2] octan-3-ol,3-(Mercaptomethyl)

3.2 Common Name : Thiol

3.3 Structural Formula



3.4	**Molecular Formula**	**:**	$C_8H_{15}NOS$
3.5	**Molecular Weight**	**:**	173.27
3.6	**CAS number**	**:**	[107220-26-8]

4.0 CHARACTERIZATION BY INFRARED SPECTROPHOTOMETER (FT-IR)

The Infrared spectra of Thiol was obtained by IR-Prestige-21 instrument.
The FT-IR spectra for Thiol are depicted in **Exhibits below** and interpretation of functional groups are presented in **table-2.**

Table-2 FT-IR Frequency table of Thiol

Bond	Approx. Frequency (cm^{-1})	Intensity and range
-OH	1317.38	Med (1330-1430)
-SH (Thiol)	2576.9	Week (2550-2600)
-CH2	2870.08 and 2929.87	Strong (2850-3000)

Conclusion: The IR frequencies of functional groups are in line with functional groups of Thiol.

9.0 CHROMATOGRAPHIC PURITY BY HPLC

The chromatographic purity of Thiol was determined by high performance liquid chromatograph (HPLC) using the developed test procedure. The result is depicted in **Table-3**. The chromatograms are depicted in **Exhibits.**

Table-3 Chromatographic Purity of Thiol

Name	Average % Chromatographic Purity By HPLC of Thiol
Thiol	86.14 %

10.0 CHARACTERIZATION BY MASS SPECTROMETRY

The Thiol sample was analyzed for the parent ion scan by using infusion-MS on Quattro-LC mass spectrometer. The result data is presented in the following **Table-4**. The mass spectrum is depicted in **Exhibits.**

Table-4 Molecular ion data of Thiol by infusion-MS Technique

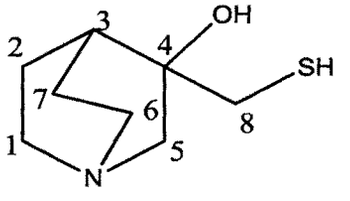
Name	m/z value	(M+H)+	Molecular weight of Thiol
Thiol	174.12	174.12	173.27

Conclusion: Confirms the Mass of Thiol

11.0 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER

The Thiol sample, was analyzed on 400 MHz Nuclear magnetic resonance spectrometer (NMR) by preparing sample in $CDCl_3$. The same was analyzed for the carbon NMR. The NMR spectra for carbon scans are depicted in **Exhibits.**

Carbon (^{13}C) NMR:



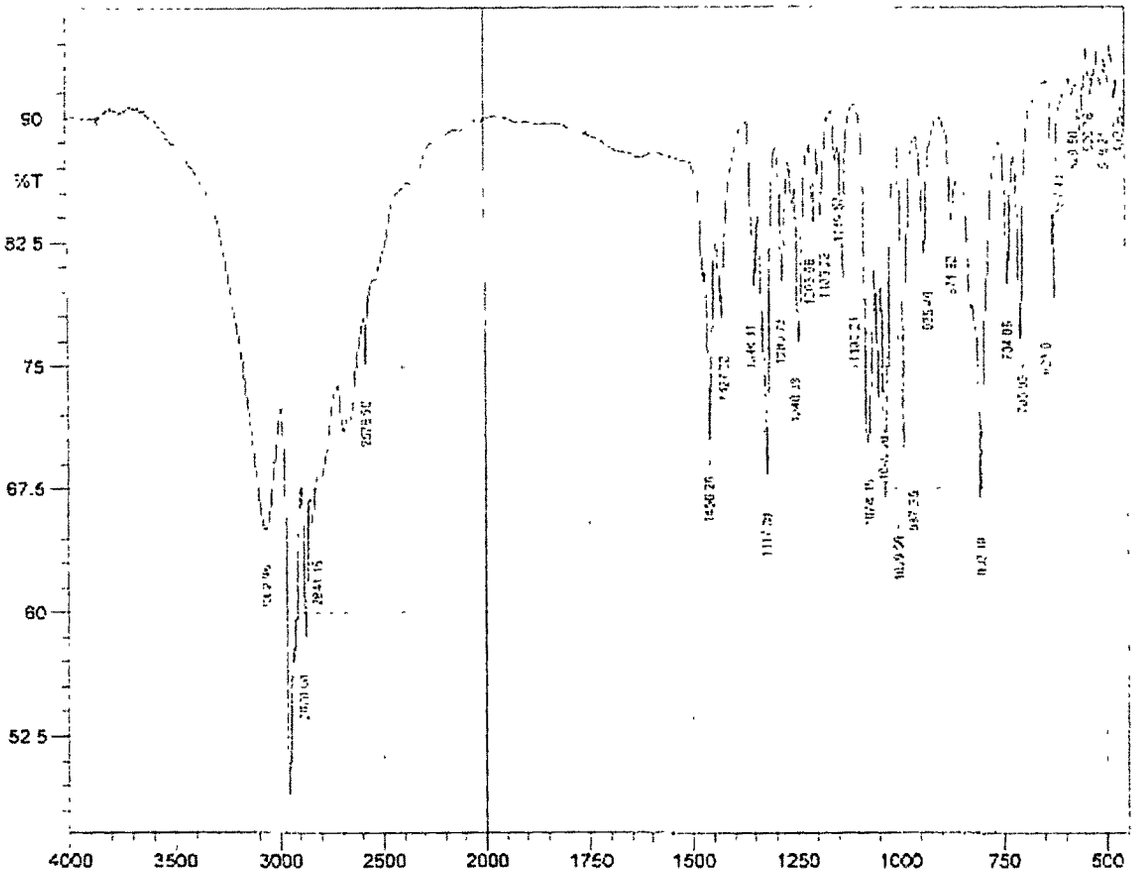
Carbon Position	δ (ppm)
C8	30.02
C7	23.51
C6	62.28
C5	70.05
C4	77.39-76.76
C3	35.28
C2	21.50
C1	46.76-46.41

Conclusion: Carbon (^{13}C) NMR confirms the structure of Thiol.

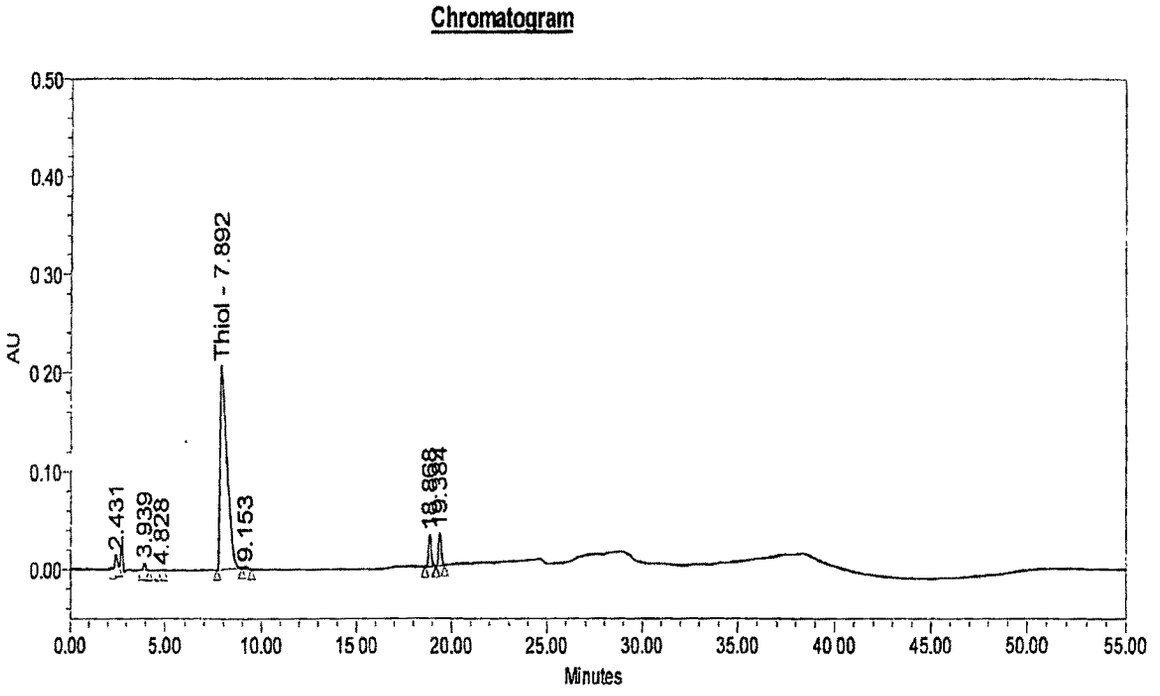
12.0 STORAGE CONDITION

Store at room temperature

IR Spectra of Thiol



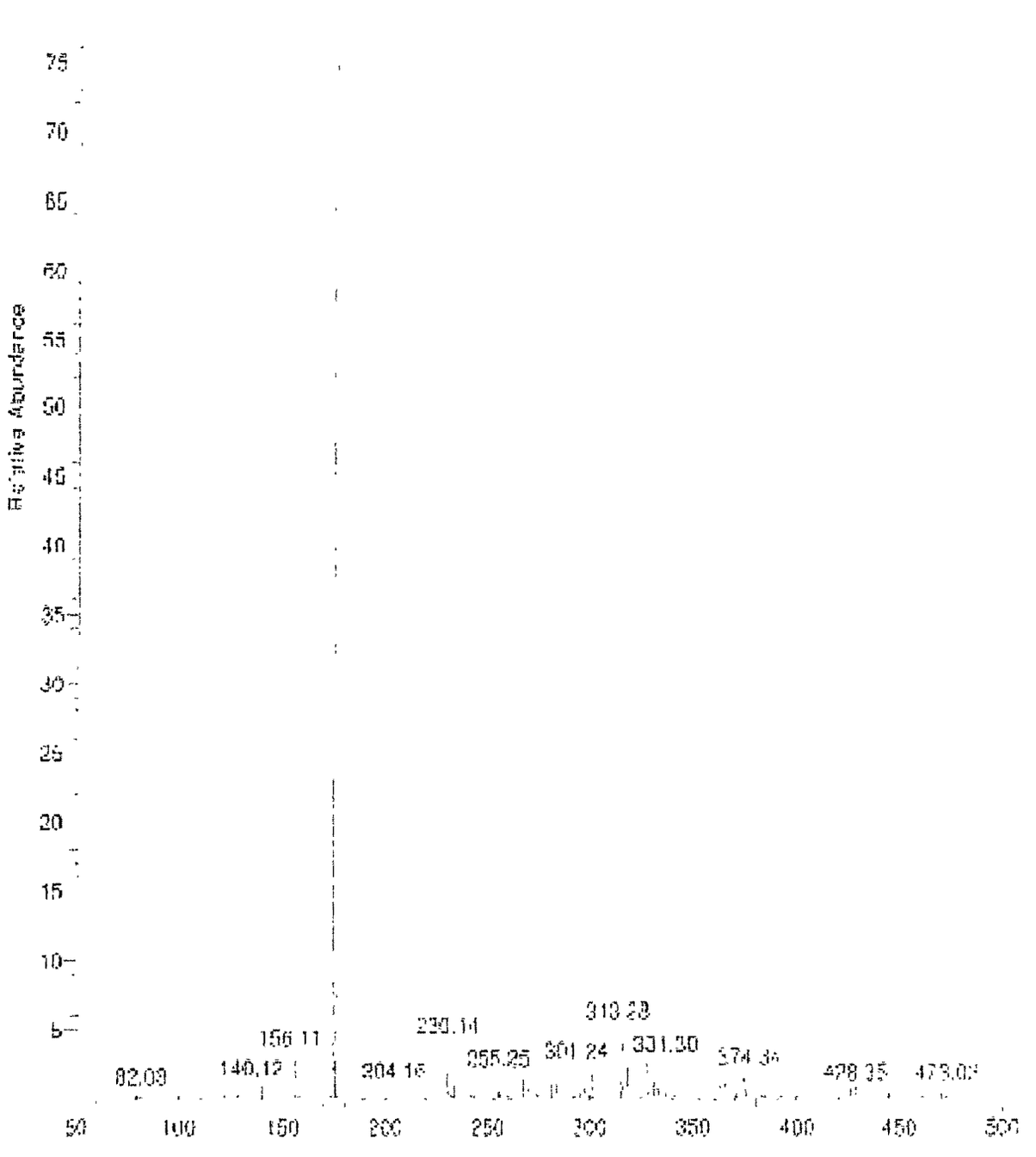
HPLC Chromatogram of Thiol



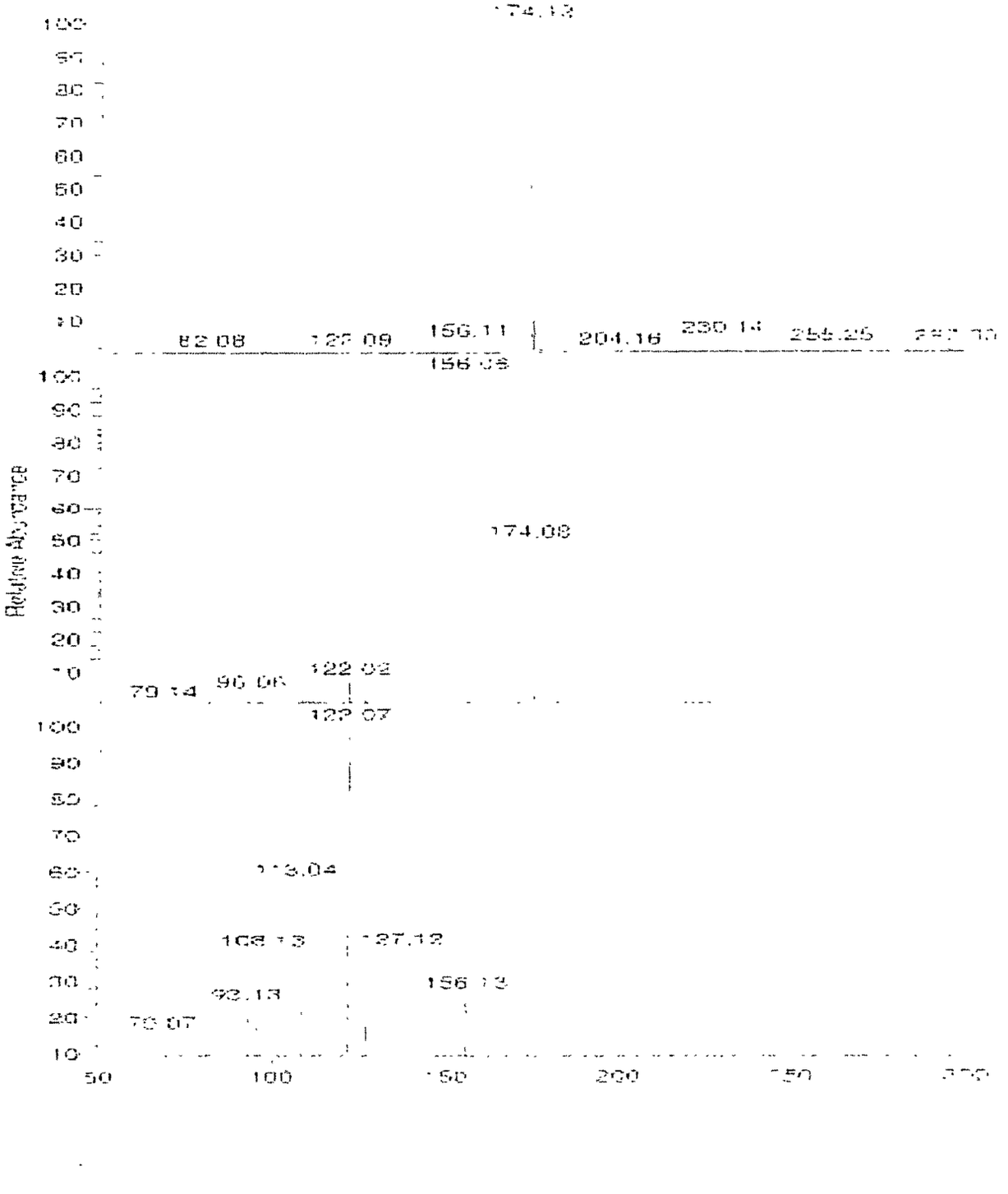
Peak Table

	Peak Name	RT	Area	% Area
1		2.43	109127	1.81
2		3.94	51426	0.85
3		4.83	3155	0.05
4	Thiol	7.89	5211717	86.47
5		9.15	22946	0.38
6		18.87	318034	5.28
7		19.38	310809	5.16
Sum			6027215	100.00

Mass Spectra of Thiol

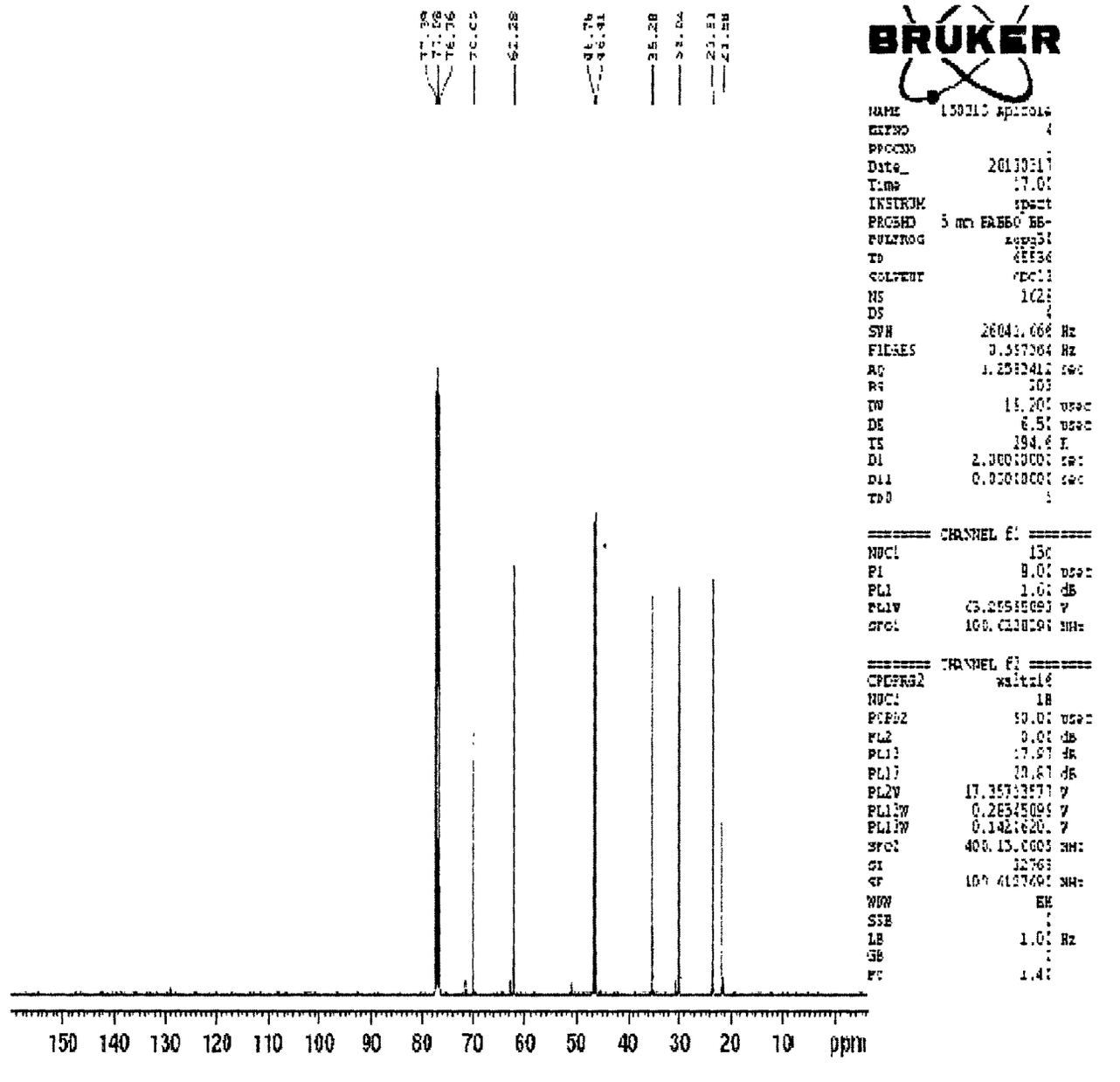


Mass Spectra (MS/MS) of Thiol



NMR Spectra of Thiol

Carbon



PHYSICO-CHEMICAL CHARACTERIZATION OF TRANS CEVIMELINE HYDROCHLORIDE [TRANS ISOMER]

1.0 INTRODUCTION

The physico-chemical characterization of Trans cevimeline hydrochloride [Trans isomer] was established by analytical techniques such as FT-IR, HPLC, MASS and NMR analysis. The chromatographic purity of trans cevimeline hydrochloride [Trans isomer] was determined by HPLC.

2.0 PHYSICAL PROPERTIES

2.1 APPEARANCE

Table-1 Appearance of trans-cevimeline hydrochloride [Trans Isomer]

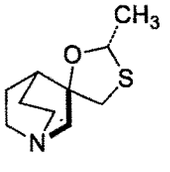
Name	Appearance
Trans Isomer	White powder

3.0 CHEMICAL PROPERTIES

3.1 Chemical Name: Trans -2-methylspiro [1, 3-oxathiolane-5, 3-quinuclidine] Hydrochloride Hemihydrate

3.2 Common Name: Trans Cevimeline Hydrochloride [Trans Isomer]

3.3 Structural Formula



3.4	**Molecular Formula**	:	$C_{10}H_{18}ClNOS.1/2\ H_20$
3.5	**Molecular Weight**	:	244.77
3.6	**CAS number**	:	Not Applicable
3.7	**Status**	:	Known Impurity for Cevimeline Hydrochloride

4.0 INFRARED SPECTRUM

The Infrared spectra of Trans Cevimeline hydrochloride [Trans Isomer] was obtained using FT-IR IR-Prestige-21 instrument.

The FT-IR spectra for Trans Cevimeline hydrochloride [Trans Isomer] reference standard batch are depicted in Exhibits below.

Table-2 FT-IR Frequency table of Trans Cevimeline hydrochloride [Trans Isomer]

Bond	Approx. Frequency (cm^{-1})	Intensity and range
-C-O	1643.35	Strong (1630-1695)
-CH3	2929.87	Strong (2850-3000)

Conclusion: The IR frequencies of functional groups are in line with the Trans Cevimeline hydrochloride.

5.0 CHROMATOGRAPHIC PURITY

The chromatographic purity of Trans Cevimeline hydrochloride [Trans Isomer] was determined by High performance liquid chromatograph (HPLC) using developed test procedure. The result is depicted in Table-3. The chromatograms are depicted in Exhibits.

Table-3 Chromatographic Purity of Trans Cevimeline hydrochloride [Trans Isomer]

Name	% Chromatographic Purity By HPLC of Trans Cevimeline hydrochloride [Trans Isomer]
Trans isomer	44.3 %

6.0 MELTING RANGE

The Melting range of Trans Cevimeline hydrochloride [Trans Isomer] was determined using Veego make melting point apparatus. The result is depicted in **Table-4.**

Table-4 Melting range of Trans Cevimeline hydrochloride [Trans Isomer]

Name	Melting range
Trans isomer	205.5 °C to 206.9 °C

7.0 CHARACTERIZATION BY MASS SPECTROMETRY

The Mass of Trans Cevimeline hydrochloride [Trans isomer] was performed for the parent ion scans using infusion-MS and infusion-MS/MS techniques on Quattro-LC mass spectrometer (Micromass- LC/MS/MS system). The result is depicted in Table-5. The mass spectrum is depicted in Exhibits.

Table-5 Molecular ion data for Trans Cevimeline hydrochloride [Trans isomer] by infusion-MS Technique

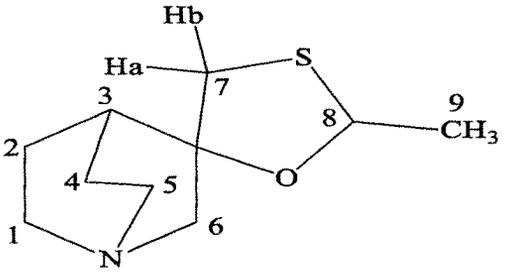
Name	m/z value	(M+H)+	Molecular weight of Trans Cevimeline
Trans Isomer	200.36	200.36	199.31

Conclusion: Confirms the Mass of Trans Cevimeline hydrochloride [Trans isomer]

8.0 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER (INOVA-500, NMR SYSTEM)

^{1}H – NMR Spectroscopy

The 1H – NMR spectrum of Trans Cevimeline HCl was obtained by INOVA-500 NMR Spectrometer at 499.81 MHz by preparing the sample in DMSO-d6. The Proton NMR assignments of Trans-Cevimeline are listed in the table and NMR reports are attached.



Position	^{1}H	δ (ppm)	Multiplicity
1, 5 & 6	6H	3.298-3.433	multiplet
2	2H	3.056-3.158	multiplet
3	1H	2.005-2.062	multiplet
4	2H	2.258-2.437	multiplet
7	Ha	3.544-3.549	doublet
	Hb	3.517-3.522	doublet
8	1H	5.340-5.347	quartet
9	3H	1.528-1.564	doublet

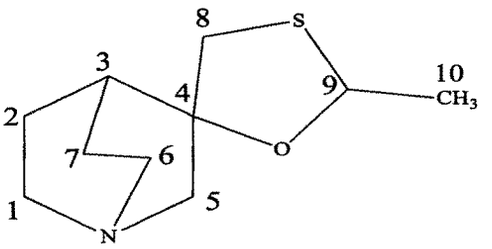
In the above proton assignment it clearly indicates that in the 8th position, observed chemical shift around 5.340-5.347ppm with quartet. If compared with cis-isomer (δ value for 8th position 5.232-5.267ppm), trans-isomer having more δ value at this position. This happens due to less steric hindrance observed in trans-isomer.

Further, trans-isomer of Cevimeline having maximum trans-form and very small amount of Cis-form, hence two different peaks are observed at 8th position (available in the attached Proton NMR report). 5.232-5.267ppm indicates that form is Cis-isomer and 5.340-5.347ppm indicates trans-isomer.

Therefore, based on above theoretical and experimental evaluations it confirms that the observed form is trans-form. The NMR spectra for Proton are depicted in Exhibits.

^{13}C – NMR Spectroscopy

The 13C – NMR spectrum of Trans Cevimeline HCl was obtained by INOVA-500 NMR Spectrometer at 125.67 MHz by preparing the sample in DMSO-d6. The carbon NMR assignments of Trans-isomer are listed in the table and NMR reports are attached. The NMR spectra for carbon scans are depicted in Exhibits.



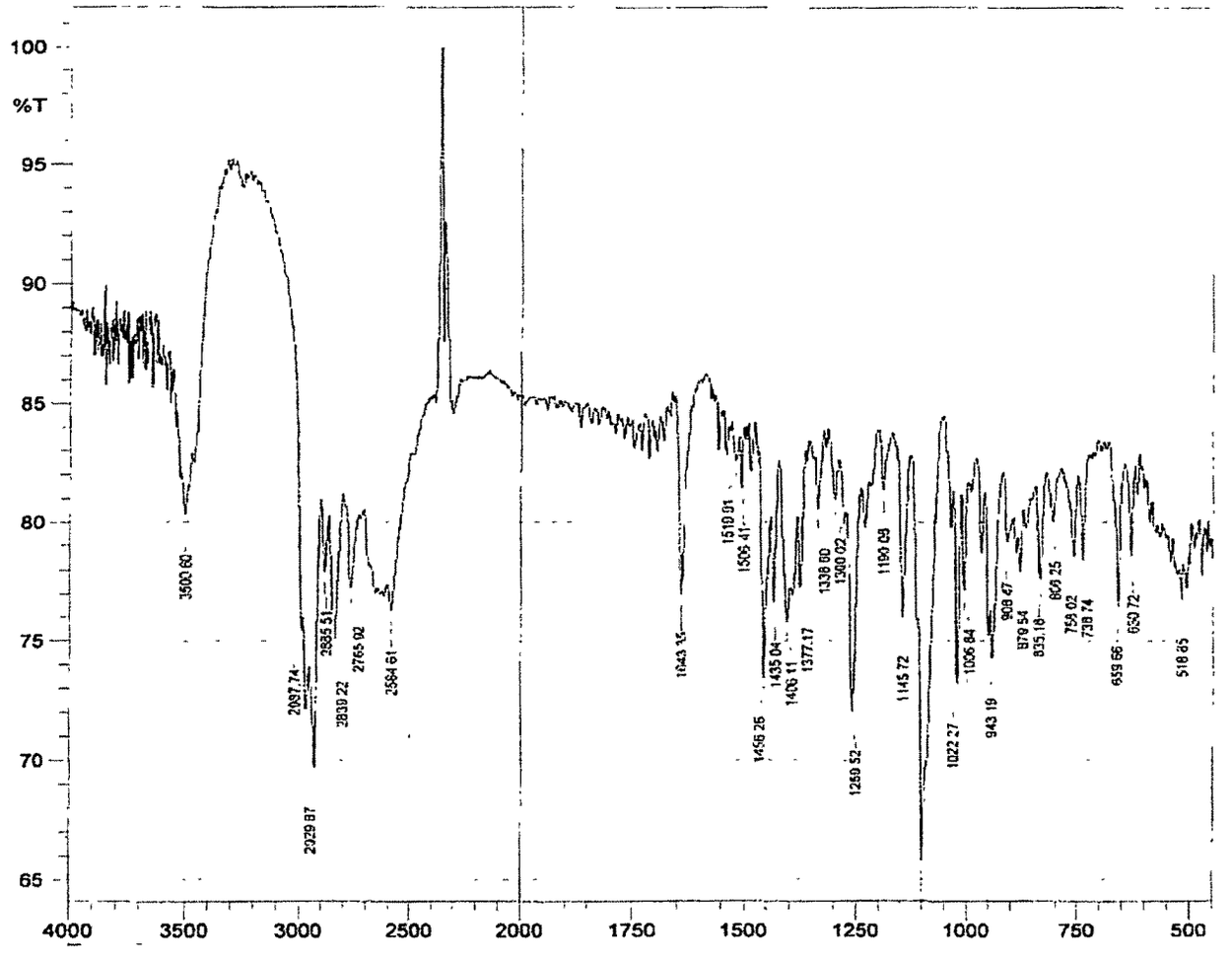
Carbon No.	δ (ppm)
1 & 6	49.731
2	19.757
3	41.438
4	85.027
5	79.942 – 81.216
7	20.146
8	40.869
9	81.216
10	18.964

Conclusion: Proton (1H) and carbon (13C) NMR confirms the structure of Trans Cevimeline Hydrochloride [Trans Isomer].

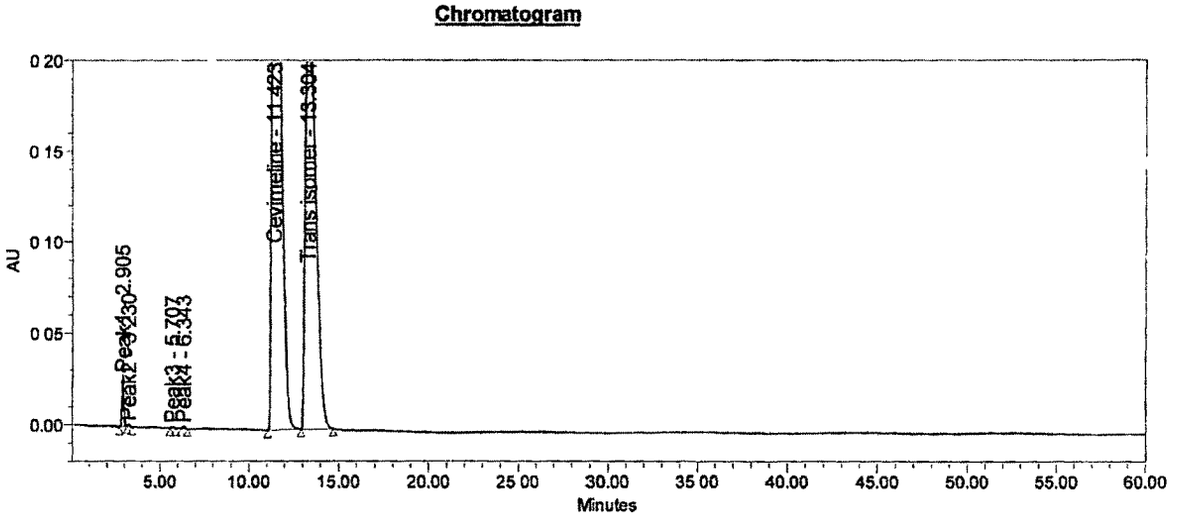
9.0 STORAGE CONDITION

Preserve in tight closed container and store at room temperature.

Infrared Spectrum of Trans Cevimeline hydrochloride [Trans isomer]



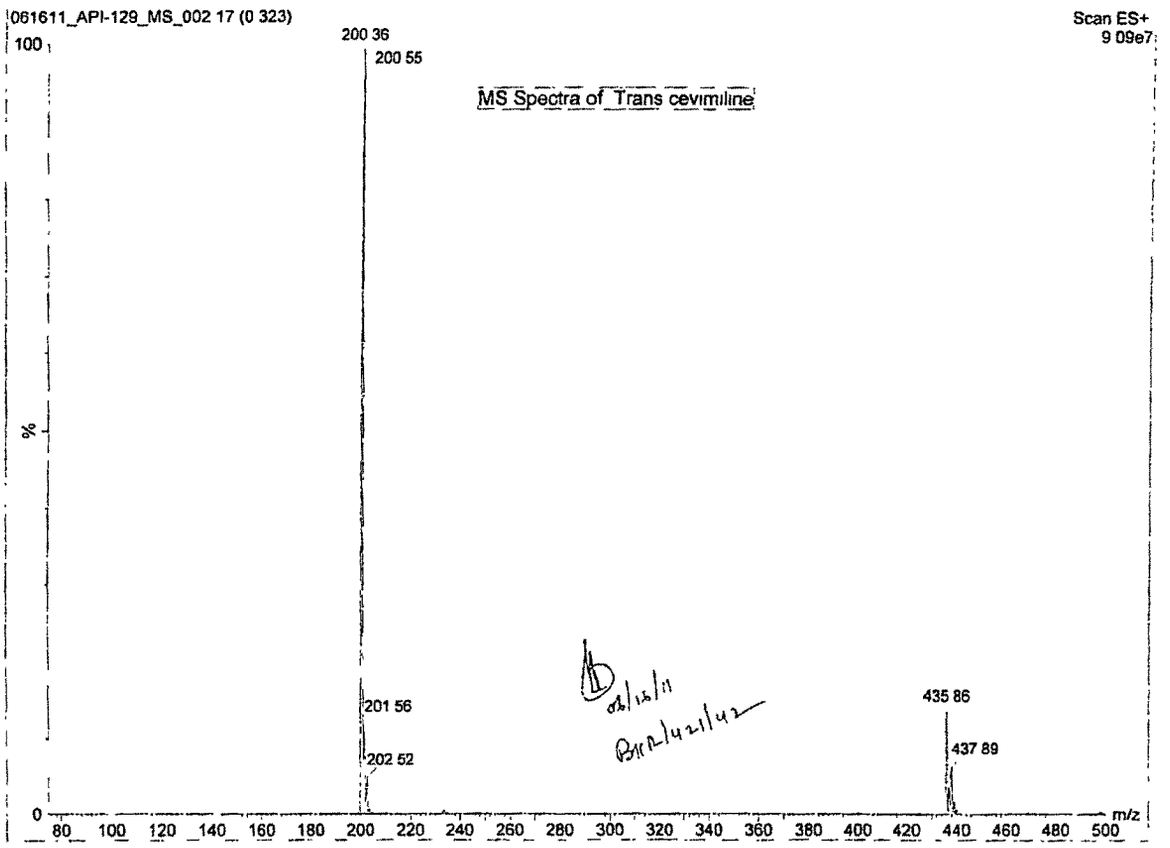
HPLC Chromatograms of Trans Cevimeline hydrochloride [Trans isomer]



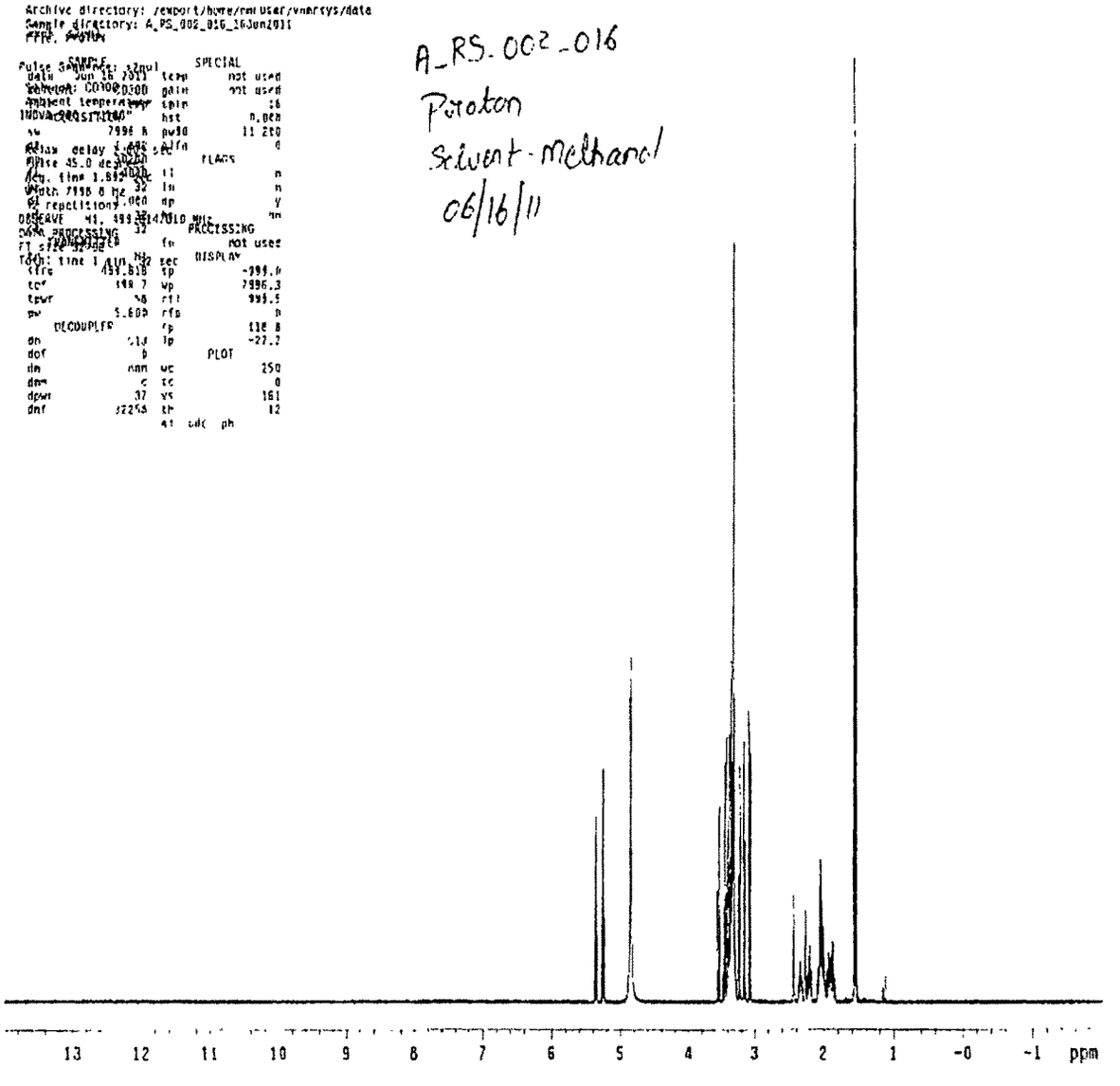
Peak Table

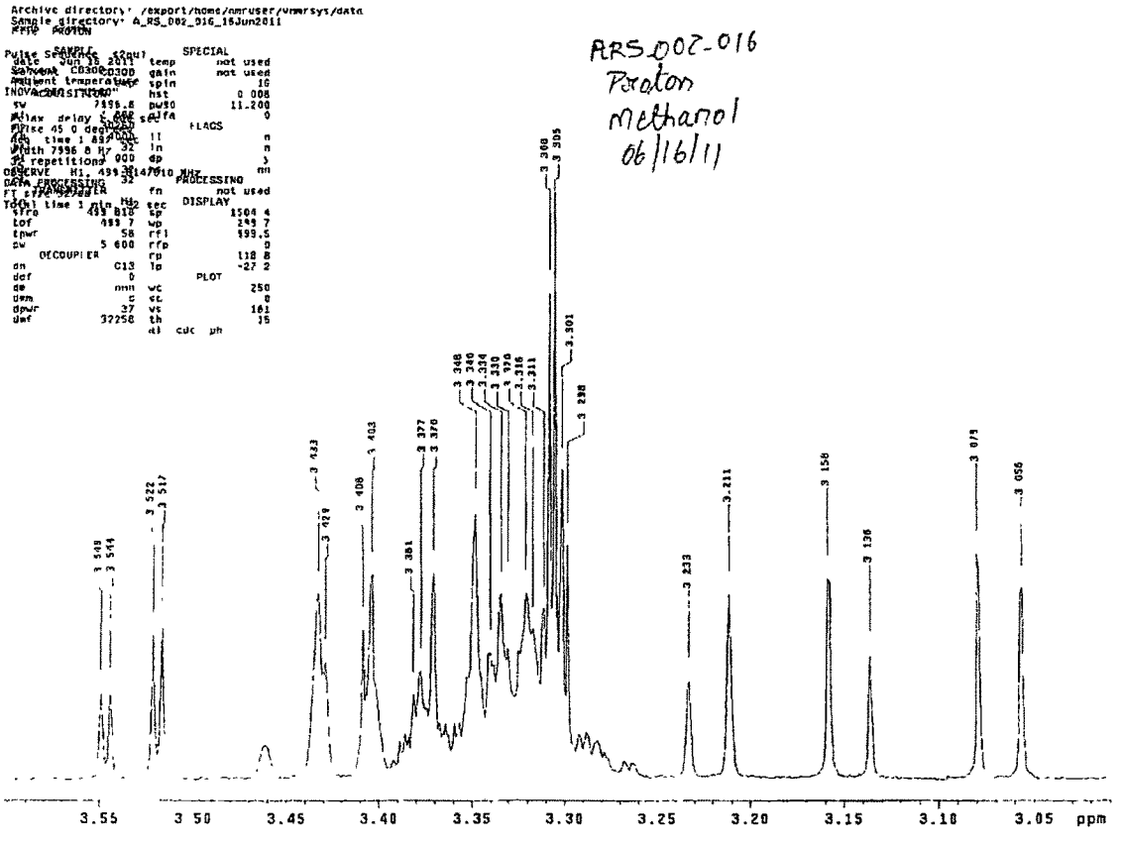
	Peak Name	RT	Area	% Area
1	Peak1	2.91	194104	0.91
2	Peak2	3.23	6136	0.03
3	Peak3	5.71	5685	0.03
4	Peak4	6.34	11117	0.05
5	Cevimeline	11.42	11707356	54.77
6	Trans isomer	13.30	9450264	44.21
Sum			21374661	100.00

Mass Spectra (Molecular ion) of Trans Cevimeline hydrochloride [Trans isomer]



^{1}H NMR of Trans Cevimeline hydrochloride [Trans isomer]

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06/16/11
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3.45
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3.35
3.30
3.25
3.20
3.15
3.10
3.05
ppm

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d1          1 000     dp                   Y
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tof          499 7     wp           983 2
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pw           5 800     rfp                0
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dn              C13     lp           -27 2
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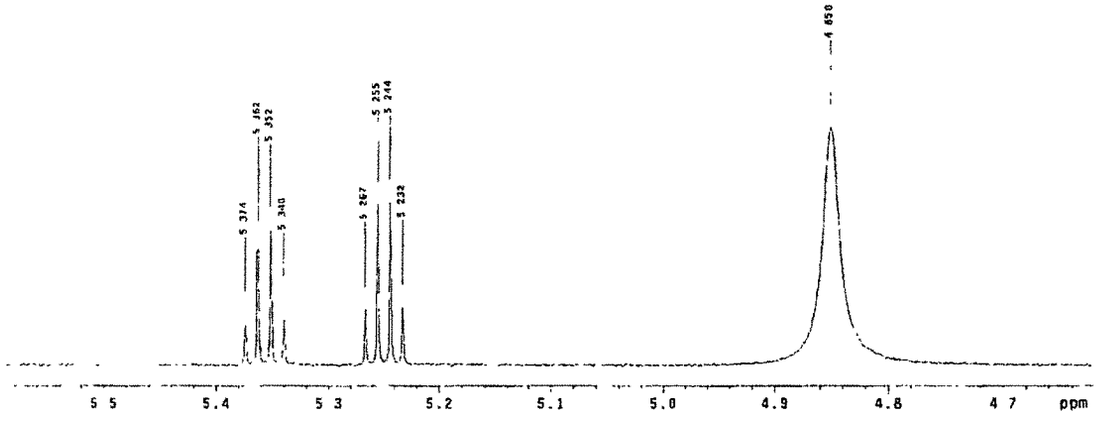
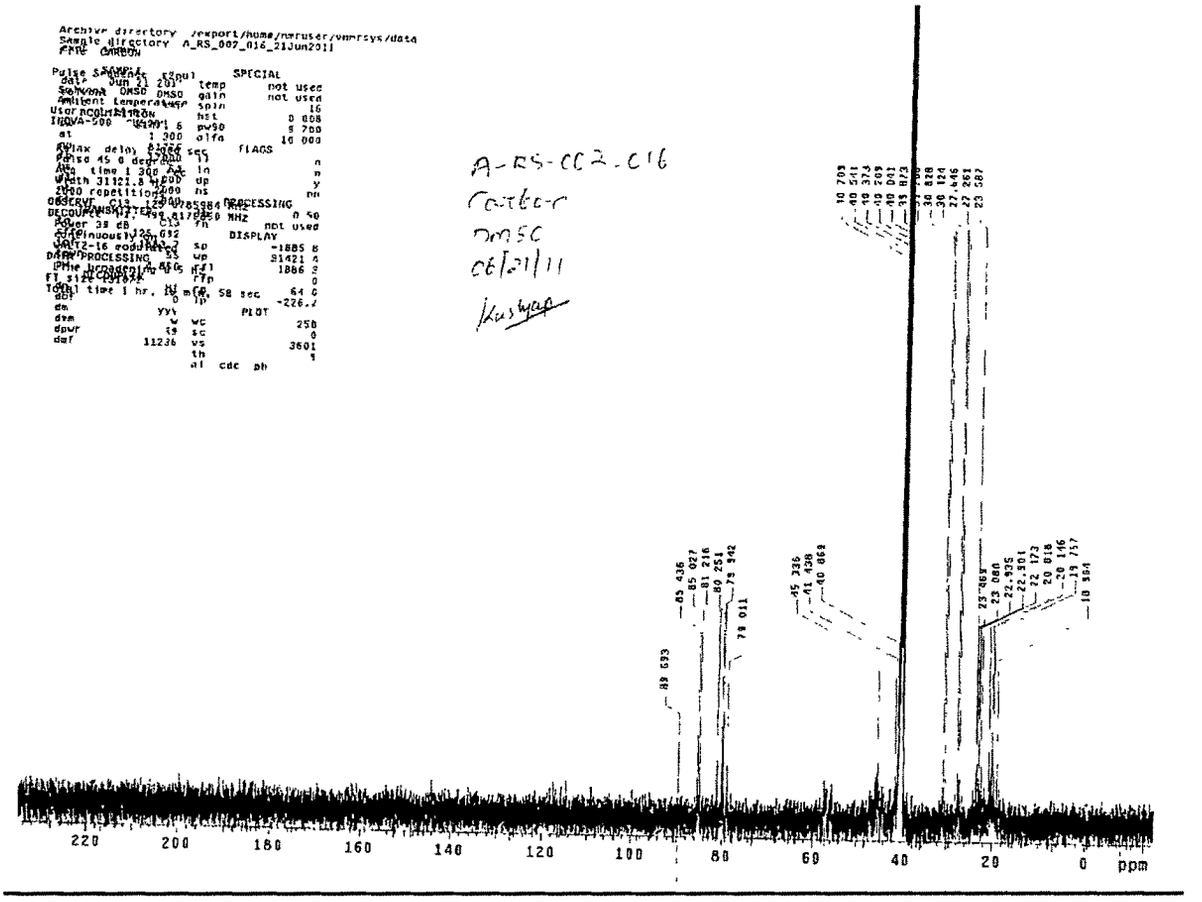
A_RS-002_016
Proton
Methanol
06/16/11



CARBON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DMSO
06/21/11
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200
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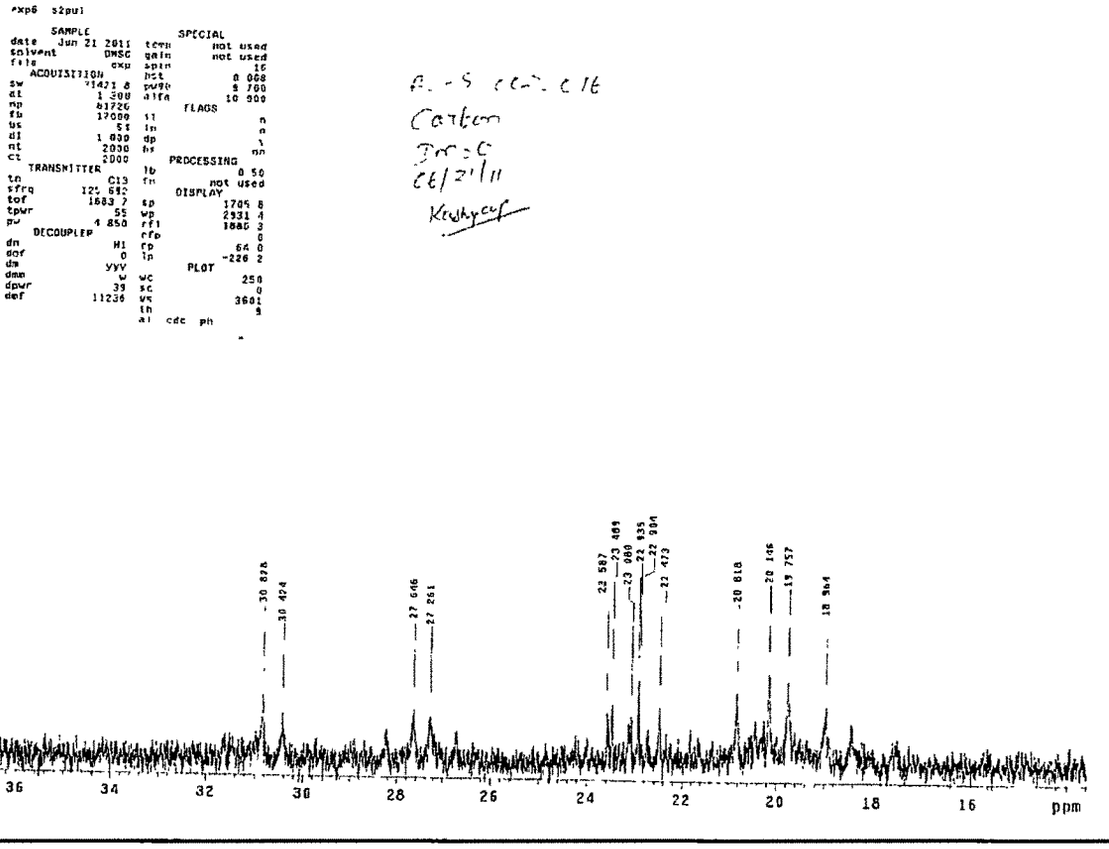
160
140
120
100
80
60
40
20
0
ppm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date Jun 21 2011
solvent DMSO
file exp
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d1 1.000
nt 2000
ct 2000
TRANSMITTER
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pw 4.850
DECOUPLER
dn H1
dof 0
dm yyy
dmm w
dpwr 39
dof 11236
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gain not used
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hst 0.000
pw90 9.700
alfa 10.900
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in n
dp y
hs nn
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DISPLAY
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wp 2931.4
rf1 1885.3
rfp 0
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lp -226.2
PLOT
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sc 0
vs 3601
th 9
al cdc ph
A. - S ccc. c 16
Carbon
DMSO
6/21/11
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27.261
23.587
23.469
23.080
22.835
22.804
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ppm

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ct             2006   PROCESSING
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dmf            11236     vs                 3601
                         th                    9
                      nl  cdc  ph
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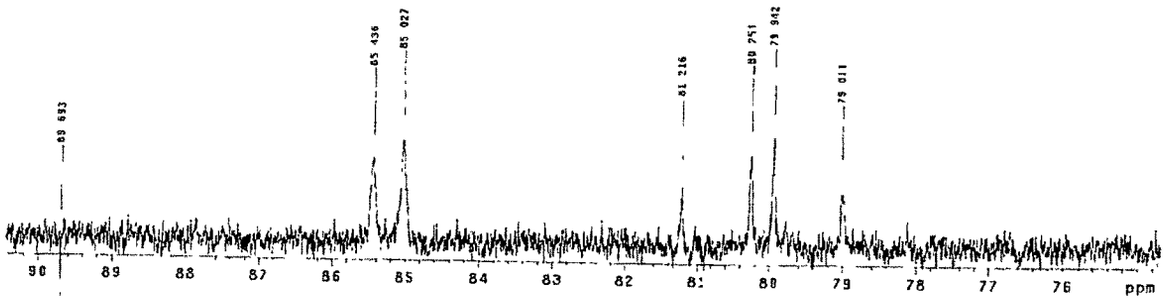
cc/=2/11
Kathy



Conclusions

Based on the studies performed to study extensively the various degradation pathways we have observed that different impurities can be formed due to the stress effects on the Cevimeline drug substance and drug product. Not all stress conditions are generating the same impurities.

Degradation pathways for Cevimeline

Oxidative pathway is the major degradation pathway for Cevimeline HCL converting the drug to the Thiol impurity with the chemical name "3-Hydroxy-3-mercaptomethyl quinuclidine".

The hydrolysis pathway is also another degradation pathway which leads to the same Thiol impurity, however the conversion rate of the drug to the impurity is very slow and the strength of the acid or the alkali and the temperature determines the rate of degradation to the thiol impurity.

There is no second order degradation observed once the thiol impurity is formed, it is pretty stable and does not undergo any further degradation. There are other impurities like Ceveimeline N-Oxide, Diol etc.

When the exercise was initiated on drug product also, we have observed the similar trend in the impurity profiles of the Cevimeline.

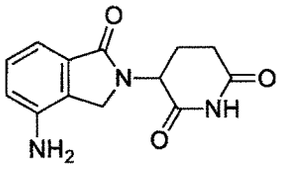
The impurity isolations and characterizations of the same, led to develop a better HPLC method which is a stability indicating method in order to monitor the related substances present in the drug substances and drug products. This method can be used at Quality Control laboratories which are required to test and release the Cevimeline drug substance and drug products for human use.

The knowledge of the possible degradation pathways achieved in this project prompted to understand the impurities behavior in the Humans when administered either orally or systemic, and to get the toxicology information of the same. After thorough literature search, we have found that all the impurities found via forced degradation studies were similar to the metabolites found in human patients and animal studies performed on Cevimeline as a part of clinical studies.

LENALIDOMIDE

Lenalidomide

REVLIMID (lenalidomide) , a thalidomide analogue, is an immunomodulatory agent with anti-angiogenic and anti-neoplastic properties. The chemical name is 3-(4-amino-1-oxo 1,3-dihydro-2H-isoindol-2-yl) piperidine-2,6-dione and it has the following chemical structure:



3-(4-amino-1-oxo 1,3-dihydro-2H-isoindol-2-yl) piperidine-2,6-dione

The empirical formula for lenalidomide is $C_{13}H_{13}N_3O_3$, and the gram molecular weight is 259.3. Lenalidomide is an off-white to pale-yellow solid powder. It is soluble in organic solvent/water mixtures, and buffered aqueous solvents. Lenalidomide is more soluble in organic solvents and low pH solutions. Solubility was significantly lower in less acidic buffers, ranging from about 0.4 to 0.5 mg/ml. Lenalidomide has an asymmetric carbon atom and can exist as the optically active forms S(-) and R(+), and is produced as a racemic mixture with a net optical rotation of zero.

REVLIMID (lenalidomide) is available in 5 mg, 10 mg, 15 mg and 25 mg capsules for oral administration. Each capsule contains Lenalidomide as the active ingredient and the following inactive ingredients: lactose anhydrous, microcrystalline cellulose, Croscarmellose sodium, and magnesium stearate. The 5 mg and 25 mg capsule shell contains gelatin, titanium dioxide and black ink. The 10 mg capsule shell contains gelatin, FD&C blue #2, yellow iron oxide, titanium dioxide and black ink. The 15 mg capsule shell contains gelatin, FD&C blue #2, titanium dioxide and black ink.

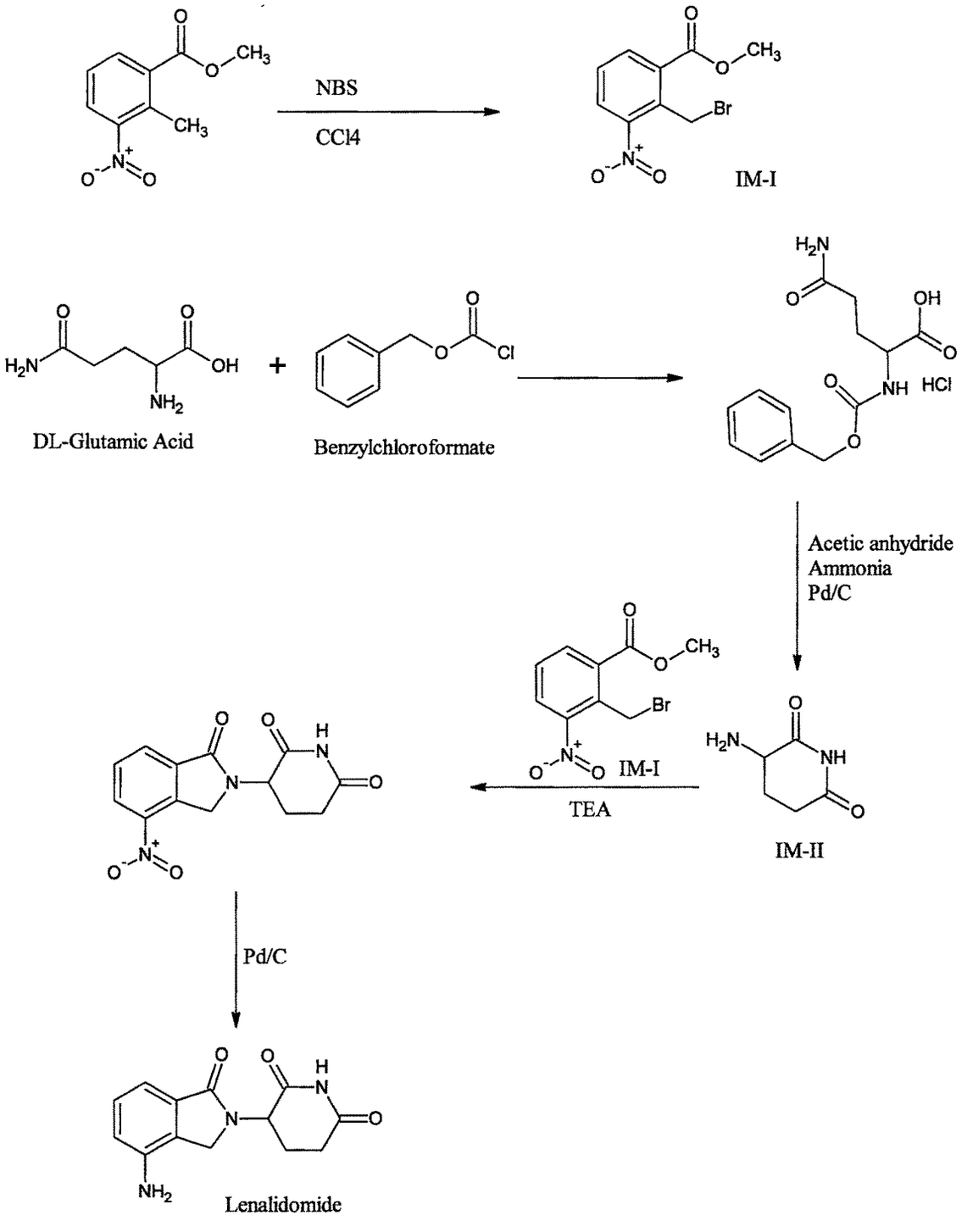
Multiple Myeloma

REVLIMID (lenalidomide) in combination with dexamethasone is indicated for the treatment of patients with multiple myeloma (MM) who have received at least one prior therapy.

Myelodysplastic Syndromes

REVLIMID (lenalidomide) is indicated for the treatment of patients with transfusion-dependent anemia due to low- or intermediate-1-risk myelodysplastic syndromes (MDS) associated with a deletion 5q cytogenetic abnormality with or without additional cytogenetic abnormalities.

Lenalidomide Synthesis



Lenalidomide can be synthesized by the similar synthetic pathways as given in the prior-art literature.

Process related Impurities

The impurity profiling study has been conducted for the above synthetic process. The review of the manufacturing procedure reveals that palladium metal could be the likely inorganic impurity that could be carried over to the final compound during the chemical transformations from the starting material to the finished API.

The organic impurities include the N-1 starting material which is "3-(4-nitro-1,3-dihydro-1-oxo-2H-isoindol-2-yl)-2,6-piperidinedione". This nitro impurity is the portion of the penultimate step material getting carried over to the final API without taking part in the reaction. There are some more organic impurities which are possible impurities in the drug substance are acetic acid, ammonia and triethylamine.

There are no enantiomeric impurities since the drug is the racemic mixture of S(-) and R(+) isomer. Both the optically active forms have similar therapeutic activity.

The residual solvents impurities are the solvents which are used in the process and also which are associated with the reagents like IPA.HCl, where in the isopropanol will be the residual solvent.

The solvents which could be potentially present in the final API of Lenalidomide as per the manufacturing process given above are as follows.

1) Acetone
2) N,N-Dimethylformamide
3) Dichloromethane or Carbon Tetrachloride

Degradation Impurities

Since the drug substances and drug products will be stored for long durations in the pharmaceutical companies and the pharmacy retail outlets, before they are consumed by the patient, it becomes imperative and prudent to study the degradation products which could be formed due to various stress factors on the drug substance either during shipment or storage. So to establish the degradation pathways and the degradation products, several studies were performed on

Lenalidomide by applying external stress factors like Acid, Base, heat, light etc. and characterized the structures of the same.

Stress studies (Induced degradation):

The following stress conditions were applied on the Lenalidomide to understand the degradation profiles and identify the degradation impurities.

- Hydrolytic degradation with Acid
- Hydrolytic degradation with Alkali
- Thermal degradation
- Photolytic degradation
- Sunlight degradation
- Oxidative degradation

The forced degradation conditions and the sample preparations during the study are similar to the ones given under the Felbamate study.

Stability indicating Analytical method (By HPLC)

The following stability indicating HPLC method has been developed to separate all the known and unknown impurities in the Lenalidomide API based on the degradation studies conducted.

A stainless steel column of length 250 mm, internal diameter 4.6 mm and filled with Phenyl groups chemically bonded to porous silica particles of 5 µ diameter.(Zorbax Eclipse XDB Phenyl (250 x 4.6 mm), 5µ or equivalent).

Preparation of buffer solution (Mobile phase A)

2.30 g of ammonium dihydrogen phosphate was weighed and transferred into a 2000 mL beaker and dissolved in 2000 mL of water and mixed well. Filtered through 0.45µ or finer porosity membrane filter.

Preparation of Mobile phase B

Used filtered (through 0.45µ or finer porosity membrane filter) and degassed Acetonitrile.

Elution Program

Time (min.)	Mobile phase A (% v/v)	Mobile phase B (% v/v)
0.0	88.0	12.0
10.0	88.0	12.0
30.0	70.0	30.0
40.0	70.0	30.0
50.0	88.0	12.0
60.0	88.0	12.0

Chromatographic parameters

Flow rate : 1.0 mL/minute

Detection : UV at 230 nm

Injection Volume : 20 µL

Column Oven Temperature : 25°C

Runtime time : 60 minutes

Preparation of Diluent

Prepared a mixture of Buffer and Acetonitrile in the ratio of 60:40. The diluent is used to prepare sample preparations

The samples which are subjected to stress conditions are analyzed by the stability indicating HPLC method given above and the percentage degradation observed in each of the condition is provided in the table below.

Table: % Degradation and peak purity data for Lenalidomide during forced degradation study

Condition	% of Lenalidomide	Purity Angle	Purity Threshold
Control Sample	100.0	1.158	2.485
Base hydrolysis-1N NaOH-5 mL	90.4	0.851	1.885
Oxidative degradation- Hydrogen peroxide 10%-5 mL-80°C-1 hour	44.5	0.140	0.261
UV light-137 hours	97.8	0.867	2.254
Sun light-7 hrs.	100.2	1.185	2.151
Thermal-80°C-7 hrs	101.0	0.990	2.035
Acid hydrolysis-1N HCl-5 mL-80°C-1 hour	93.7	1.619	2.912

Purity angle should be less than the Purity Threshold for the peak to be considered as pure with no co-elution of the impurities.

The information given in the table above points to the stability indicating nature of the HPLC method developed for estimating the impurity content in felbamate drug substance or drug product.

Lenalidomide Impurity Profile/ Degradation pathways

Lenalidomide subjected to the stress factors did not undergo any degradation at all under the sunlight, UV-Light and Thermal conditions. However the drug undergoes significant degradation under the acid and base degradation conditions. Severe degradation was observed to the extent of 50% loss in oxidative degradation with Hydrogen peroxide.

Acid and base hydrolysis gave rise to the same impurity, which is very hydrophilic in nature when compared to the drug at the retention time of 3.12min as per the LC/MS/MS method developed to be compatible with the mass detector. The polar functional groups are added to the drug moiety. The oxidative degradation also generates the same impurity at 3.12 min along with other minor impurity at 6.8 min retention time. The LC/MS/MS experiments were conducted on the degradation samples and the impurity mass data was established. Impurity had a m/z value of 279

which is a (M+H)+ ion. The molecular weight of the drug is 259.2. The difference of 18 mass unit addition observed between the drug and the impurity at RT=3.12 min.

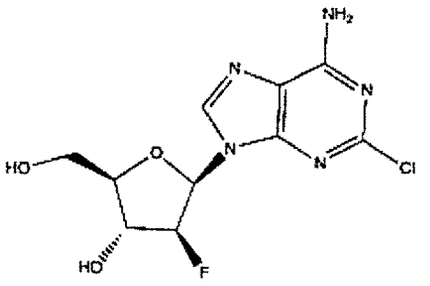
The structure elucidation of the impurity at RT=3.12min was done based on the mass data and the impurity is the hydrolysis or the oxidative product of Lenalidomide with piperidine-dione getting cleaved to the free Acid and the free amide groups. The second impurity at RT=6.8 min is unknown.

CLOFARABINE

CLOFARABINE

Clofarabine is a purine nucleoside antimetabolite marketed in the U.S. and Canada as **Clolar**. In Europe and Australia/New Zealand the product is marketed under the name **Evoltra**. It is FDA-approved for treating relapsed or refractory acute lymphoblastic leukaemia (ALL) in children after at least two other types of treatment have failed. It is not known if it extends life expectancy. Some investigations of effectiveness in cases of acute myeloid leukaemia (AML) and juvenile myelomonocytic leukaemia (JMML) have been carried out. Ongoing trials are assessing its efficacy, if any, for managing other cancers.

Clolar (clofarabine) Injection contains clofarabine, a purine nucleoside metabolic inhibitor. Clolar (1 mg/mL) is supplied in a 20 mL, single-use vial. The 20 mL vial contains 20 mg clofarabine formulated in 20 mL unbuffered normal saline (comprised of Water for Injection, USP, and Sodium Chloride, USP). The pH range of the solution is 4.5 to 7.5. The solution is sterile, clear and practically colorless, and is preservative-free.



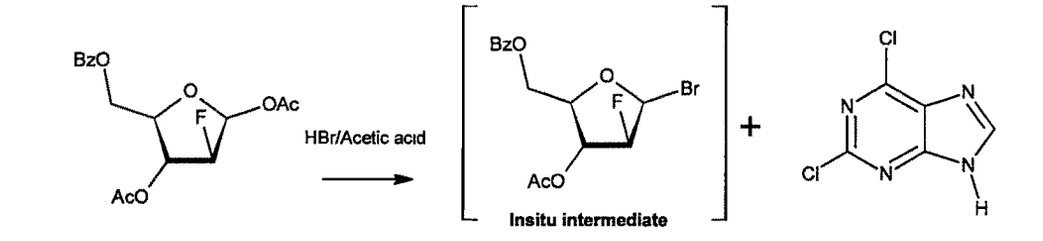
IUPAC Name: 5-(6-amino-2-chloro-purin-9-yl) -4-fluoro-2- (hydroxymethyl)oxolan-3-ol

The scheme of synthetic procedure is as follows

1.0 Route of Synthesis (ROS)-Schematic

Synthetic Scheme and Details of Clofarabine synthesis

Stage-I: Preparation of Protected Clofarabine

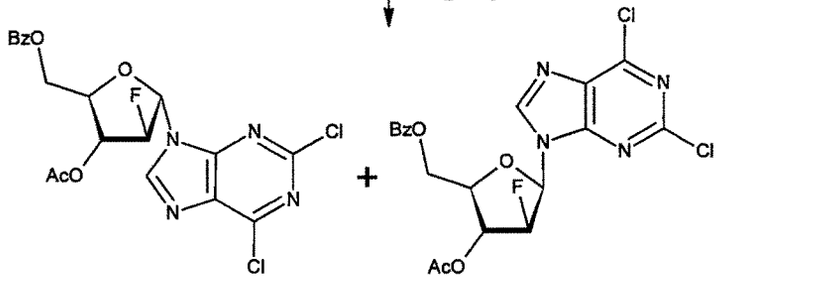


1,3-di-o-acetyl-5-o-benzoyl-2-deoxy-2-fluoro-D-arabinofuranose

3-o-acetyl-5-o-benzoyl-2-deoxy-2-fluoro-D-arabinofuranosyl Bromide

2,6-dichloro purine

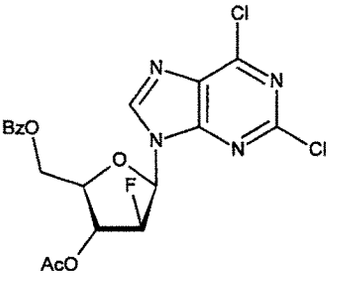
Benzyl triethyl ammonium chloride
K_2CO_3



Protected Clofarabine (α - Anomer)

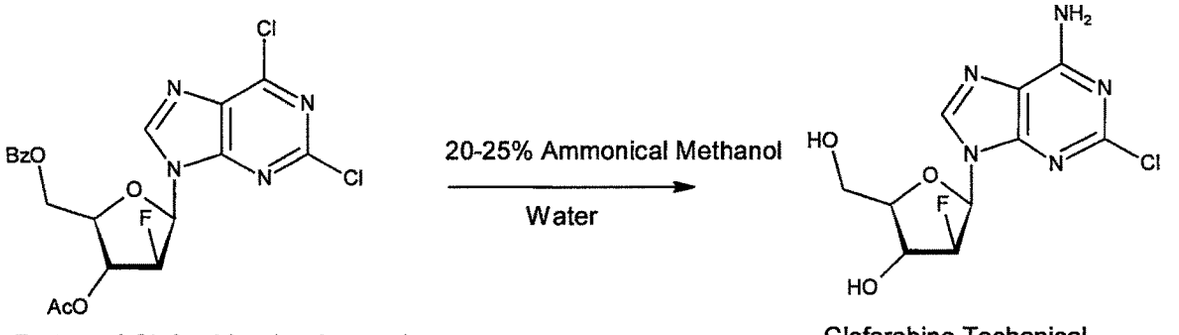
Protected Clofarabine (β - Anomer)

Column purification
Ethyl acetate
N-Hexane
Methanol



Protected Clofarabine (β - Anomer)

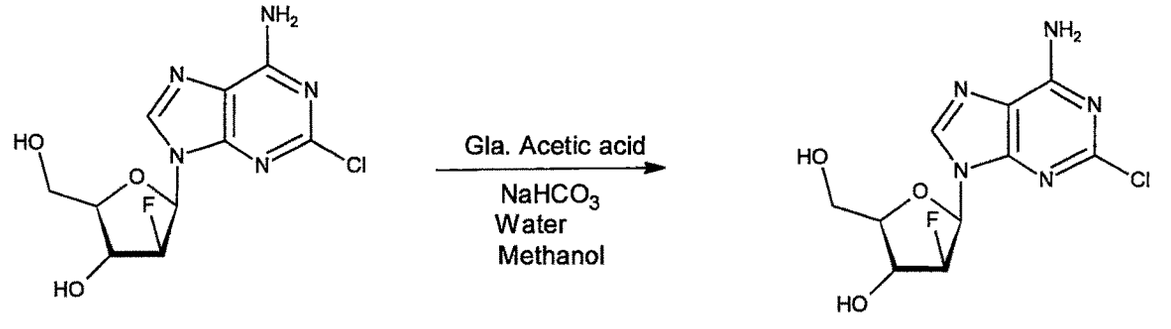
Stage –II: Preparation of Clofarabine Crude



Protected Clofarabine (β -Anomer)

Clofarabine Techanical

Stage –III: Preparation of Clofarabine



Clofarabine Crude

Clofarabine API

Stage-I: Preparation of Protected Clofarabine

The Stage-I intermediate namely protected Clofarabine is synthesized by the bromination of 1, 3-di-O-acetyl-5-O-benzoyl-2- deoxy-2-Fluoro-D-arabinofuranose using Hydro bromic acid/Acetic acid mixture at 25°C to 30°C for 6 hrs and the brominated product was considered as such for the next step without isolation. The insitu bromo intermediate is condensed with 2,6-Dichlorpurine using benzyl triethyl ammonium chloride and potassium carbonate at 25°C to 35°C for 3 to 4 hrs .The reaction mass was then worked up to get the alpha and beta anomers of protected Clofarabine .The alpha and beta anomers are then separated by conventional column separation using the polarity difference of the anomers .The anomers are separated by a silcagel column separation using ethyl acetate/Hexane solvent system and the isolated crude material is slurred with methanol to get the pure required beta anomer.

Stage-II Preparation of Clofarabine Crude

The Clofarabine crude is prepared by the simultaneous deprotection and amination of protected Clofarabine using Methanolic ammonia in an autoclave at 60°C to 65°C for 16 to 18hrs. After completion of the reaction, the crude product is isolated by adjusting the pH of the reaction mass to pH 7.1 to 7.2 with dilute hydrochloric acid solution.

Stage-III Preparation of Clofarabine drug substance.

The Clofarabine API is prepared from Clofarabine Crude using acetic acid crystallisation. The crude material is dissolved in acetic acid and stirred at 50 to 60 °C for 15 mins and the clear solution is cooled to 25°C to 30°C to precipitate the pure product. The pure product is then made free from acetic acid by neutralizing it with sodium bicarbonate solution in a water medium and the isolated product is slurried with methanol to get Clofarabine API.

1.0 ASSAY (% W/W ON ANHYDROUS BASIS) AND RELATED SUBSTANCE (% W/W ON AS IS BASIS BY HPLC)- METHOD DEVELOPED

1.1 Instrumentation

A High Performance Liquid Chromatograph equipped with Gradient elution capability, Ultraviolet Spectrophotometer as detector and an auto sampler.
(Shimadzu LC 2010 system or equivalent).

Data handling system

LC Solution Software or equivalent chromatographic software

Column

A stainless steel column of length 250 mm, internal diameter 4.6 mm and filled with Octyl Silane chemically bonded to porous silica particles of 5 µ diameter.
(Luna or Symmetry C8 (250 x 4.6 mm x 5µ) or equivalent.

1.2 Reagents

1) Distilled Water
2) Monobasic potassium phosphate
   (Potassium dihydrogen orthophosphate, KH_2PO_4)
3) Triethyl amine
4) Orthophosphoric acid

1.3 Preparation of buffer [Mobile phase-A]

1.36 g of Potassium dihydrogen orthophosphate, KH_2PO_4 was weighed accurately and dissolved in 1000 mL distilled water, it is mixed well to dissolve the solid. 1 ml of triethyl amine was added and mixed well. The pH was adjusted to 6.8 ± 0.1 with Ortho-phosphoric acid. The solution is filtered through 0.45µ or finer porosity membrane filter.

1.4 Preparation of Mobile phase B

Used filtered (through 0.45µ or finer porosity membrane filter) and degassed Acetonitrile.

1.5 Elution Program (Gradient Composition)

Elution program: -1: For Clofarabine standards and sample solution for assay injection

Initial program: Mobile Phase A : Mobile phase B :::: 90 : 10

Time in minutes	Mobile phase A (% v/v)	Mobile phase B (% v/v)
0.01	90	10
20.00	90	10

Elution program : -2 : For Blank and sample solution for related substances (RS)

Initial program: Mobile Phase A : Mobile phase B :::: 90 : 10

Time in minutes	Mobile phase A (% v/v)	Mobile phase B (% v/v)
0.01	90	10
20.00	90	10
45.00	70	30
50.00	70	30
55.00	90	10
60.00	90	10

1.6 Chromatographic parameters

Flow rate : 1.3 mL/minute
Detection : UV at 210 nm
Injection Volume : 10 µL
Column Oven Temperature : 35°C
Runtime time : Not less than 20 minutes for Elution program-1 (Standards and sample solution for assay)
Not less than 60 minutes for Elution program-2 (Blank and sample solution for related substances (RS))

1.7 Preparation of diluent

Water: Acetonitrile:: 90 : 10

1.8 Preparation of Clofarabine standard solution for assay and related substances

25 mg of Clofarabine standard was accurately weighed and transferred into a 25 mL volumetric flask. 20 mL of diluent was added and sonicated to dissolve the solid with

occasional shaking. It is diluted to volume with diluent and mixed well. This is stock standard solution-S

5.0 mL of above stock solution-S was transferred into 50 mL volumetric flask, and diluted to volume with diluent and mixed. This is diluted standard solution-S_1

1.9 Preparation of Sample solution for assay

250 mg of Clofarabine sample was accurately weighed and transferred in duplicate into individual 25 mL volumetric flask. 20 mL of diluent was added into each volumetric flask and sonicated to dissolve the solid with occasional shaking. It is diluted to volume with acetonitrile and mixed well. This is stock sample solution-A and B

Transferred accurately 1.0 mL of each stock sample solution-A and B into individual 10 mL volumetric flask and diluted to volume with diluent and mixed well. This is diluted sample solution-A_1 and B_1

1.0 mL of each stock sample solution- A_1 and B_1 was transferred accurately into individual 10 mL volumetric flask and diluted to volume with diluent and mixed well. This is diluted sample solution-A_2 and B_2.

1.10 Preparation of Sample solution for related substances (RS)

250 mg of Clofarabine sample was weighed accurately and transferred into 25 mL volumetric flask. 20 mL of diluent was added to the volumetric flask and sonicated to dissolve the solid with occasional shaking. It was diluted to volume with acetonitrile and mixed well. This is sample solution-C for related substances.

1.0 mL of sample solution-C was transferred accurately into 10 mL volumetric flask and diluted to volume with diluent and mixed well. This is diluted sample solution-D.

Or use either solution A_1 or B_1 prepared in the section 1.9.

1.11 Procedure

Separately injected the diluent, standard preparation and sample preparation into the chromatograph. Recorded the chromatograms. The peak responses were measured only for the major peak in the chromatogram of standard and sample for assay. The peak responses of all eluting peaks in the chromatogram of the sample solution for related substances was determined. The diluent chromatogram was examined for any extraneous peaks, and disregarded the corresponding peaks observed in the chromatogram of the sample solution for related substances. Follow the injection sequence as mentioned below:

Sr. no.	Sample	No. of injections	Elution program
1	Diluent	1	Elution program-2
2	Diluted Standard S_1 (system suitability)	6	Elution program-1
3	Sample preparation for Assay – 1	1	Elution program-1
4	Sample preparation for Assay – 2	1	Elution program-1
5	Sample preparation (RS)	1	Elution program-2

2.0 System Suitability Parameter

- **% Relative Standard deviation (RSD) for area of Clofarabine**

  Not more than 2.0 %

- **Theoretical plates (Column efficiency)**

  Not less than 3000

- **Tailing factor (Asymmetry)**

  Not more than 2.0

2.1 Calculation

Calculated Assay (% w/w, on anhydrous) for both samples as per formula given below:

2.1.1 **Assay of Clofarabine** (% w/w, on anhydrous basis) =

$$= \frac{At}{As} \times \frac{\text{Std. wt (mg)}}{25} \times \frac{5}{50} \times \frac{25}{\text{Sample wt (mg)}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{100}{(100-Z)} \times P$$

Where,

At	:	Peak area of Clofarabine in the chromatogram of sample preparation
As	:	Average peak area of Clofarabine in the chromatogram of standard preparation
Std. wt.	:	Weight of Clofarabine standard in mg
Sample wt.	:	Weight of Clofarabine sample preparation in mg
P	:	% Potency /Assay of Clofarabine standard (As is basis)
Z	:	% Water content

2.1.2 **Related substances**

Percentage individual known / unknown impurity

$$= \frac{At}{As} \times \frac{\text{Std. wt (mg)}}{25} \times \frac{5}{50} \times \frac{25}{\text{Sample wt (mg)}} \times \frac{10}{1} \times P$$

Where,

At	:	Peak area of known/unknown impurity in sample preparation
As	:	Average peak area of Clofarabine in standard preparation
Std. wt	:	Weight of Clofarabine standard in mg
Sample wt	:	Weight of Clofarabine sample preparation in mg
P	:	% Potency / Assay of Clofarabine standard (As is basis)

For known impurity followed the below RRT table

Name of compound	Retention time (Minutes)	Relative retention time (RRT)
Clofarabine	8.9	1.00
Clofarabine Related Compound – A	10.5	1.17
Clofarabine Related Compound – B	4.4	0.49

2.1.3 Unspecified unknown impurity

= Report unspecified unknown impurity obtained from calculation.

2.1.4 Percentage total Impurities:

= Report summation of the all impurities

3.0 Report

The average of the results obtained from the analysis of sample preparation in duplicate as assay (% w/w, on anhydrous basis)

4.0 Specification

4.1 Assay

Not less than 98.0 % w/w & not more than 102.0 % w/w (On anhydrous basis)

Related impurities

Clofarabine Related Compound – A	:	Not more than 0.15 %
Clofarabine Related Compound – B	:	Not more than 0.15 %
Unspecified unknown impurity	:	Not more than 0.10 %
Total impurities	:	Not more than 0.50 %

Where,

Clofarabine Related Compound – A : Benzoic acid amide

Clofarabine Related Compound – B : 2-Chloro-6-aminopurine

4.2 STRESS STUDIES (FORCED DEGRADATION / INDUCED DEGRADATION)

The Clofarabine drug substance was subjected to forced degradation under the following stress conditions.

4.2.1 Preparation of Clofarabine Standard solution for assay and related substances

25.01 mg of Clofarabine standard was accurately weighed and transferred in to a 25 mL volumetric flask. Added 20 mL of diluent sonicated to dissolve the solid and diluted to volume with diluent and mixed well. (Clofarabine stock standard solution).

5.0 mL of above Clofarabine stock standard solution was transferred in to a 50 mL volumetric flask, and diluted to volume with diluent and mixed well. (Clofarabine diluted standard solution)

4.2.2 Preparation of Stock Sample solution-X

100.37 mg of Clofarabine sample was accurately weighed and transferred into 10 mL volumetric flask. 8 mL of diluent was added and 2 mL of acetonitrile and sonicated to dissolve the solid with occasional shaking.

4.2.3 Preparation of Control sample solution for related substances (RS)

1.0 mL of stock sample solution-X prepared under section **4.2.2** was transferred into a 10 mL volumetric flask, diluted to volume with diluent and mixed well. (Control sample solution for related substances).

4.2.4 Preparation of Control sample solution for assay

1.0 mL of control sample solution for related substances (RS) prepared under section **4.2.3** was transferred into a 10 mL volumetric flask, diluted to volume with diluent and mixed well. (Diluted control sample solution for assay).

4.2.5 Preparation of sample solution for Hydrolytic degradation with acid (For Related Substances).

1.0 mL of stock sample solution-X prepared under section **4.2.2** was transferred into a 10 mL volumetric flask. 2.0 mL of 1 N Hydrochloric acid was added. Treated solution was heated at 80°C for 1 hour. Cooled the solution to room temperature. Neutralized with 1 N

Sodium hydroxide. The diluent was added and mixed well. It was diluted to volume with diluent and mixed well. (Stock acid degradation sample solution for Related Substances).

4.2.6 Preparation of sample solution for Hydrolytic degradation with acid (For Assay).

1.0 mL of stock acid degradation sample solution prepared under section **4.2.5** was transferred into a 10 mL volumetric flask, diluted to volume with diluent and mixed well. (Diluted acid degradation sample solution for Assay).

4.2.7 Preparation of blank solution for Hydrolytic degradation with acid (For Related Substances).

A blank solution for acid hydrolysis of Related Substances was prepared by following the same procedure given under section **4.2.5** omitting the sample.

4.2.8 Preparation of blank solution for Hydrolytic degradation with acid (For Assay).

A blank solution for acid hydrolysis of Assay was prepared by following the same procedure given under section **4.2.6** omitting the sample.

4.2.9 Preparation of sample solution for Hydrolytic degradation with base (For Related Substances).

1.0 mL of stock sample solution-X prepared under section **4.2.2** was transferred into a 10 mL volumetric flask. 2.0 mL of 1 N Sodium hydroxide was added. The treated solution was heated at 80°C for 1 hour. Cooled the solution to room temperature. Neutralized with 1 N Hydrochloric acid. The diluent was added to volume and mixed well. (Stock base degradation sample solution for Related Substances).

4.2.10 Preparation of sample solution for Hydrolytic degradation with base (For Assay).

1.0 mL of stock base degradation sample solution prepared under section **4.2.9** was transferred into a 10 mL volumetric flask, diluted to volume with diluent and mixed well. (Diluted base degradation sample solution for Assay).

4.2.11 Preparation of blank solution for Hydrolytic degradation with base (For Related Substances).

A blank solution for base hydrolysis of Related Substances was prepared by following the same procedure given under section **4.2.9** omitting the sample.

4.2.12 Preparation of blank solution for Hydrolytic degradation with base (For Assay).

A blank solution for base hydrolysis of Assay was prepared by following the same procedure given under section **4.2.10** omitting the sample.

4.2.13 Preparation of sample solution for Thermal degradation (Sample) (For Related Substances).

25.40 mg of Clofarabine sample was accurately weighed and transferred into a 25 mL volumetric flask. Exposed the volumetric flask to 80°C for 4 hours in oven. Cooled the volumetric flask to room temperature. 20 mL of diluent was added and sonicated to dissolve the solid and diluted to volume with diluent and mixed well. (Stock Thermal degradation (sample) sample solution for Related Substances).

4.2.14 Preparation of sample solution for Thermal degradation (Sample) (For Assay).

5.0 mL of stock thermal degradation sample solution prepared under section **4.2.13** was transferred into a 50 mL volumetric flask, diluted to volume with diluent and mixed well. (Diluted Thermal degradation (sample) sample solution for Assay).

4.2.15 Preparation of sample solution for Thermal degradation (Solution) (For Related Substances).

1.0 mL of stock sample solution-X prepared under section **4.2.2** was transferred into a 10 mL volumetric flask. The volumetric flask was exposed to 80° for 4 hours in oven. The solution was cooled to room temperature. The solution was further diluted to volume with diluent and mixed well. (Stock Thermal degradation (solution) sample solution for Related Substances).

4.2.16 Preparation of sample solution for Thermal degradation solution (For Assay).

1.0 mL of stock thermal degradation sample solution prepared under section **4.2.15** was transferred into a 10 mL volumetric flask, diluted to volume with diluent and mixed well. (Diluted Thermal degradation (solution) sample solution for Assay).

4.2.17 Preparation of sample solution for Oxidative degradation with Hydrogen peroxide (For Related Substances).

1.0 mL of stock sample solution-X prepared under section **4.2.2** was transferred into a 10 mL volumetric flask. 2.0 mL of 10% Hydrogen peroxide was added and mixed well. The treated solution was heated at 80°C for 1 hour. The solution was cooled to room temperature and diluted to volume with diluent and mixed well. (Stock Oxidative degradation sample solution for Related Substances).

4.2.18 Preparation of sample solution for Oxidative degradation with Hydrogen peroxide (For Assay).

1.0 mL of stock oxidative degradation sample solution prepared under section **4.2.17** was transferred into a 10 mL volumetric flask, diluted to volume with diluent and mixed well. (Diluted Oxidative degradation sample solution for Assay).

4.2.19 Preparation of blank solution for Oxidative degradation with Hydrogen peroxide (For Related Substances).

A blank solution for oxidation degradation of Related Substances was prepared by following the same procedure given under section **4.2.17** omitting the sample.

4.2.20 Preparation of blank solution for Oxidative degradation with Hydrogen peroxide (For Assay).

A blank solution for oxidation degradation of Assay was prepared by following the same procedure given under section **4.2.18** omitting the sample.

4.2.21 Preparation of sample solution for Photolytic degradation using UV light (254 nm). (For Related Substances).

1.0 mL of stock sample solution-X prepared under section **4.2.2** was transferred into a 10 mL volumetric flask. The volumetric flask was exposed to UV light for 12 hours and

diluted to volume with diluent and mixed well. (Stock UV light degradation sample solution for Related Substances).

4.2.22 Preparation of sample solution for Photolytic degradation using UV light (254 nm). (For Assay).

1.0 mL of stock UV light degradation sample solution prepared under section **4.2.21** was transferred into a 10 mL volumetric flask, diluted to volume with diluent and mixed well. (Diluted UV light degradation sample solution for Assay).

4.2.23 Preparation of sample solution for Photolytic degradation with Sun light. (For Related Substances).

1.0 mL of stock sample solution-X prepared under section **4.2.2** was transferred into a 10 mL volumetric flask. The volumetric flask was exposed to Sun light for 12 hours and diluted to volume with diluent and mixed well. (Stock Sun light degradation sample solution for Related Substances).

4.2.24 Preparation of sample solution for Photolytic degradation with Sun light. (For Assay).

1.0 mL of stock UV light degradation sample solution prepared under section **4.2.23** was transferred into a 10 mL volumetric flask, diluted to volume with diluent and mixed well. (Diluted Sun light degradation sample solution for Assay).

4.2.25 % Assay of Clofarabine and peak purity data

Table-1 Assay of Clofarabine in Forced degradation samples

(EXHIBITS)- provided below

Mode of Degradation	**Condition**	**Area**	**%Assay of Clofarabine**
Control Sample	- As such	3339225	**99.7**
Hydrolytic degradation with acid	- Drug + 2 mL 1 N Hydrochloric acid, heated the solution for 1 hour at 80° C temperature in water bath	2524838	***75.4***
Hydrolytic degradation with base	- Drug + 2 mL 1 N Sodium hydroxide, heated the solution for 1 hour at 80° C temperature in water bath	758107	***22.6***
Oxidative degradation with Hydrogen peroxide	- Drug + 2 mL 10 % Hydrogen peroxide, heated the solution for 1 hour at 80° C temperature in water bath	3282039	**98.0**
Photolytic degradation using UV light (254 nm)	- Exposed the drug solution for 12 hours under UV light	3285545	**98.1**
Photolytic degradation with Sun light	- Exposed the drug solution for 12 hours in sun light	3283218	**98.0**
Thermal degradation (Sample)	- Heated the drug for 4 hour at 80° C temperature under oven.	3417533	**100.8**
Thermal degradation (Solution)	- Drug solution heated for 4 hour at 80° C temperature under oven.	3400847	**101.5**

Table-2 Peak purity data of Clofarabine for Assay

(EXHIBITS)-provided below

Mode of Degradation	**Condition**	**Peak purity data**	
		*** PA**	**** TH**
Control Sample	- As such	0.108	0.300
Hydrolytic degradation with acid	- Drug + 2 mL 1 N Hydrochloric acid, heated the solution for 1 hour at 80° C temperature in water bath	0.164	0.358
Hydrolytic degradation with base	- Drug + 2 mL 1 N Sodium hydroxide, heated the solution for 1 hour at 80° C temperature in water bath	0.309	0.483
Oxidative degradation with Hydrogen peroxide	- Drug + 2 mL 10 % Hydrogen peroxide, heated the solution for 1 hour at 80° C temperature in water bath	0.106	0.293
Photolytic degradation using UV light (254 nm)	- Exposed the drug solution for 12 hours under UV light	0.078	0.262
Photolytic degradation with Sun light	- Exposed the drug solution for 12 hours in sun light	0.083	0.271
Thermal degradation (Sample)	- Heated the drug for 4 hour at 80° C temperature under oven.	0.071	0.261
Thermal degradation (Solution)	- Drug solution heated for 4 hour at 80° C temperature under oven.	0.074	0.264

Note: * PA : Purity Angle, ** TH : Purity Threshold

4.2.26 % Degradation of Clofarabine and peak purity data

Table 3 - % Degradation in Forced degradation samples for Related Substances

(EXHIBITS)-provided below

Mode of Degradation	Condition	% Degradation of Clofarabine
Hydrolytic degradation with acid	- Drug + 2 mL 1 N Hydrochloric acid, heated the solution for 1 hour at 80° C temperature in water bath	**20.7 %**
Hydrolytic degradation with base	- Drug + 2 mL 1 N Sodium hydroxide, heated the solution for 1 hour at 80° C temperature in water bath	**76.2 %**
Oxidative degradation with Hydrogen peroxide	- Drug + 2 mL 10 % Hydrogen peroxide, heated the solution for 1 hour at 80° C temperature in water bath	**-1.8 %**
Photolytic degradation using UV light (254 nm)	- Exposed the drug solution for 12 hours under UV light	**2.1 %**
Photolytic degradation with Sun light	- Exposed the drug solution for 12 hours in sun light	**1.9 %**
Thermal degradation (Sample)	- Heated the drug for 4 hour at 80° C temperature under oven.	**-3.6 %**
Thermal degradation (Solution)	- Drug solution heated for 4 hour at 80° C temperature under oven.	**-4.3 %**

Table 4 Peak purity data of Clofarabine for Related Substances

(EXHIBITS)-Provided below

Mode of Degradation	Condition	Peak purity data	
		* PA	** TH
Control Sample	- As such	0.108	0.300
Hydrolytic degradation with acid	- Drug + 2 mL 1 N Hydrochloric acid, heated the solution for 1 hour at 80° C temperature in water bath	0.201	0.290
Hydrolytic degradation with base	- Drug + 2 mL 1 N Sodium hydroxide, heated the solution for 1 hour at 80° C temperature in water bath	0.085	0.243
Oxidative degradation with Hydrogen peroxide	- Drug + 2 mL 10 % Hydrogen peroxide, heated the solution for 1 hour at 80° C temperature in water bath	0.171	0.354
Photolytic degradation using UV light (254 nm)	- Exposed the drug solution for 12 hours under UV light	0.256	0.331
Photolytic degradation with Sun light	- Exposed the drug solution for 12 hours in sun light	0.247	0.330
Thermal degradation (Sample)	- Heated the drug for 4 hour at 80° C temperature under oven.	0.225	0.388
Thermal degradation (Solution)	- Drug solution heated for 4 hour at 80° C temperature under oven.	0.282	0.382

Note: * PA : Purity Angle, ** TH : Purity Threshold

4.2.27 Acceptance criteria

1) There should not be any interference between principle and degradation peaks during forced degradation of Clofarabine.

2) Peak purity of the principle peak should pass in all the conditions (Purity angle should be less than purity threshold).

4.2.28 Conclusion

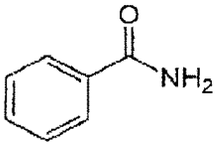
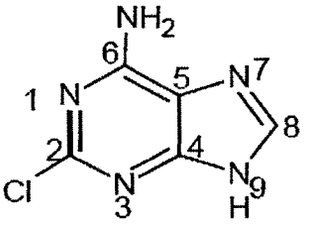
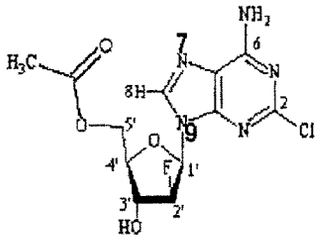
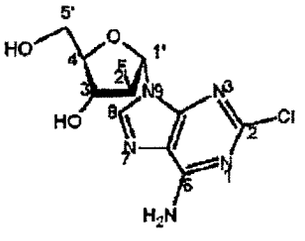
The acceptance criteria for stress studies were met, which shows the stability indicating nature of the method developed for estimating the potency and the related substances of Clofarabine drug substance and drug product Cloral

The major degradation of Clofarabine was found in Hydrolytic degradation with base.

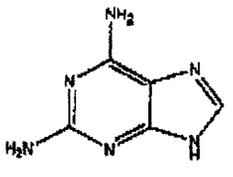
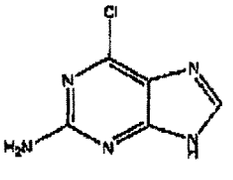
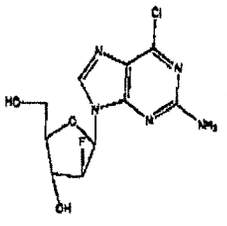
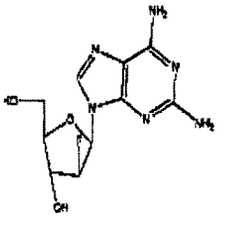
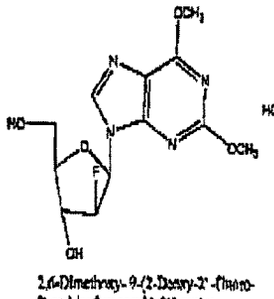
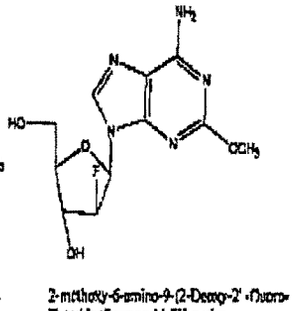
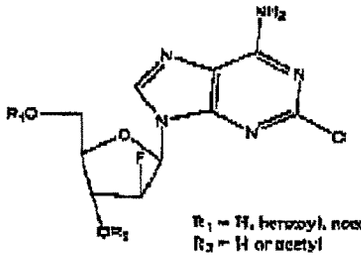
The results of the forced degradation studies conducted on Clofarabine drug substance are summarized in **Table 1** and **Table3** for Assay and Related Substances respectively.

CLOFARABINE IMPURITY PROFILE

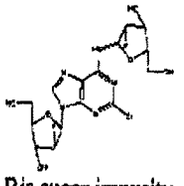
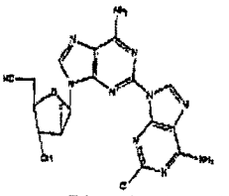
The route of synthesis for Clofarabine was evaluated for potential impurities. The impurities. which have been identified based on the route of synthesis of Clofarabine. are listed below.

Name	Chemical Name, Structure, Molecular Formula and Molecular Weight	Source	Limits
Clofarabine Impurity A	Benzoic acid amide  Molecular Formula: C_7H_7NO Molecular Weight: 121.14	Process	NMT 0.15%
Clofarabine Impurity B	2-Chloro-6-aminopurine  Molecular Formula: $C_5H_4ClN_5$ Molecular Weight: 169.57	Process	NMT 0.15%
Clofarabine Impurity C	6-Amino-2-chloro-9-(2-deoxy-2-fluoro-5-O-acetyl-β-D-arabinofuranosyl)-9H-purine  Molecular Formula: $C_{12}H_{13}ClFN_5O_4$ Molecular Weight: 345.71	Process	NMT 0.15%
Clofarabine Impurity D	6-Amino-2-chloro-9-(2`-deoxy-2- fluoro-α-D-arabinofuranosyl)-9H-purine  Molecular Formula: $C_{10}H_{11}ClFN_5O_3$ Molecular Weight: 303.68	Process	NMT 0.15%

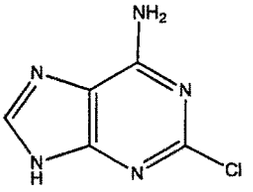
Clofarabine Impurities

Additional Clofarabine Impurities	Origin
 9*H*-purine-2,6-diamine  6-chloro-9*H*-purin-2-amine	formed due to reaction of residual 2.6-dichloro purine from stage-I) with ammonia solution
  a)2.6-Diamino-9-(2' -fluoro-β-D-arabinofuranosyl)-9H-purine b)2-Amino-6-chloro-9-(2'-f1uoro-β–Dsarabinofuranosyl)-9H-purine	Impurities formed by substitution at 2.6-dichloro position and 2-chloro position by ammonia
 2,6-Dimethoxy-9-(2-Deoxy-2'-fluoro-D-arabinofuranosyl)-9H-purine  2-methoxy-6-amino-9-(2-Deoxy-2'-fluoro-D-arabinofuranosyl)-9H-purine	Methanol undergo substitution reaction and replace the chloro moiety at 2-position and / or 6-position to form a methoxy derivatives of the Clofarabine.
 R_1 = H, benzoyl, acetyl R_2 = H or acetyl	Possible impurities 3' O-aacetyl or 5' and 3' O-acetylated impurity.(Similar to that of impurity C)

Clofarabine Impurities

 Bis sugar impurity  Bispurine impurity	Possibility of the amino group (-NH2) in the purine ring reacting with any carried over bromosugar moiety (3-O-acetyl-5-O-benzoyl-2-deoxy-2-fluoro-D-arabinofuranosyl Bromide) to form Bis-sugar (Impurity-1) or the amino group (-NH2) in the purine ring system substituting the 2-chloro moiety to form the corresponding impurity-2)

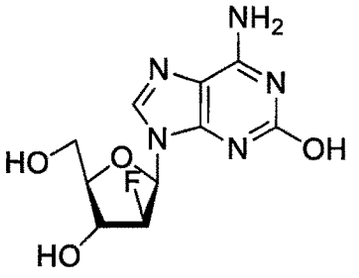
Possible Acid degradation impurities:-



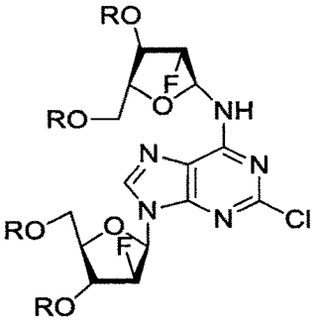
2-chloro-9*H*-purin-6-amine

Possible Base Degradation impurities:-

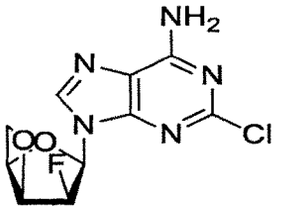
Impurity-A



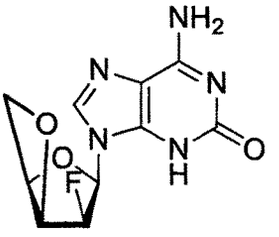
Impurity-B



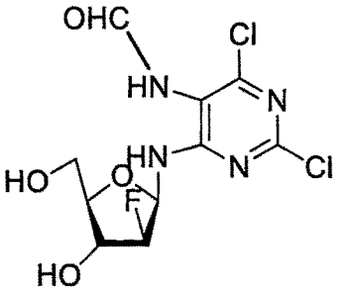
Impurity-C



Impurity-D



Impurity-E (Alpha or Beta both)



Possible Hydrogen peroxide degradation impurities:-

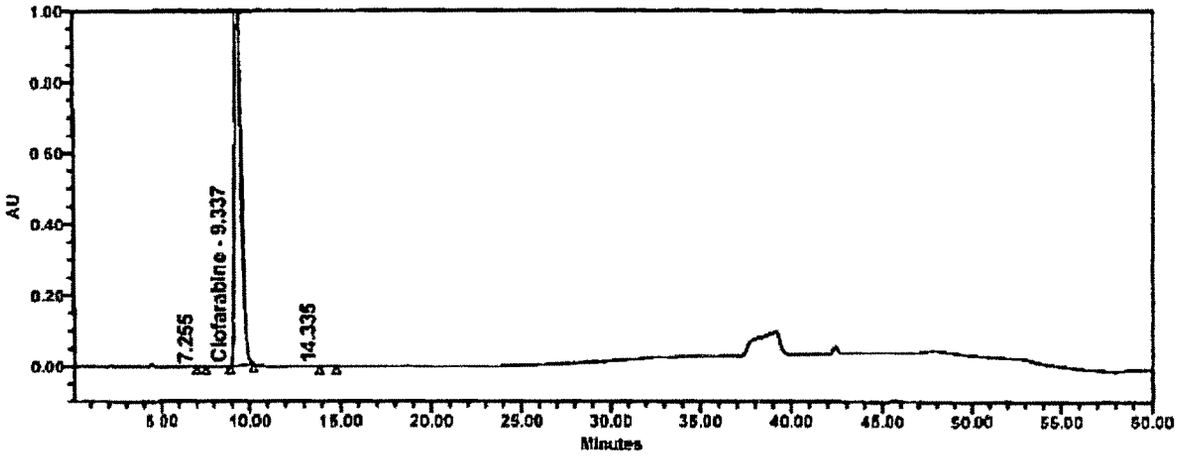
Degradation not observed.

EXHIBITS

The HPLC chromatograms are depicted below:

Control sample Chromatogram

Chromatogram



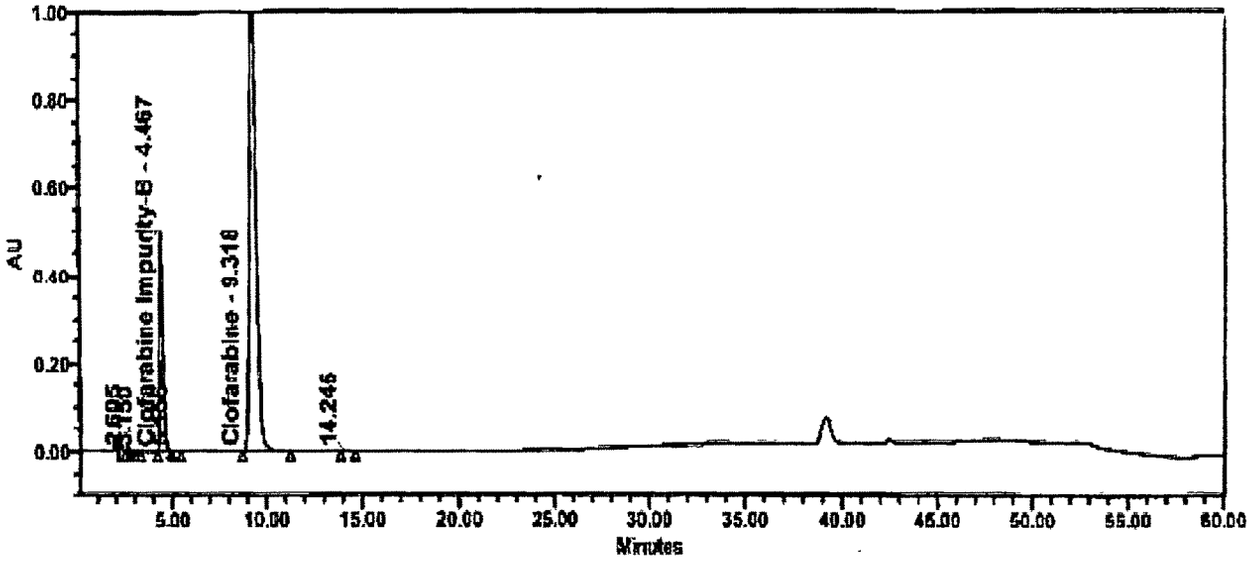
Peak Table

	Peak Name	RT	Area	% Area
1	Clofarabine Impurity-B	4.30		
2		7.25	6136	0.02
3	Clofarabine	9.34	32001015	99.91
4	Clofarabine Impurity-A	10.80		
5		14.34	24084	0.08
Sum			32031235	100.00

Acid degradation sample

Chromatogram

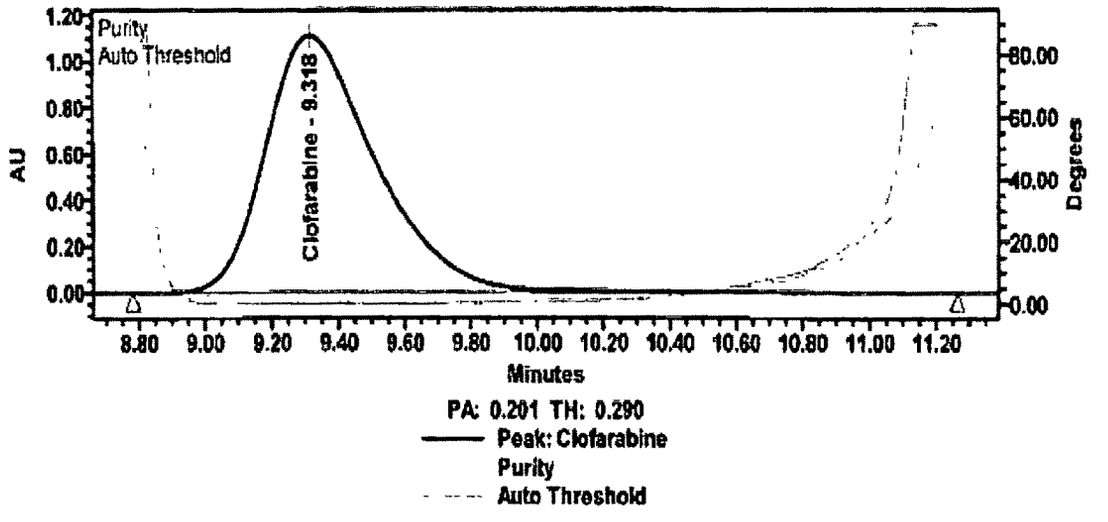
Chromatogram

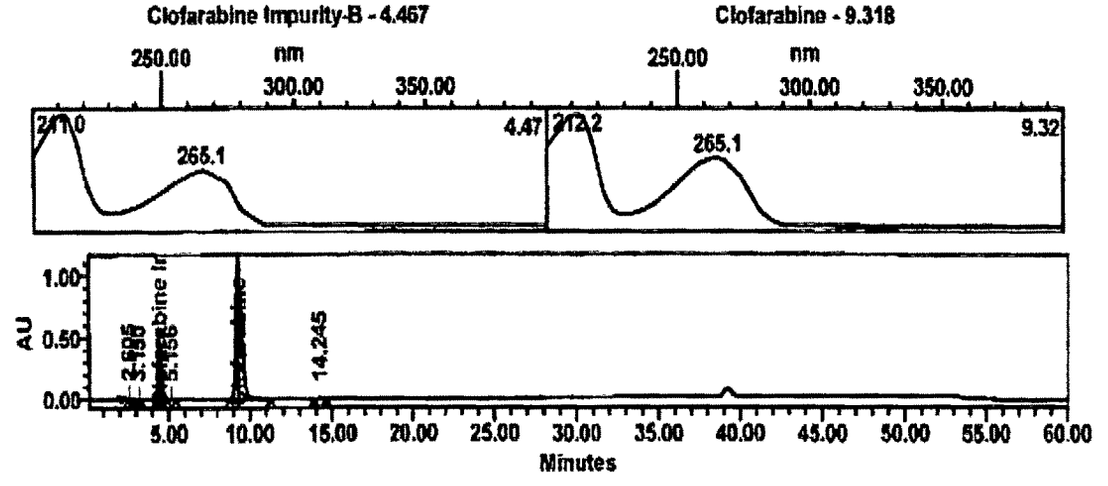


Peak Table

	Peak Name	RT	Area	% Area	RT Ratio
1		2.60	67572	0.22	
2		3.15	13319	0.04	
3	Clofarabine Impurity-B	4.47	5514206	17.79	0.48
4		5.16	14041	0.05	
5	Clofarabine	9.32	25373022	81.87	
6	Clofarabine Impurity-A	10.80			
7		14.25	10066	0.03	
Sum			30992226	100.00	

Peak Purity Data

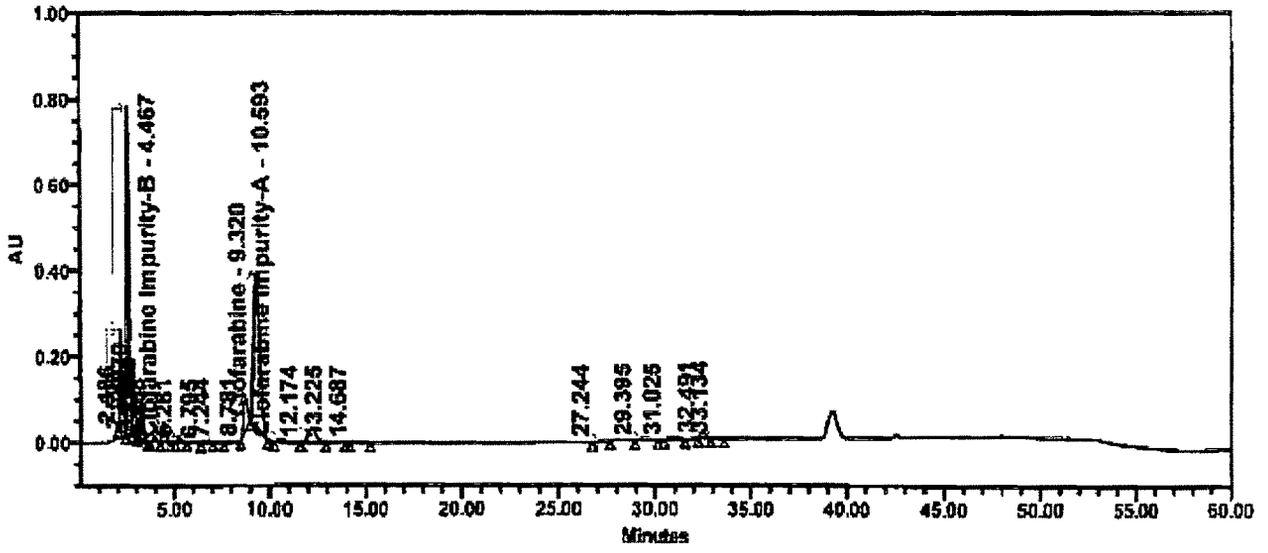




Base degradation chromatogram

Chromatogram

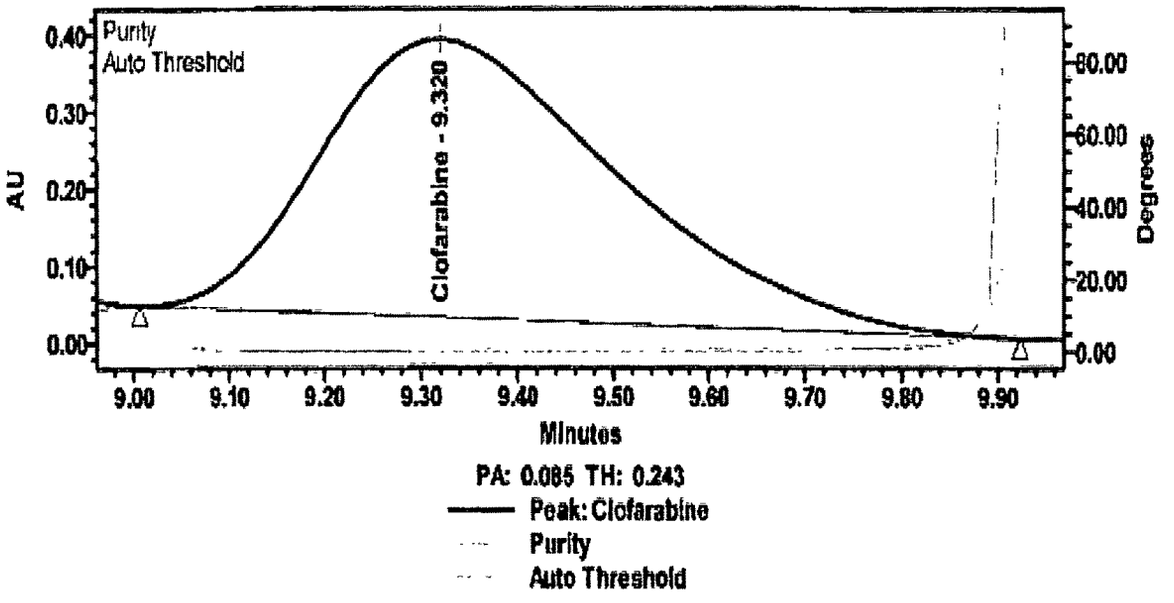
Chromatogram

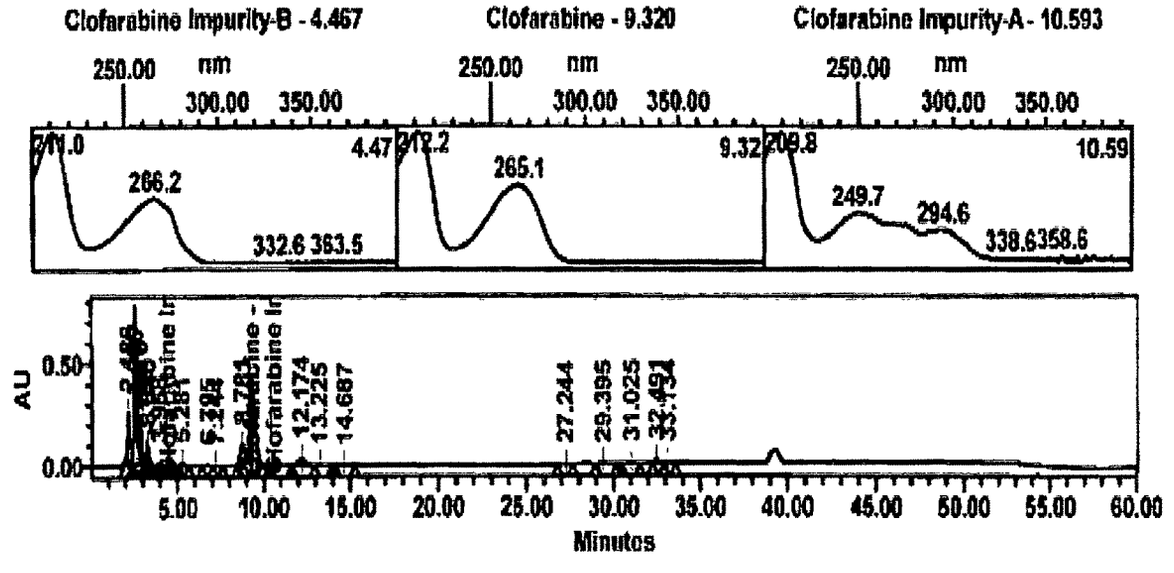


Peak Table

	Peak Name	RT	Area	% Area	RT Ratio
1		2.19	2123165	9.61	
2		2.60	6055321	27.42	
3		2.87	1475597	6.68	
4		3.16	307156	1.39	
5		3.31	858904	3.89	
6		3.97	174619	0.79	
7	Clofarabine Impurity-B	4.47	484085	2.19	0.48
8		5.28	153403	0.69	
9		6.79	60067	0.27	
10		7.24	31163	0.14	

Peak Purity Data

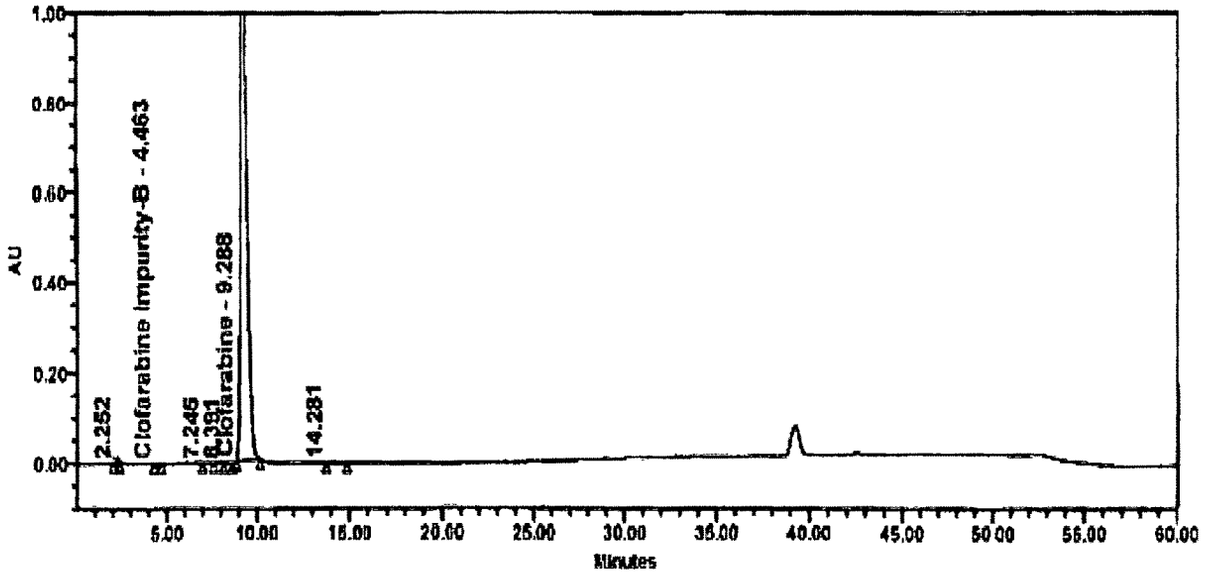




Thermal Degradation chromatogram

Chromatogram (Solid form Degradation)

Chromatogram

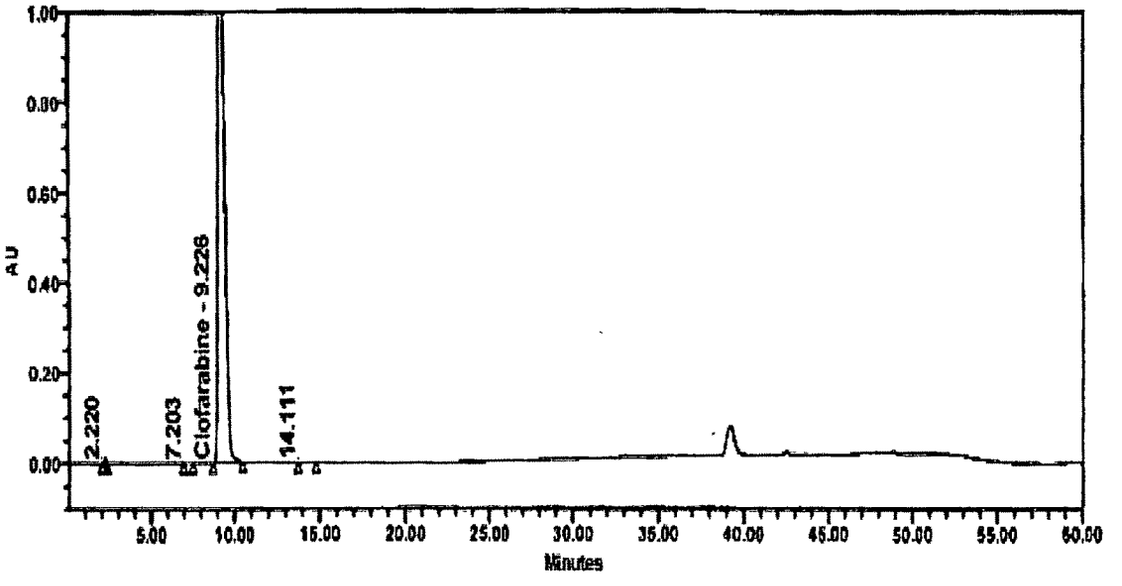


Peak Table

	Peak Name	RT	Area	% Area	RT Ratio
1		2.25	69119	0.21	
2	Clofarabine Impurity-B	4.46	6596	0.02	0.48
3		7.25	10284	0.03	
4		8.39	7474	0.02	
5	Clofarabine	9.29	33545904	99.63	
6	Clofarabine Impurity-A	10.80			
7		14.28	31738	0.09	
Sum			33671115	100.00	

Chromatogram (Solution form Degradation)

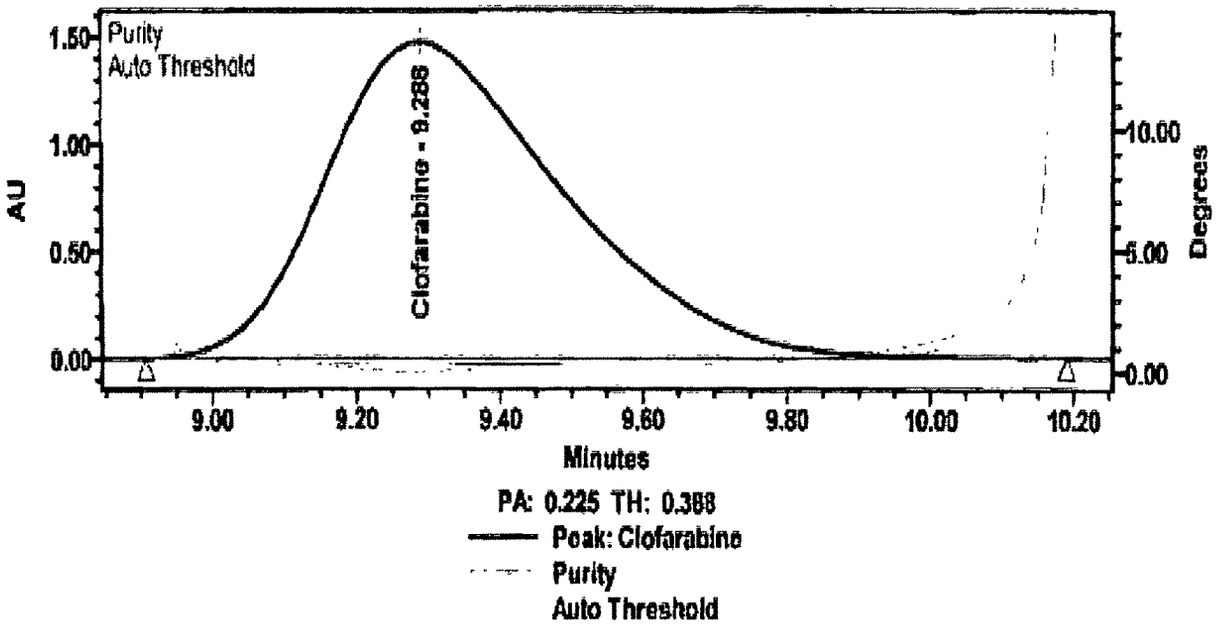
Chromatogram

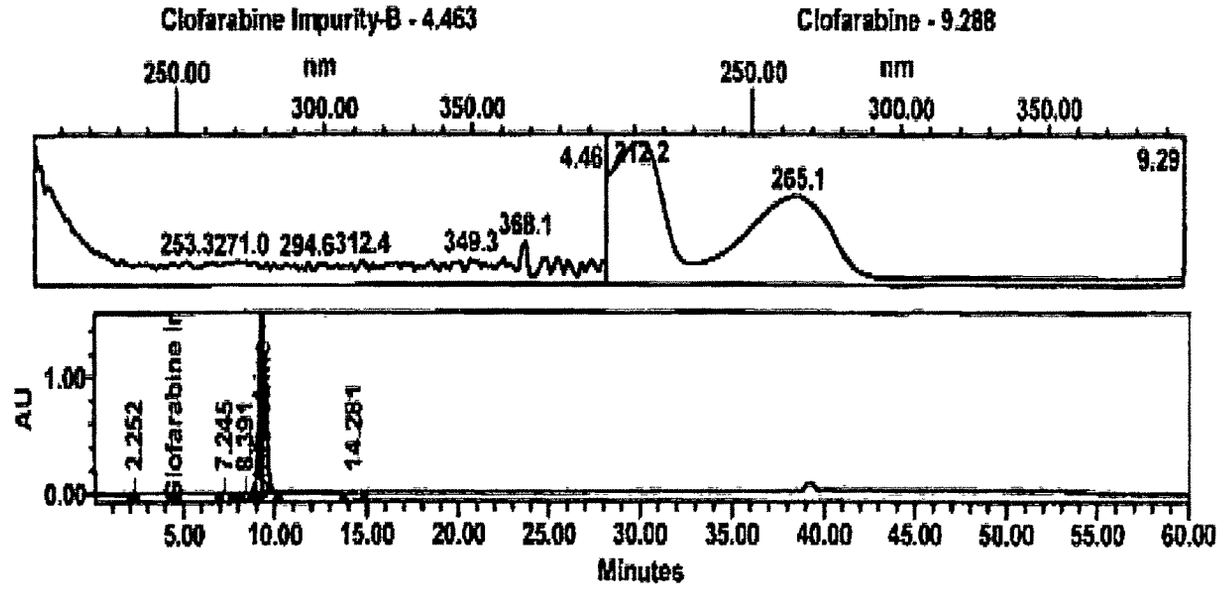


Peak Table

	Peak Name	RT	Area	% Area
1		2.22	104427	0.31
2	Clofarabine Impurity-B	4.30		
3		7.20	5514	0.02
4	Clofarabine	9.23	33367637	99.58
5	Clofarabine Impurity-A	10.80		
6		14.11	30180	0.09
Sum			33507758	100.00

Peak Purity Data

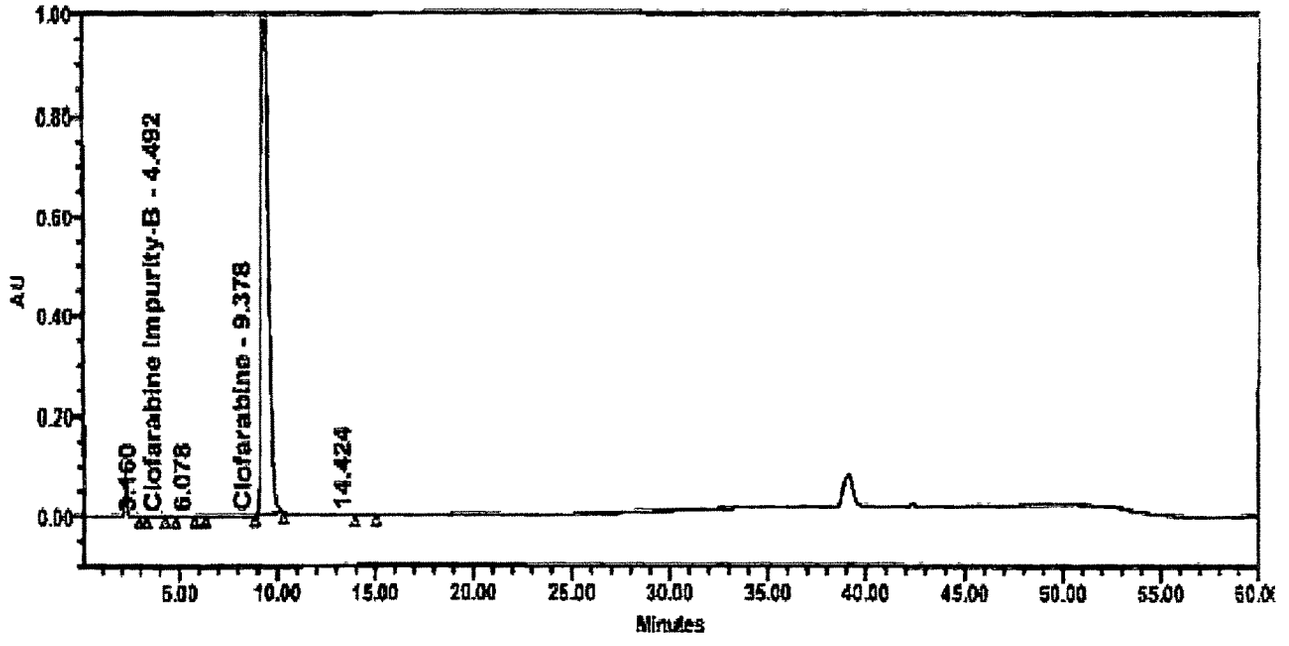




Oxidative degradation chromatogram

Chromatogram

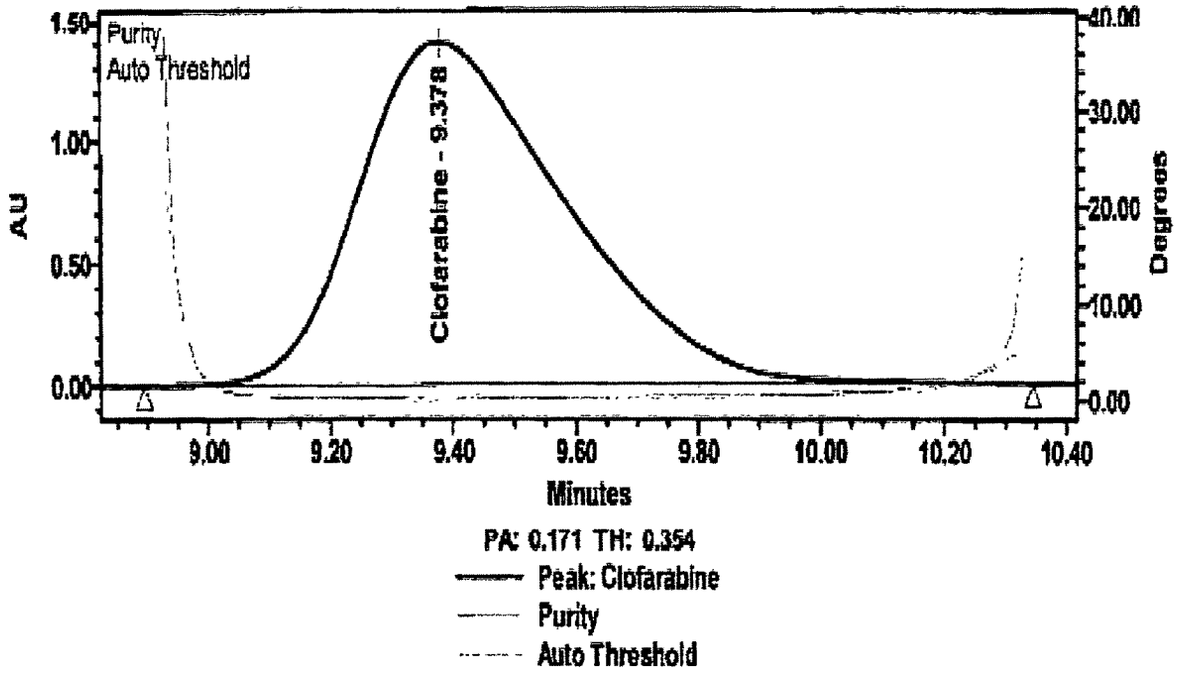
Chromatogram

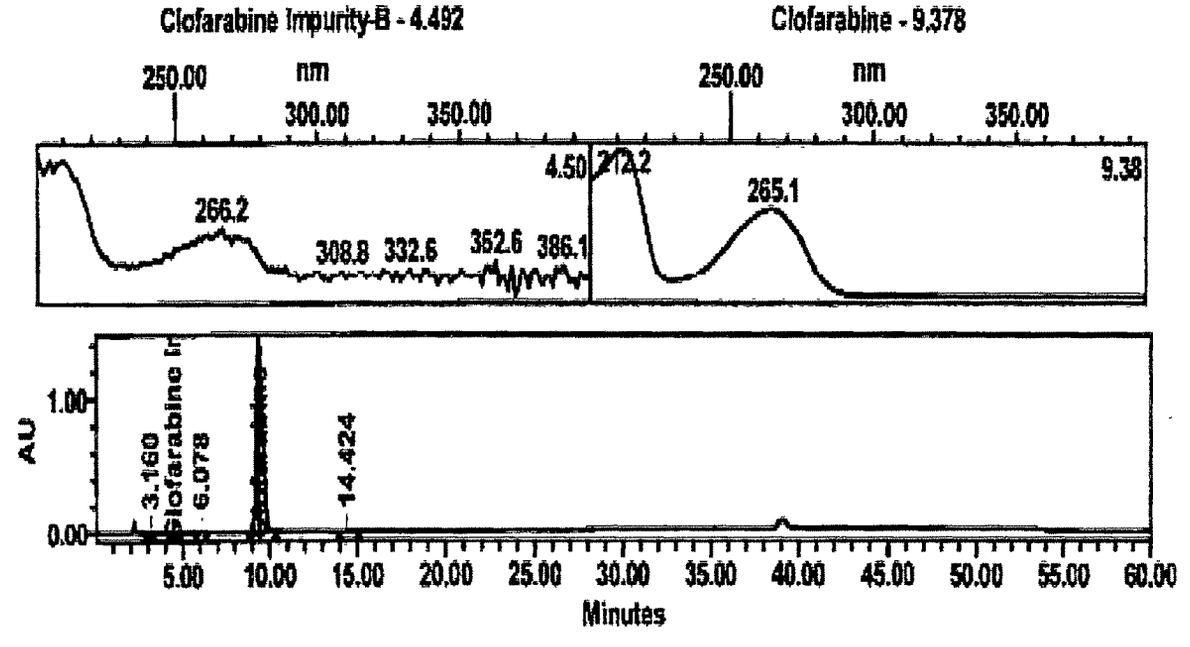


Peak Table

	Peak Name	RT	Area	% Area	RT Ratio
1		3.16	6268	0.02	
2	Clofarabine Impurity-B	4.49	17093	0.05	0.48
3		6.08	8287	0.03	
4	Clofarabine	9.38	32578213	99.80	
5	Clofarabine Impurity-A	10.80			
6		14.42	34372	0.11	
Sum			32644233	100.00	

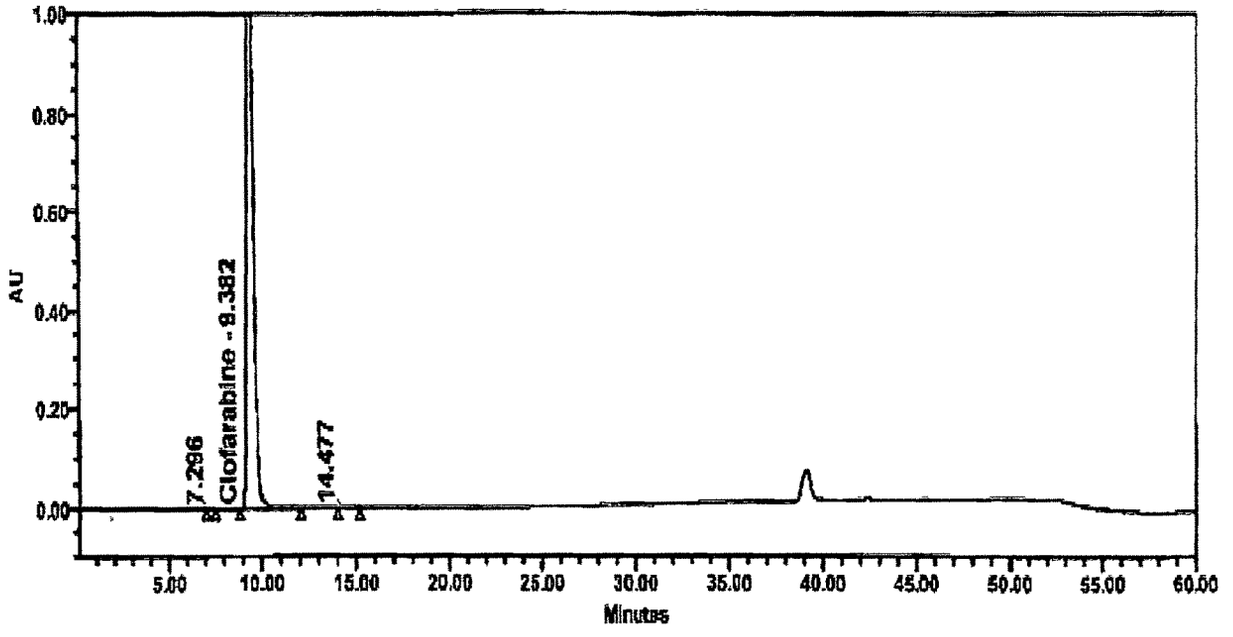
Peak Purity Data





UV Light Degradation

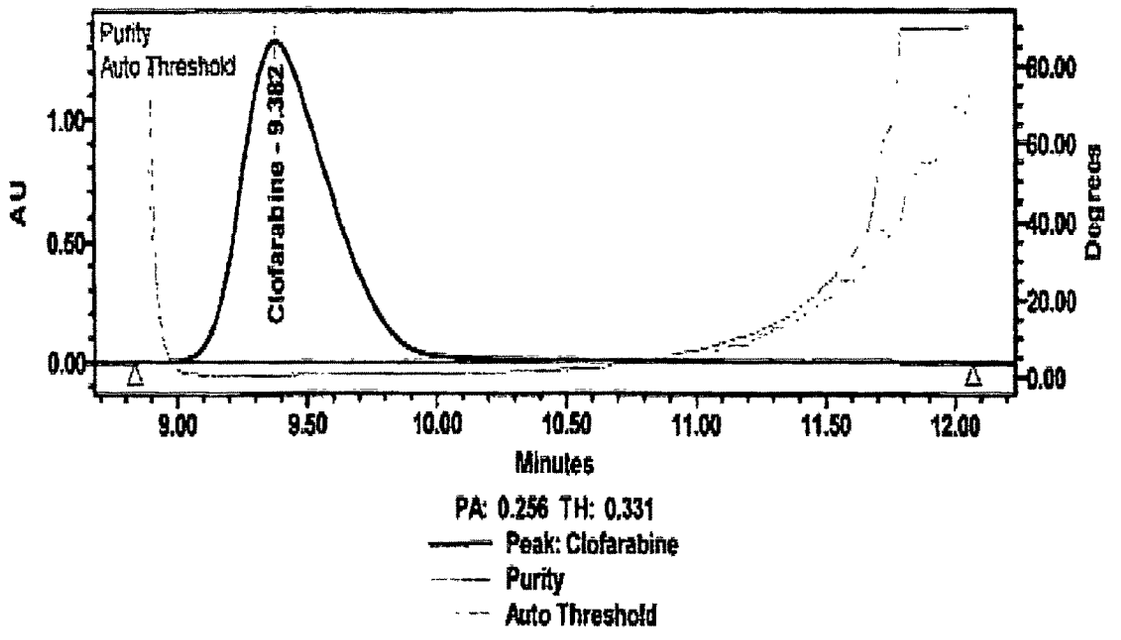
Chromatogram

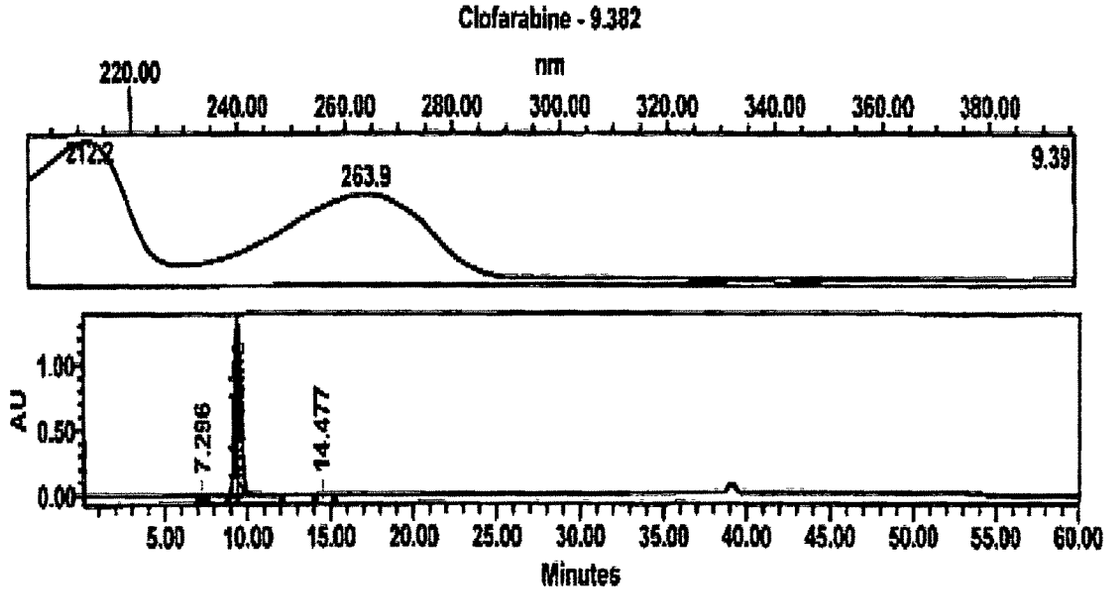


Peak Table

	Peak Name	RT	Area	% Area
1	Clofarabine Impurity-B	4.30		
2		7.30	5112	0.02
3	Clofarabine	9.38	31330780	99.88
4	Clofarabine Impurity-A	10.80		
5		14.48	32497	0.10
Sum			31368389	100.00

Peak Purity

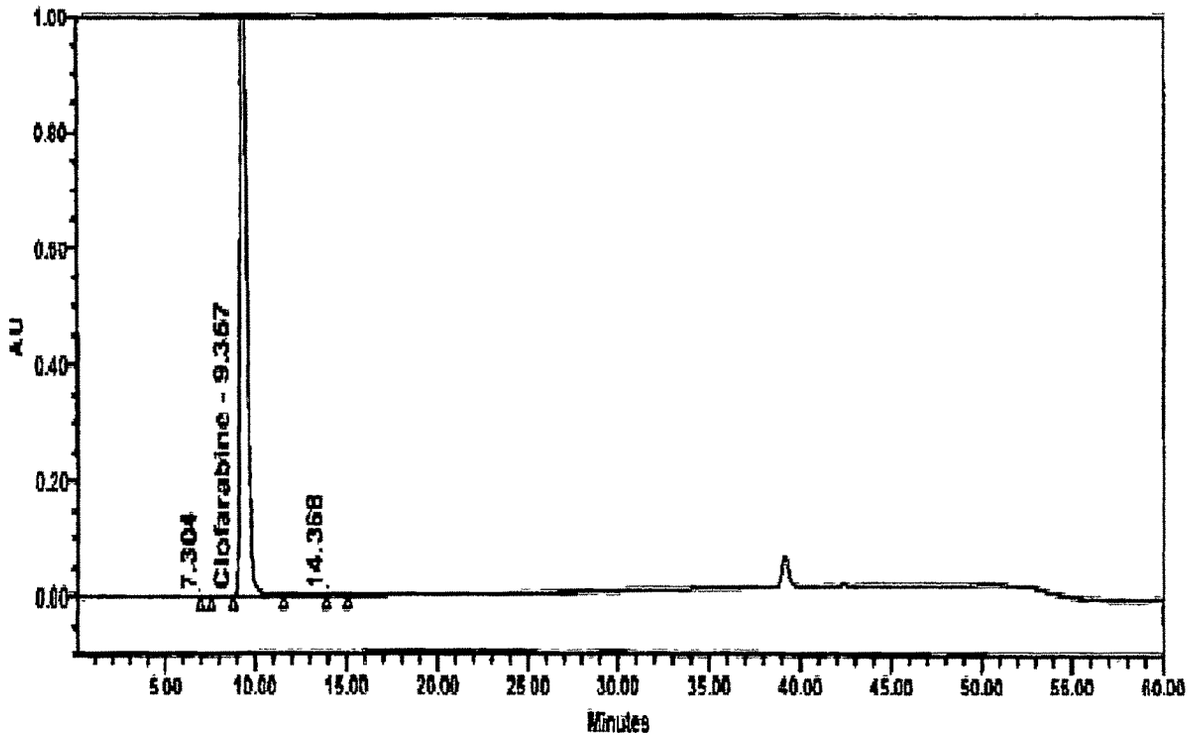

Purity
Auto Threshold
Clofarabine - 9.382
AU
Degrees
Minutes
PA: 0.256 TH: 0.331
Peak: Clofarabine
Purity
Auto Threshold


Clofarabine - 9.382
nm
212.2
263.9
9.39
7.296
14.477
AU
Minutes

Sunlight Degradation

Chromatogram

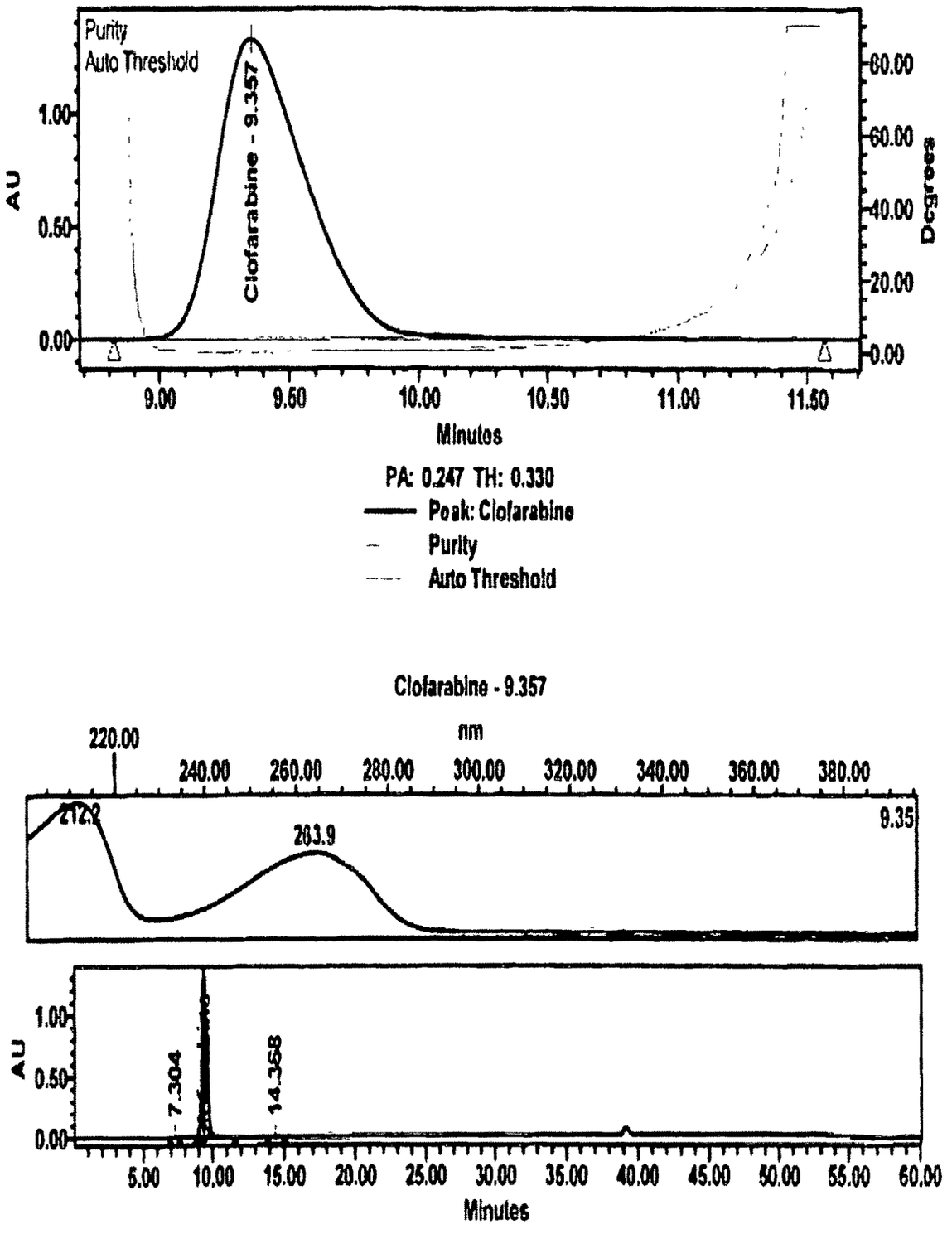
Chromatogram



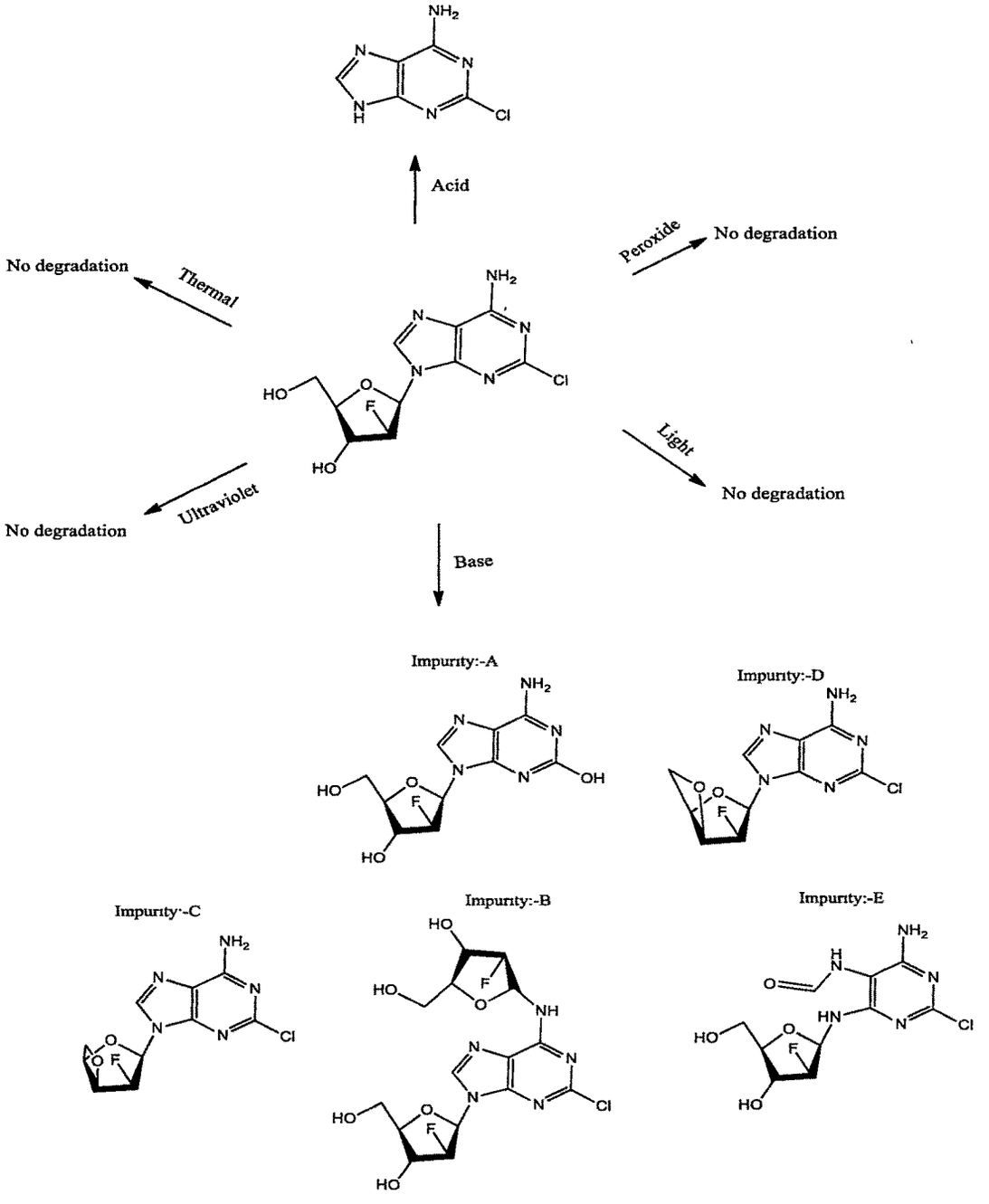
Peak Table

	Peak Name	RT	Area	% Area
1	Clofarabine Impurity-B	4.30		
2		7.30	7387	0.02
3	Clofarabine	9.36	31395766	99.88
4	Clofarabine Impurity-A	10.80		
5		14.37	29905	0.10
Sum			31433059	100.00

Peak Purity


Purity
Auto Threshold
Clofarabine - 9.357
AU
Degrees
Minutes
PA: 0.247 TH: 0.330
Peak: Clofarabine
Purity
Auto Threshold
Clofarabine - 9.357
nm
212.2
263.9
9.35
7.304
14.368
AU
Minutes

Clofarabine Degradation Pathways



CHARACTERIZATION AND STRUCTURE ELUCIDATION

All the possible process and degradation individual impurities either synthesized or isolated by enriching the impurity in the mother liquors and characterized by various analytical techniques and confirmed the structure of the impurities. Later these impurities were spiked and confirmed the retention times (RT's) and relative retention times (RRT's). The full physico-chemical characterization of each individual impurity is provided below.

PHYSICO-CHEMICAL CHARACTERIZATION OF BENZOIC ACID AMIDE

The physico-chemical characterization of Benzoic acid amide was established by analytical techniques such as FT-IR, HPLC and NMR analysis. The chromatographic purity of Benzoic acid amide was determined by HPLC.

1.0 PHYSICAL PROPERTIES

2.0 APPEARANCE

Table-1 Appearance of Benzoic acid amide

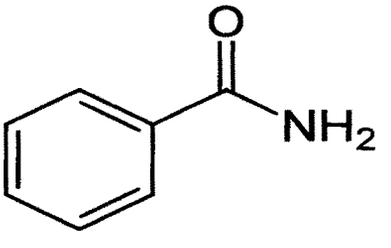
Name	Appearance
Benzoic acid amide	White Crystalline powder

3.0 CHEMICAL PROPERTIES

3.1 Chemical Name: Benzamide

3.2 Common Name: Benzoic acid amide (Clofarabine Related Compound-A)

3.3 Structural Formula



3.4 Molecular Formula: $C_6H_5CONH_2$

3.5 Molecular Weight: 121.14

3.6 Melting range: 127.6 °C to 128.8°C [By Melting point apparatus]

4.0 CHROMATOGRAPHIC PURITY BY HPLC

The Chromatographic purity of Benzoic acid amide was determined by High performance liquid chromatography (HPLC) using in-house test procedure. The results are depicted in **Table-2**. The chromatograms are depicted in **Exhibits below**.

Table-2 Chromatography purity

Name	% Chromatography purity by HPLC (Average)
Benzoic acid amide	96.1 %

The percentage chromatographic purity of Benzoic acid amide is 96.1% and remaining all the other peaks are unknown peaks.

5.0 CHARACTERIZATION BY FT-IR

The Infrared spectra of Benzoic acid amide was obtained using FT-IR.
The FT-IR spectra of Benzoic acid amide is depicted in **Table-3** and **Exhibits.**

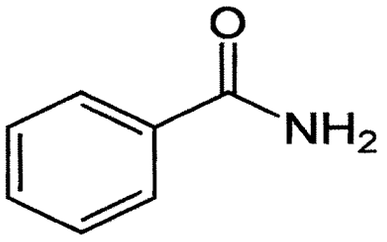
Table-3 FT-IR Frequency table

Bond	Approx. Frequency (cm^{-1})	Intensity and range
-C-C-	1448.54,1494.83 & 1577.77	Medium
N-H	3367.71	Medium
C-O-C	1072.42	Medium

Conclusion: The IR frequencies are in-line with the functional groups associated with the Benzoic acid amide structure and chemical name.

6.0 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER

The Benzoic acid amide was analyzed on 400 MHz Nuclear magnetic resonance spectrometer (NMR) by preparing sample in DMSO. The lot was analyzed for the proton (^1H) NMR and carbon (^{13}C) NMR. The NMR spectra for proton scan and carbon scans are depicted in **Exhibits.**



1H NMR

Proton Position	Chemical Shift (ppm)
3H	7.45
1H	7.52
1H	8.02
2H	7.90

13C NMR

Carbon Position	Chemical Shift (ppm)
CO	167.90
Aromatic ring carbons	127.41 to 134.19

Conclusion

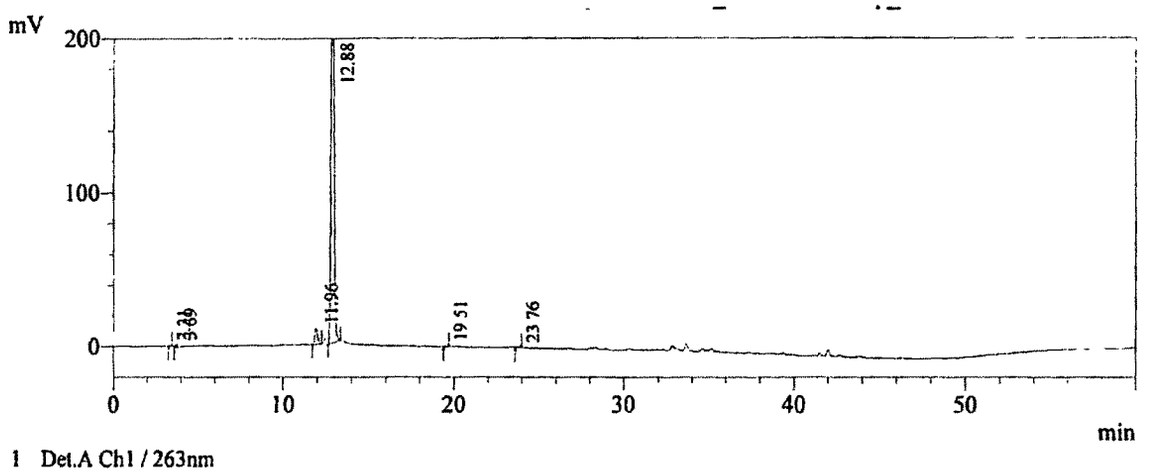
From ^1H NMR, it is clear that there are five aromatic protons present in the structure and From ^{13}C NMR, it is also clear that there are five aromatic carbons and a carbonyl group in the structure. These spectral data shows that the structure is benzoic acid amide.

7.0 STORAGE CONDITION

Store in a well closed container at room temperature

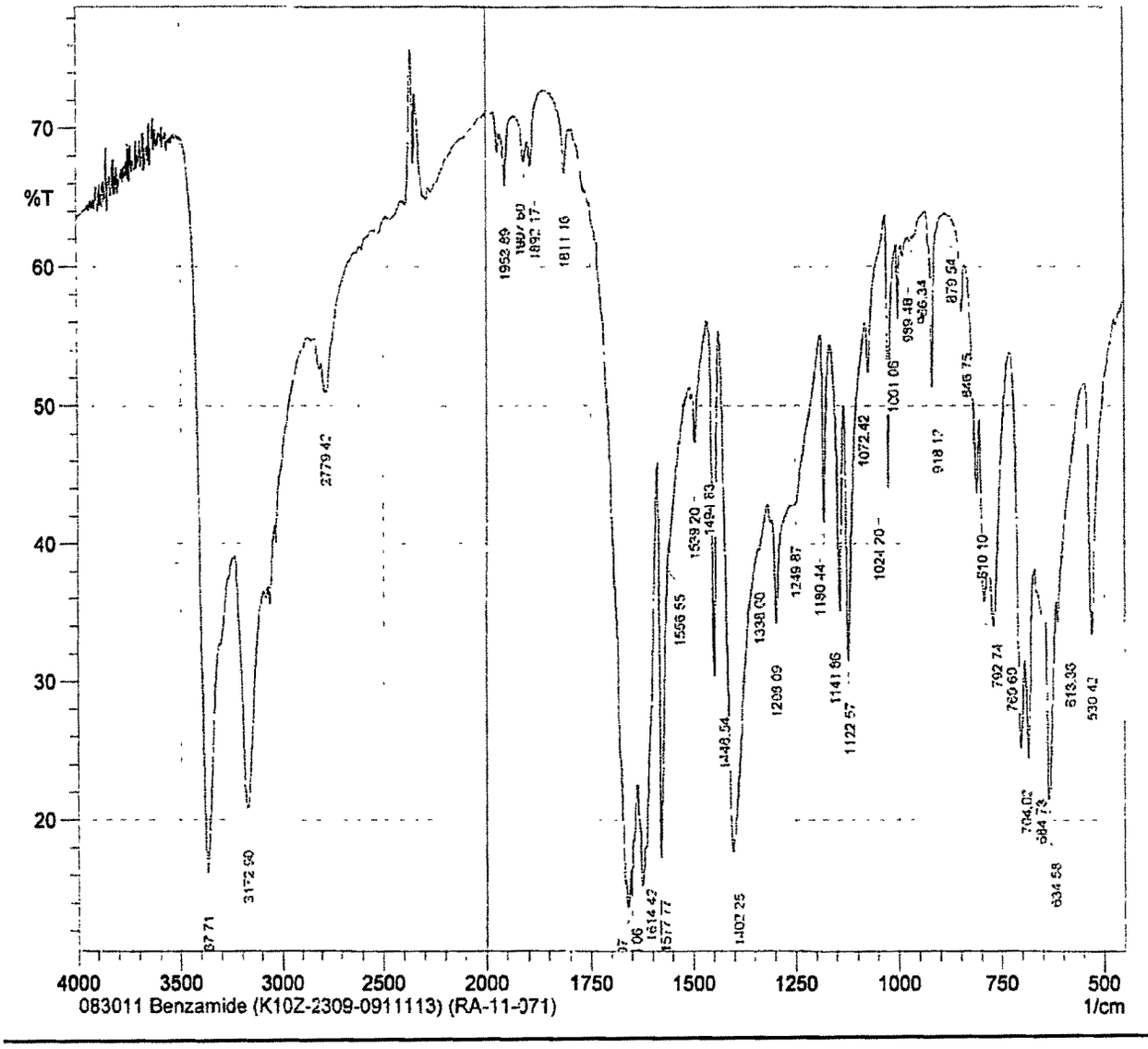
EXHIBITS

Chromatogram of chromatographic purity by HPLC

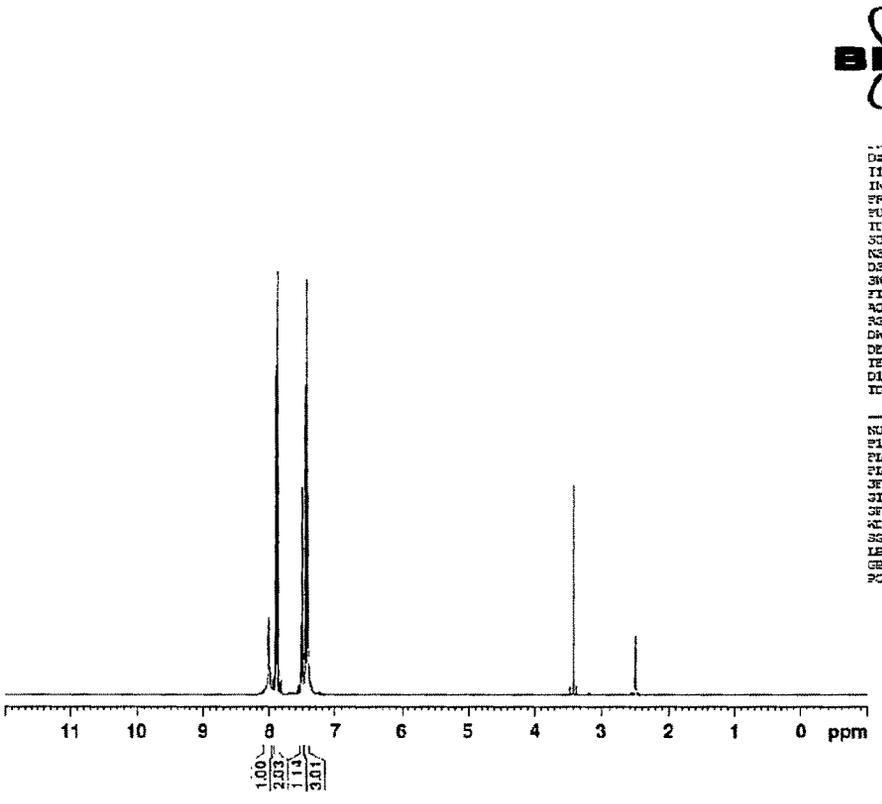


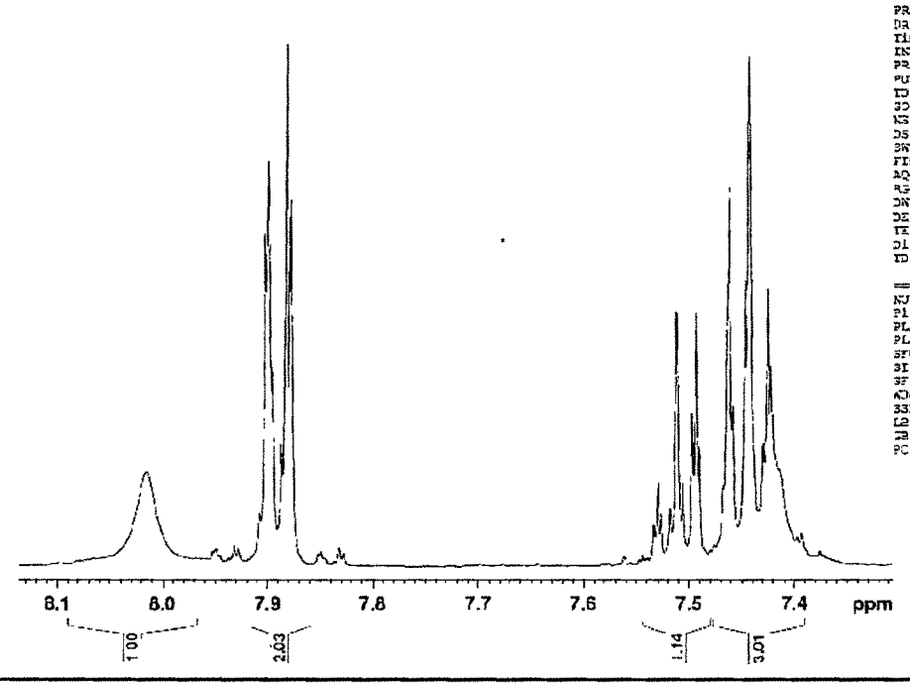
Peak#	Ret. Time	Area	Area %	Name
1	3.31	3204	0.10	Unknown impurity
2	3.69	6108	0.18	Unknown impurity
3	11.96	112545	3.35	Unknown impurity
4	12.88	3224878	96.12	Benzamide
5	19.51	4911	0.15	Unknown impurity
6	23.76	3474	0.10	Unknown impurity
Total		3355121	100.00	

Infrared Spectrum of Benzoic acid amide

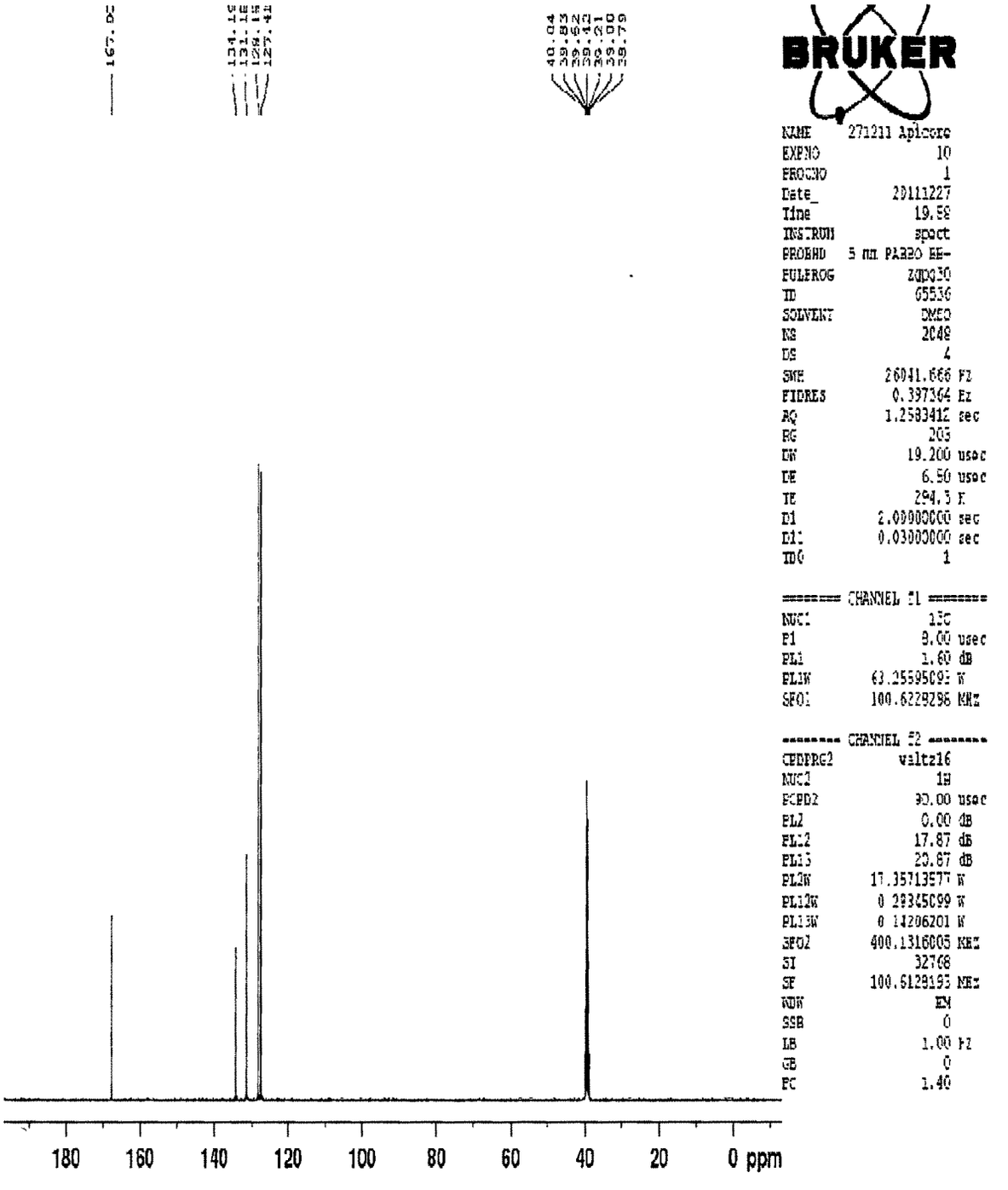


Proton (^{1}H) NMR of Benzoic acid amide





Carbon (^{13}C) NMR of Benzoic acid amide



PHYSICO-CHEMICAL CHARACTERIZATION OF 2-CHLORO-6-AMINOPURINE

The Physico-chemical characterization of 2-Chloro-6-aminopurine was established by analytical techniques such as FT-IR, HPLC and NMR analysis. The Chromatographic purity of 2-Chloro-6-aminopurine reference standard was determined by HPLC.

1.0 PHYSICAL PROPERTIES

2.0 APPEARANCE

Table-1 Appearance of 2-Chloro-6-aminopurine

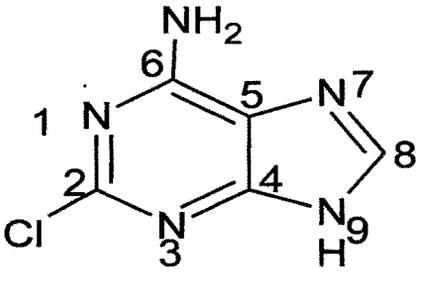
Name	Appearance
2-chloro-6-aminopurine	Off-white powder

3.0 CHEMICAL PROPERTIES

3.1 Chemical Name: 2-Chloroadenine or 6-Amino-2-chloro purine

3.4 Common Name: 2-Chloro-6-aminopurine (Clofarabine Related Compound-B)

3.5 Structural Formula



3.4 Molecular Formula: $C_5H_5ClN_5$

3.5 Molecular Weight: 169.5

3.6 Melting range: Above 250°C [By Melting point apparatus]

4.0 CHROMATOGRAPHIC PURITY BY HPLC

The Chromatographic purity of 2-Chloro-6-aminopurine was determined by High performance liquid chromatography (HPLC) using in-house test procedure. The results are depicted in **Table-2**. The chromatograms are depicted in **Exhibits below**.

Table-2 Chromatography purity

Name	**% Chromatography purity by HPLC (average)**
2-chloro-6-aminopurine	96.9 %

The percentage chromatographic purity by HPLC of 2-Chloro-6-aminopurine is 96.9% and remaining all the other peaks are unknown.

5.0 CHARACTERIZATION BY FT-IR

The Infrared spectra of 2-Chloro-6-aminopurine was obtained using FT-IR.
The FT-IR spectra of 2-Chloro-6-aminopurine is depicted in **Table-3** and **Exhibits** below.

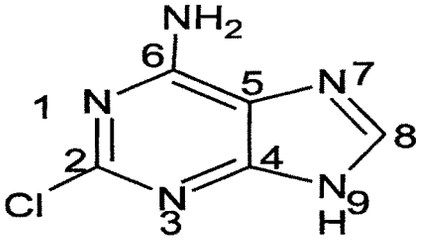
Table-3 FT-IR Frequency table

Bond	**Approx. Frequency (cm^{-1})**	**Intensity and range**
N-H	3151.69 & 3324.21	Medium
Halogen	1404.18	Medium

Conclusion: The IR frequencies are in-line with the functional groups associated with the 2-Chloro-6-aminopurine structure and chemical name.

6.0 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER

The 2-Chloro-6-aminopurine was analyzed on 400 MHz Nuclear magnetic resonance spectrometer (NMR) by preparing sample in DMSO. The lot was analyzed for the proton (^{1}H) NMR and carbon (^{13}C) NMR. The NMR spectra for Proton scan and carbon scans are depicted in **Exhibits.**



^{1}H NMR

Proton Position	Chemical Shift (ppm)
H8	8.266 (S)
NH_2	7.481 to 7.516 (br,s)

^{13}C NMR

Carbon Position	Chemical Shift (ppm)
C2	156.59
C8	152.74
C6	151.23
C4	139.48
C5	117.47

Conclusion

From ^{1}H NMR, it is clear that there is an imidazolyl proton and an amine proton in the moiety and

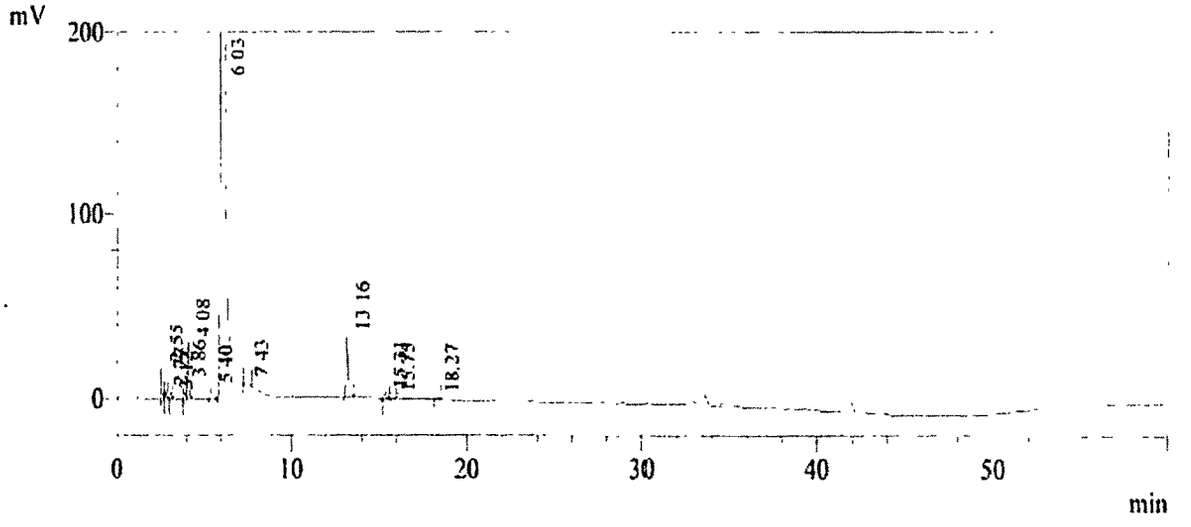
From ^{13}C NMR, the five aromatic carbons are confirmed. From these the structure of the compound is confirmed as 2-Chloro-6-aminopurine.

7.0 STORAGE CONDITION

Store in a well closed container at room temperature

EXHIBITS

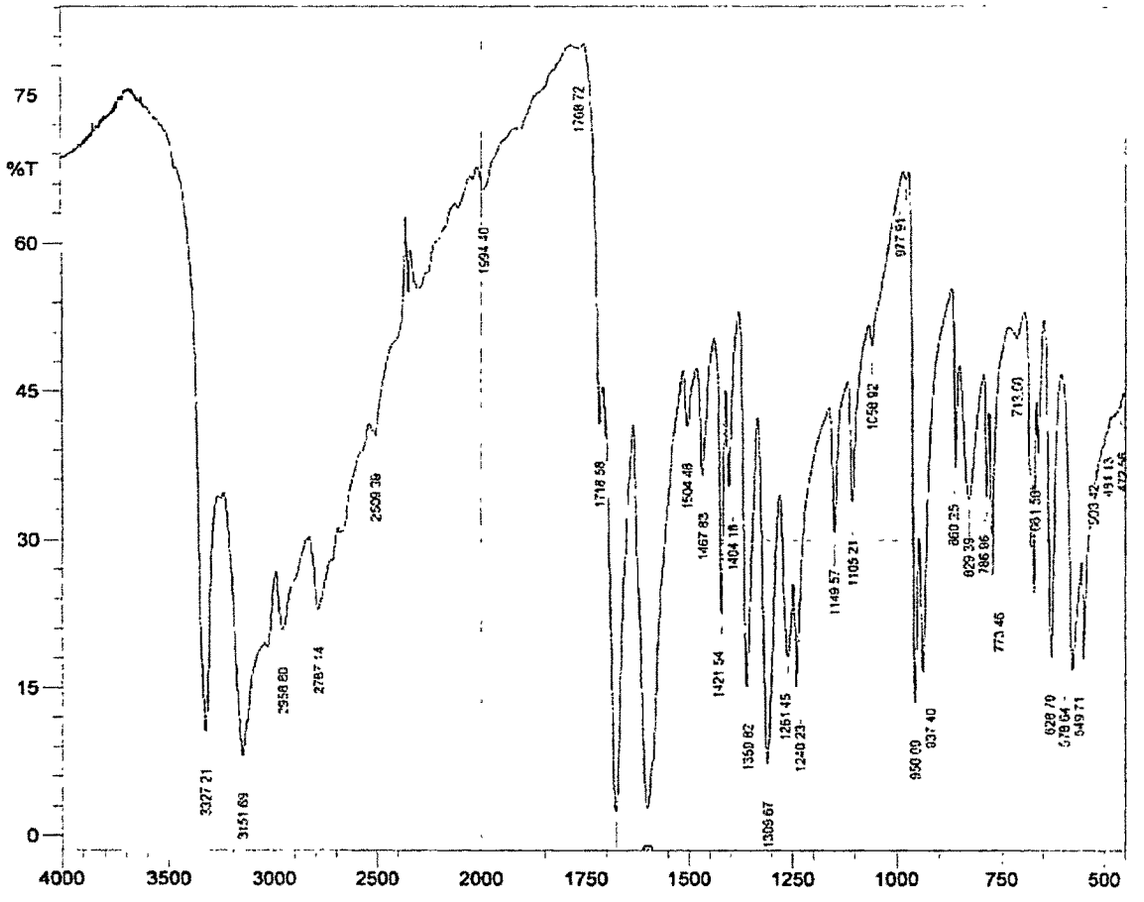
HPLC Chromatogram of related substances profile



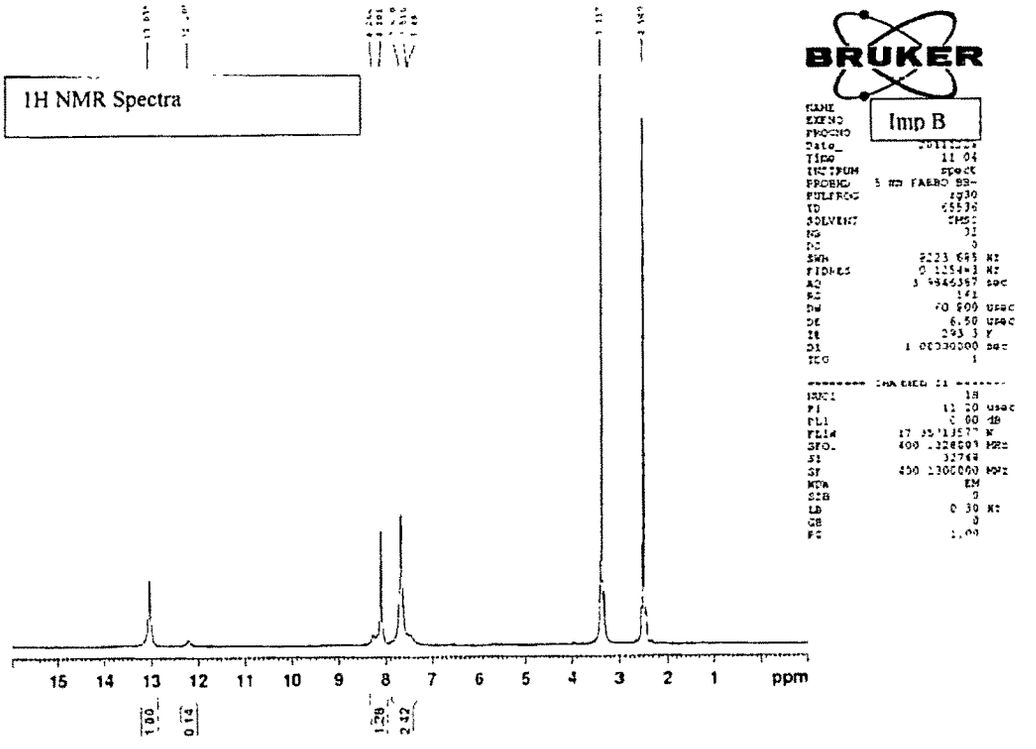
Detector A Ch1 263nm

Peak#	Ret. Time	Area	Area %	Name
1	2.55	65109	0.30	Unknown impurity
2	2.77	19401	0.09	Unknown impurity
3	3.12	10457	0.05	Unknown impurity
4	3.86	34677	0.16	Unknown impurity
5	4.08	160012	0.75	Unknown impurity
6	5.40	42327	0.20	Unknown impurity
7	6.03	20737986	96.96	2-Chloro adenine
8	7.43	16992	0.08	Unknown impurity
9	13.16	274572	1.28	Unknown impurity
10	15.31	18163	0.08	Unknown impurity
11	15.75	4470	0.02	Unknown impurity
12	18.27	4710	0.02	Unknown impurity
Total		21388876	100.00	

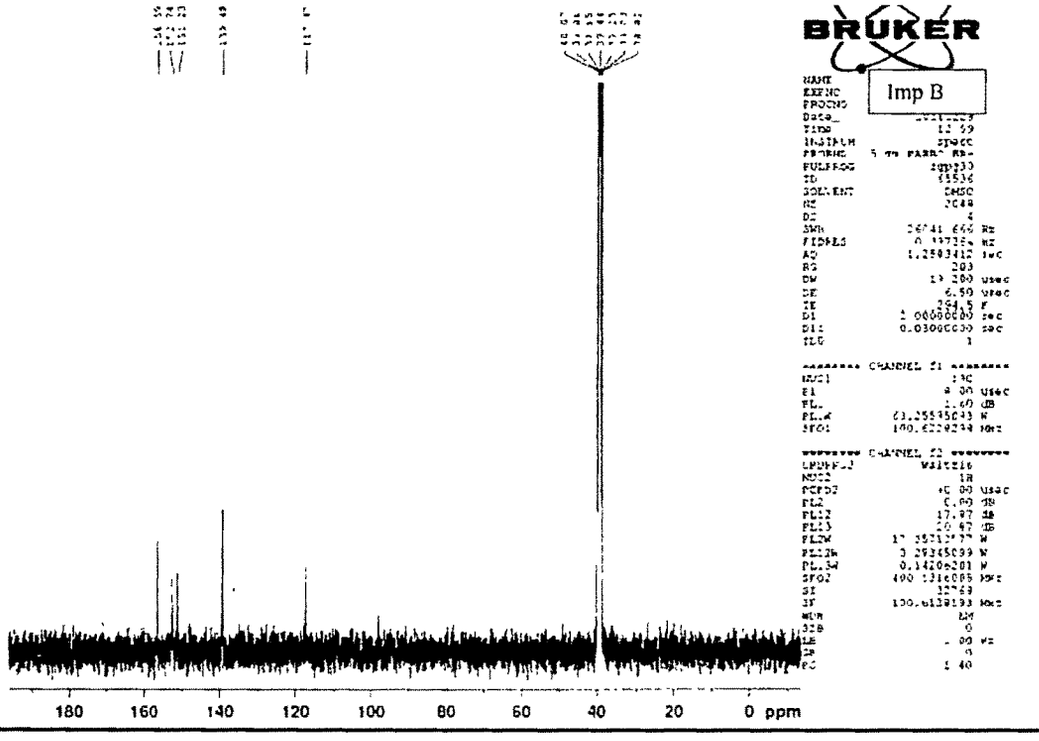
FT-IR Spectra of 2-Chloro-6-aminopurine



Proton (^{1}H) NMR of 2-Chloro-6-aminopurine



Carbon (^{13}C) NMR Spectra



PHYSICO-CHEMICAL CHARACTERIZATION OF CLOFARABINE RELATED COMPOUND-C

The physico-chemical characterization of Clofarabine Related Compound-C was established by analytical techniques such as FT-IR, HPLC, MS, NMR, DSC and TGA analysis. The Chromatographic purity of Clofarabine Related Compound-C Reference Standard was determined by HPLC.

1.0 PHYSICAL PROPERTIES

2.0 APPEARANCE

Table-1 Appearance of Clofarabine Related Compound-C

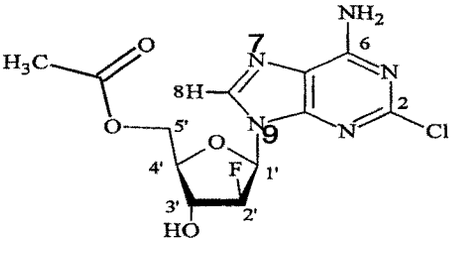
Name	Appearance
Clofarabine related compound C	Off-white powder

3.0 CHEMICAL PROPERTIES

3.1 Chemical Name: 6-Amino-2-chloro-9-(2-deoxy-2-fluoro-5-O-acetyl-β-D-arabinofuranosyl)-9H-purine

3.2 Common Name: Clofarabine Related Compound-C

3.3 Structural Formula



Clofarabine Impurity-C

3.4 Molecular Formula: $C_{12}H_{13}ClFN_5O_4$

3.5 Molecular Weight: 345.71

3.6 Melting range: Above 250 °C [By melting point apparatus]

4.0 CHROMATOGRAPHIC PURITY BY HPLC

The Chromatographic purity of Clofarabine Related Compound-C was determined by High performance liquid chromatography (HPLC) using in-house test procedure. The results are depicted in **Table-2**. The chromatograms are depicted in **Exhibits below**.

Table-2 Chromatography purity

Name	% Chromatography purity by HPLC (average)	Potency
Clofarabine related compound C	98.37 %	97.5 %

The percentage chromatographic purity by HPLC of Clofarabine Related Compound-C is 98.37% and remaining all the other peaks are unknown.

5.0 CHARACTERIZATION BY FT-IR

The Infrared spectra of Clofarabine Related Compound-C was obtained using FT-IR.
The FT-IR spectra of Clofarabine Related Compound-C is depicted in **Table-3** and **Exhibits.**

Table-3 FT-IR Frequency table

Bond	Approx. Frequency (cm^{-1})	Intensity
-C-C-	1463.97,1512.19 & 1597.06	Medium
Halogen	1386.82	Medium
C-O-C	1074.35	Medium
C=O	1743	Strong

Conclusion: The IR frequencies are in-line with the functional groups associated with the Clofarabine related compound-C structure and chemical name. The ester carbonyl at C=O confirms the presence of Acetyl ($COCH_3$) group in the moiety

6.0 CHARACTERIZATION BY MASS SPECTROMETRY

The Clofarabine Related Compound-C was analyzed for the parent ion scan by using infusion-MS on LC/MS/MS mass spectrometer. The mass data presented in following **Table-4** is acquired via negative ion scan. Therefore the $(M-H)^-$ ions are observed. The mass spectral data is presented in **Exhibits below**.

Table-4: Molecular ion data for Clofarabine Related Compound-C by infusion-MS Technique

Name	$(M-H)^-$	Molecular weight of Clofarabine Related Compound-C
Clofarabine Related Compound C	344.08	345.71

Mass fragmentation pattern for the drug substance (Clofarabine), as per the Organic Process Research & Development 2004, 8, Pages 889 to 896 is given below

Clofarabine

$(M-H)^-$ Clofarabine: 302.07

$(M\text{-Sugar Moiety})^-$ → 2-Chloro adenine: 168.03

Clofarabine related compound-C

The fragmentation studies were done on the Clofarabine Related Compound-C with parent ion at 344.08 (M - in negative mode), which produced daughter ions at different masses 283.85 (m1) and 168.02(m2). The assignments for the daughter ions are done as follows.

M = Molecular weight of Clofarabine Related Compound-C (MW=344.08)

$(M-H)^-$ Acetyl Clofarabine: 344.08

$(M\text{-Acetyl})^-$ des acetyl desflouro Clofarabine: 283.85(m1)

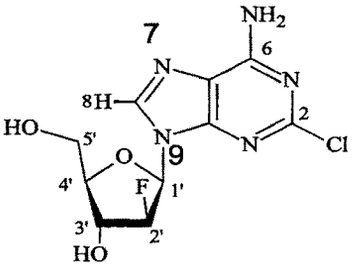
$(M\text{-Sugar Moiety})^-$ → 2-Chloro adenine: 168.02 (m2)

Conclusion

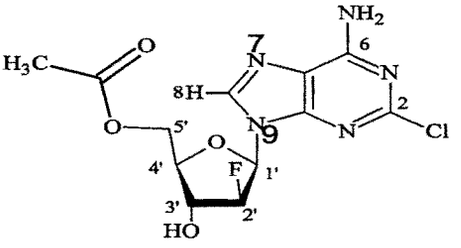
Mass fragmentation data confirms that m/z of 43 daltons is added to the Clofarabine which corresponds to the acetyl group. Based on the fragmentation data it can be concluded that the acetyl group is attached to the sugar moiety of the nucleoside and not to the chloro adenine portion of the structure of Clofarabine, as we see the daughter ions (M-sugar moiety) gives the fragment at m/z = 168.02 which is similar to the Clofarabine daughter ion. The loss of acetyl group leads to another daughter ion with m/z at 283.85 which is specific to the impurity-C and not seen in the Drug substance. Now there are two possibilities for the acetyl group addition onto the sugar moiety either at 3rd position (-OH) or the 5th position on the CH2OH. The specific position of acetyl addition could not be established on the mass spectrophotometer, since further fragmentation studies on the molecule also could not reveal the acetyl position. Therefore the acetyl position in the Clofarabine related compound-C is identified by the NMR data given below.

7.0 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER

The Clofarabine Related Compound-C & Clofarabine drug substance were analyzed on 400 MHz Nuclear magnetic resonance spectrometer (NMR) by preparing sample in DMSO. Both the Drug substance and Clofarabine Related Compound-C were analyzed for the proton (^{1}H) NMR and carbon (^{13}C) NMR Scans. The NMR spectra for Proton scans and Carbon scans are depicted in **Exhibits below**.



Clofarabine



Clofarabine Impurity-C

Table-5: NMR chemical shifts

^{1}H NMR

Proton Position	Chemical Shift (ppm)	
	Clofarabine	**Clofarabine related compound -C**
H8	8.27 (d)	8.2 (d)
NH_2	7.89 (br,s)	7.92 (br,s)
H1	6.32 (dd)	6.35 (dd)
OH3'	5.98 (d)	6.15 (d)
H2'	5.29 (dt)	5.33-5.22 (dt)
OH5' **	5.16 (t)	--
H3'	4.43 (dm)	4.52 (dm)
H4'	3.85 (m)	4.0 (m)
H5' **	3.6-3.72 (m)	4.36 (m)
CH3	-	2.04(s)

^{13}C NMR

Carbon Position	Chemical Shift (ppm)	
	Clofarabine	**Clofarabine related compound -C**
CH_3- **	--	20.53
C5'	60.28	63.31
C3'	72.56	73.15
C1'	81.31	81.38
C4'	83.46	81.22
C2'	96.24	95.76
C5	117.30	117.31
C8	139.99	140.16
C6	150.12	150.15
C4	153.22	153.28
C2	156.73	156.77
C-O **	--	170.19

**** The distinguishing proton and carbon frequencies between the drug and the impurity-C**

7.1 Structure confirmation

The Clofarabine related compound-C has been identified as 6-Amino-2-chloro-9-(2-deoxy-2-fluoro-5-O-acetyl-β-D-arabinofuranosyl)-9H-purine based on the below characterization data. The mass data shows that the mass of the compound is 345.71 and it accounts for an acetyl group addition on the clofarabine structure. This has been confirmed by the NMR data which depicts the presence of acetyl group. Again, the position of the acetyl in clofarabine impurity is specified as 5-O-acetyl based on the below NMR values which shows the presence of 3-OH protons intact but the 5-OH proton is absent. The 5-OH is acetylated, therefore the proton is missing.

Reported frequencies

The ^{1}H NMR and ^{13}C NMR values of Clofarabine as per literature -Organic Process Research & Development 2004, 8, Pages 889 to 896 are as follows.

The ^{1}HNMR reported values for Clofarabine are 8.27 (d,1H,-*NH=CH-N-*), 7.87(S,2H,*NH2*) 6.32 (dd, 1H, *-O-H*), 5.95 (d,1H, *CH-OH*) , 5.22(dt,1H, *CH-F*), 5.08 (t,1H, *CH_2-OH*), 4.43 (m,1H, *CH-OH*), 3.85 (m,1H, CH- *CH_2-OH*) 3.6-3.72 (m,2H, *CH_2-OH*).

The ^{13}C NMR reported values for Clofarabine are 60.34(C5') 72.56(C3'), 81.44(C1'), 83.50(C4'), 95.33(C2'), 117.35(C5), 140.00(C8), 150.16(C6)153.26(C4), 156.80(C2)

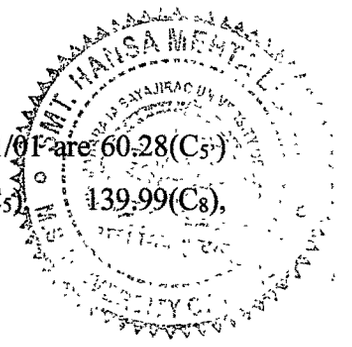
Observed Frequencies

The data observed for our samples of Clofarabine and the Clofarabine related compound-C when recorded on a 400 MHz NMR are provided above in the table-5 for comparison purposes and the individual values are given below as well.

Drug Substance (Clofarabine)

The ^{1}H NMR values of the drug substance for Lot # API-173/11/10/01 are 8.27(d,1H,$_{H8}$), 7.89(S,2H,*NH2*), 6.32 (dd, 1H, *-O-H*), 5.98 (d,1H, *CH-OH*), 5.29 (dt,1H, *CH-F*), 5.16 (t,1H, *CH_2-OH*), 4.43 (m,1H, *CH-OH*), 3.85 (m,1H, CH- *CH_2-OH*), 3.6-3.72 (m,2H, *CH_2-OH*).

The ^{13}C NMR Values of the drug substance for Lot# API-173/11/101/01 are 60.28($C_{5'}$) 72.56($C_{3'}$), 81.31($C_{1'}$), 83.46($C_{4'}$), 96.24($C_{2'}$), 117.30(C_5), 139.99(C_8), 150.12(C_6)153.22(C_4), 156.73(C_2)



Clofarabine related compound-C

The Acetyl Clofarabine impurity values from the ^1H NMR spectra are 8.22(d,1H), 7.92(br,S,2H,NH2), 6.35(dd,H1), 6.15(d,1H,OH3), 5.34,5.22(1H,H2), **5.08(t,1H,OH5) (MISSING),** 4.52(dm,1H,H3), 4.36(m,2H,H5), 4.0(m,1H4), 3.38(1.17-OH of DMSO containing water) and 2.04(s,3H,acetyl).

The Acetyl Clofarabine values from the ^{13}C NMR spectra are 20.53(CH3-CO) 63.31($C_{5'}$) 73.15($C_{3'}$), 81.38($C_{1'}$), 81.22($C_{4'}$), 95.76($C_{2'}$), 117.31(C_5), 140.16(C_8), 150.15(C_6), 153.28(C_4), 156.77(C_2), 170.19 (CO of acetyl)

Conclusion

From ^1H NMR, it is clear that the 5-OH proton is missing in the impurity and it is acetylated and whereas, the 5-OH proton is present in Clofarabine drug substance. Due to the acetylation of the 5-OH, the corresponding CH_2 proton from the CH_2OH functional group is further shifted downfield to 4.36 ppm. Whereas it is found at 3.6 to 3.72 ppm in Clofarabine.

From ^{13}C NMR , it is also clear that the signal at 20.53 ppm shows the presence of an additional CH_3 group and the signal at 170.19 ppm corresponds to the ester carbonyl of acetyl group. These frequencies are not found in the Clofarabine drug substance sample. Again, the carbon signal (C_3) of 3-OH is not affected, whereas, the 5-OH of $C_{5'}$ is shifted downfield to 63.31 from 60.28 due to the acetyl group attached to the 5-OH.

The imidazolyl proton of the purine moiety is split into two peaks showing the single doublet which confirms to the beta configuration of the moiety. So the chirality of the impurity was carried from the drug substance itself and it is in agreement with the acetyl position in the impurity on 5-OH, which should not affect the beta anomeric form.

So the impurity is identified and characterized as "6-Amino-2-chloro-9-(2-deoxy-2-fluoro-5-O-acetyl-β-D-arabinofuranosyl)-9H-purine".

8.0 CHARACTERIZATION BY THERMO GRAVIMETRIC ANALYSIS [TGA]

The TGA of Clofarabine Related Compound-C was performed. The TGA data of the impurity presented in **Exhibits** depicts only residual moisture present in the sample.

There is no hydrate form observed as there is no bound water found in the sample. Also the Clofarabine Related Compound-C sample does not exist as any solvate either.

9.0 CHARACTERIZATION BY DIFFERENTIAL SCANNING CALORIMETER [DSC]

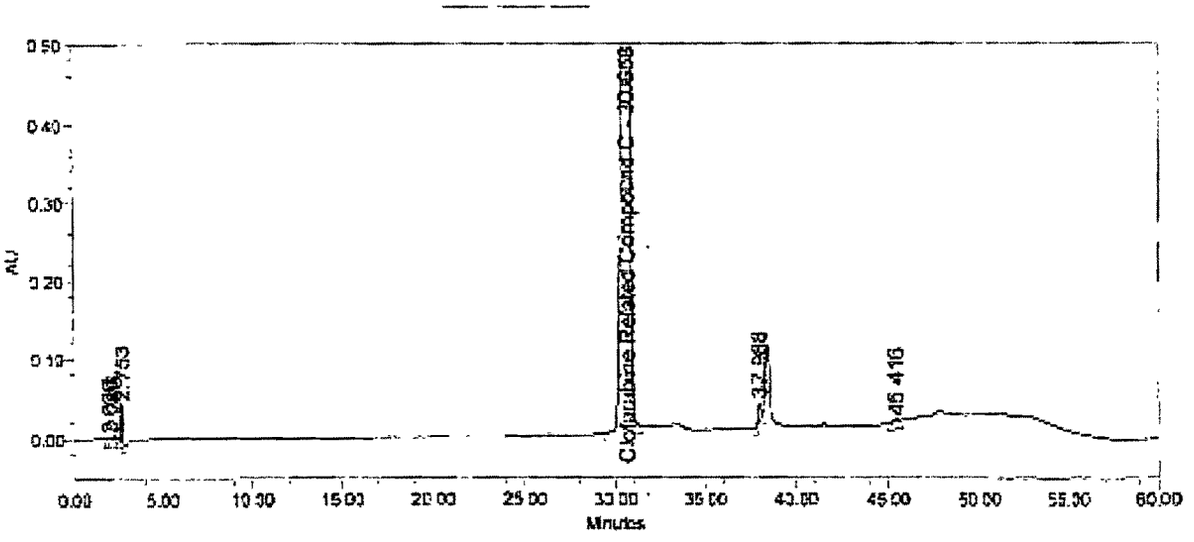
The DSC of Clofarabine Related Compound-C was performed. The DSC data of impurity presented in **Exhibits** depicts the exotherm and endotherm nature of the Clofarabine Related Compound-C

10.0 STORAGE CONDITION

Store in a well closed container at room temperature

EXHIBITS

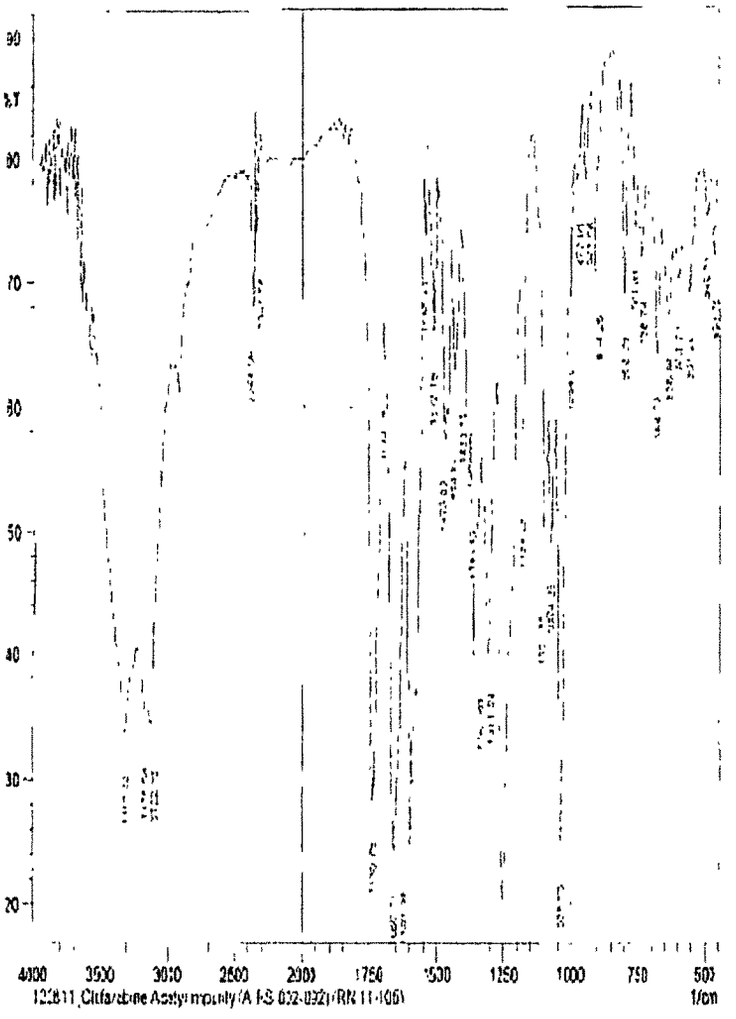
Chromatograms of chromatographic purity



Peak Table

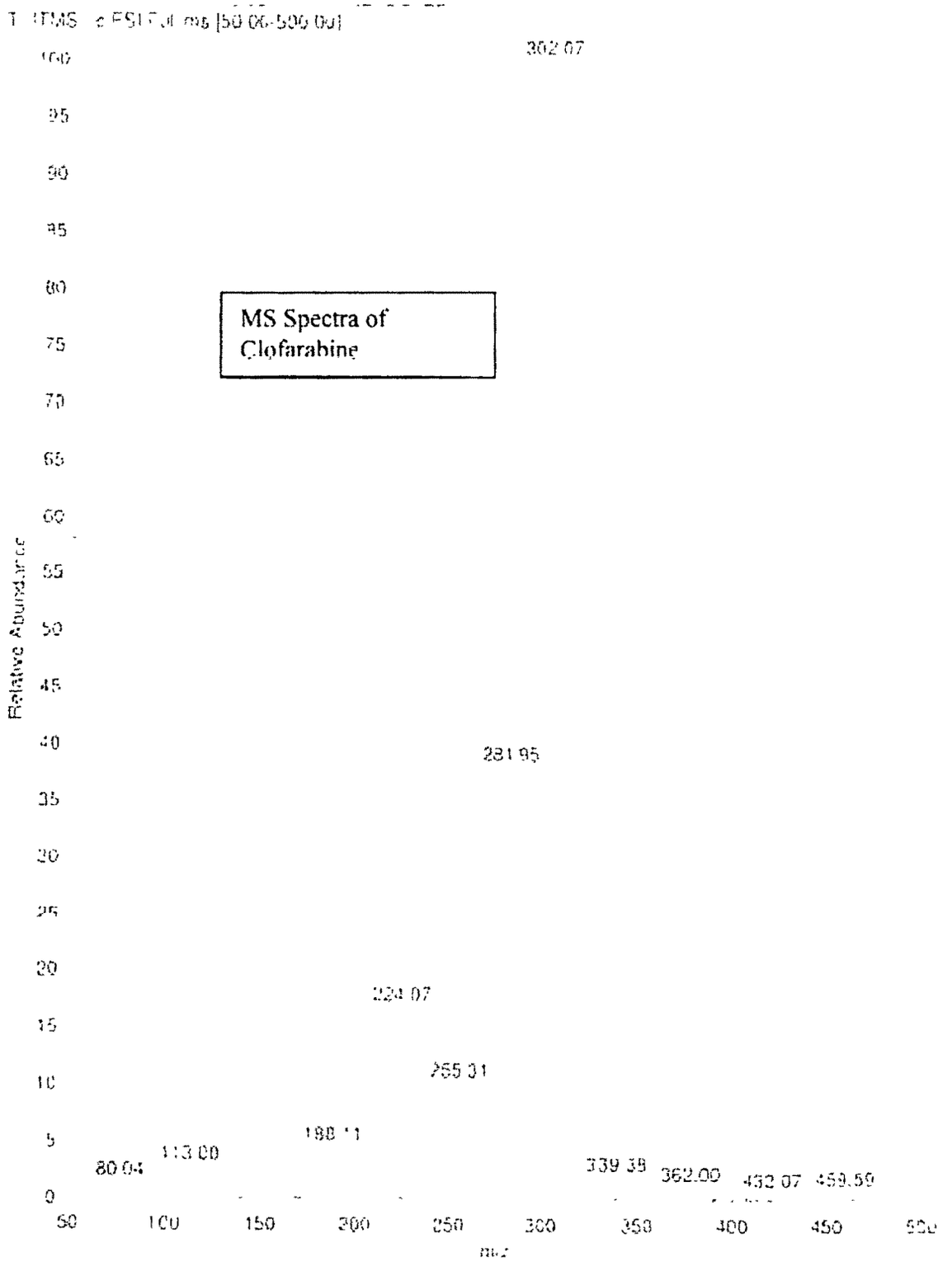
	Peak Name	RT	Area	% Area
1		2.02	19341	0.06
2		2.28	27068	0.08
3		2.75	240873	0.69
4	Clofarabine Related Compound C	30.66	34117637	98.37
5		37.99	247692	0.71
6		45.42	29673	0.09
Sum			34682285	100.00

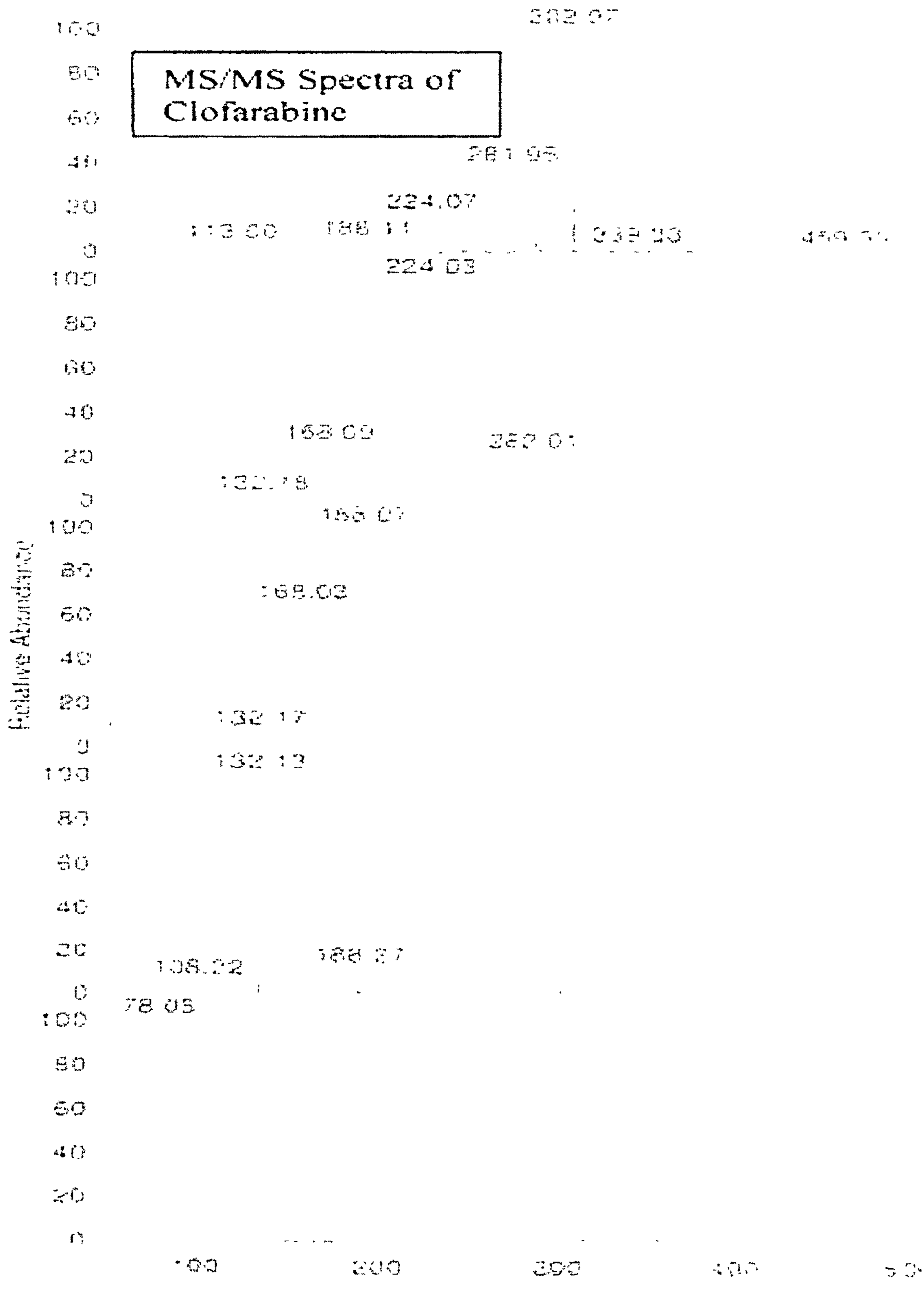
Infrared Spectrum of Clofarabine Related Compound-C



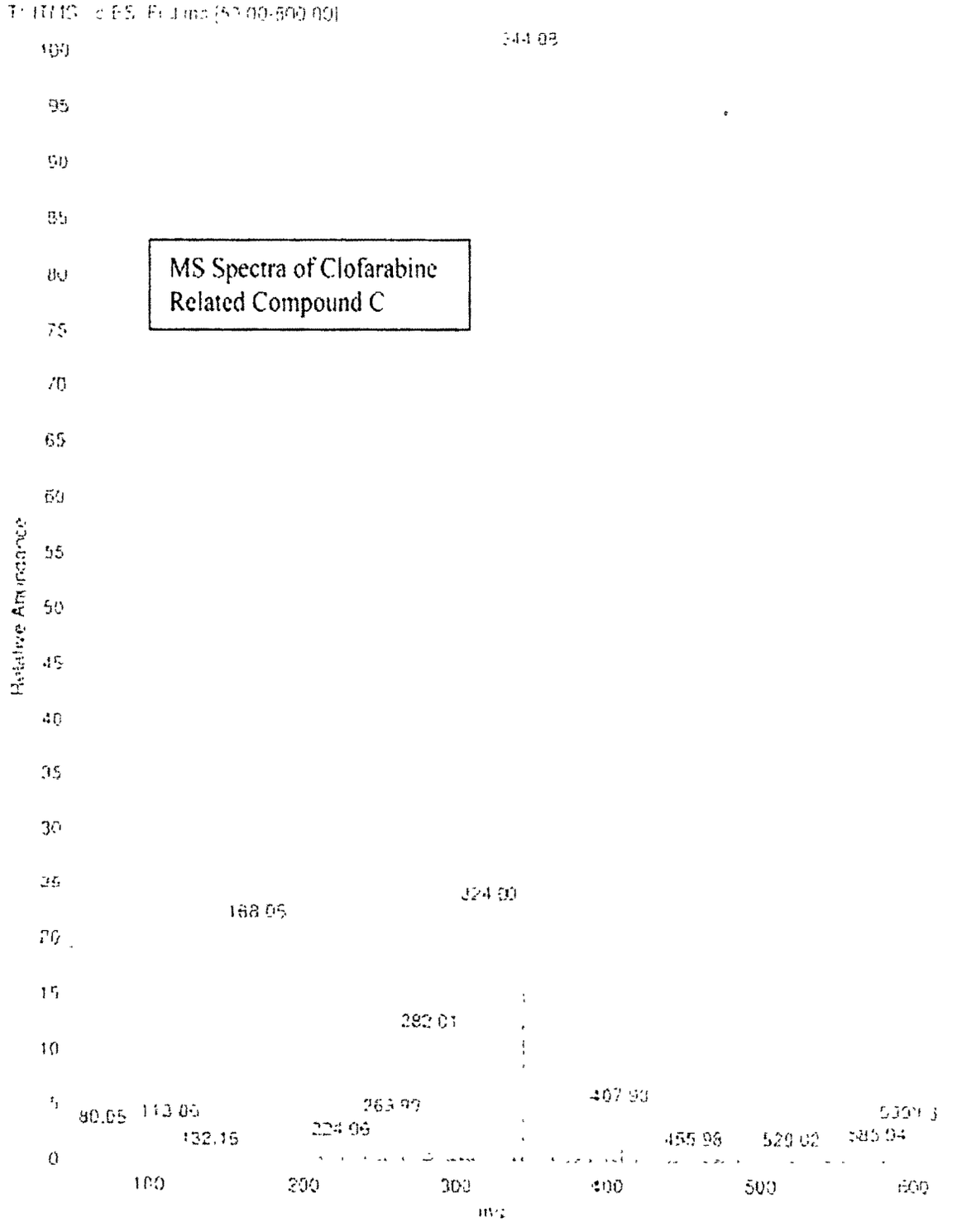
	Peak	Intensity	Area
1	468.7	71 84	4 151
2	485 71	75 098	3 103
3	551.39	68 834	6 221
4	603.72	68 561	6 031
5	638.44	67 177	6 993
6	664.73	64 054	1 924
7	738 74	71 576	3 807
8	763.81	74 169	2 481
9	802.39	68 727	4 143
10	914.28	70 409	3 304
11	940.88	77 901	2 715
12	977.91	77 839	2 48
13	1004 91	66 915	3 33
14	1035 63	24 83	16 832
15	1074 35	49 002	6 282
16	1101 35	45 388	11.17
17	1184.29	54 314	8.41
18	1255 66	20 293	33.375
19	1311 59	40 079	14.354
20	1368 G	39 614	13 399
21	1388 82	53 429	6 925
22	1433 11	62 777	5 884
23	1460 97	59 251	2 803
24	1473 62	57 381	4 234
25	1512 19	65 848	4 611
26	1546 91	73 255	1 948
27	1577 77	38 351	17 975
28	1597 08	24 297	15 746
29	1630 71	24 099	28 23
30	1703 14	62 873	1 781
31	1739 79	28 269	22 864
32	2341 58	7362	1 973
33	2368 59	6779	5 937
34	3122 76	34 017	38 431
35	3165 54	342	36 315
36	3315 63	33 651	37.873

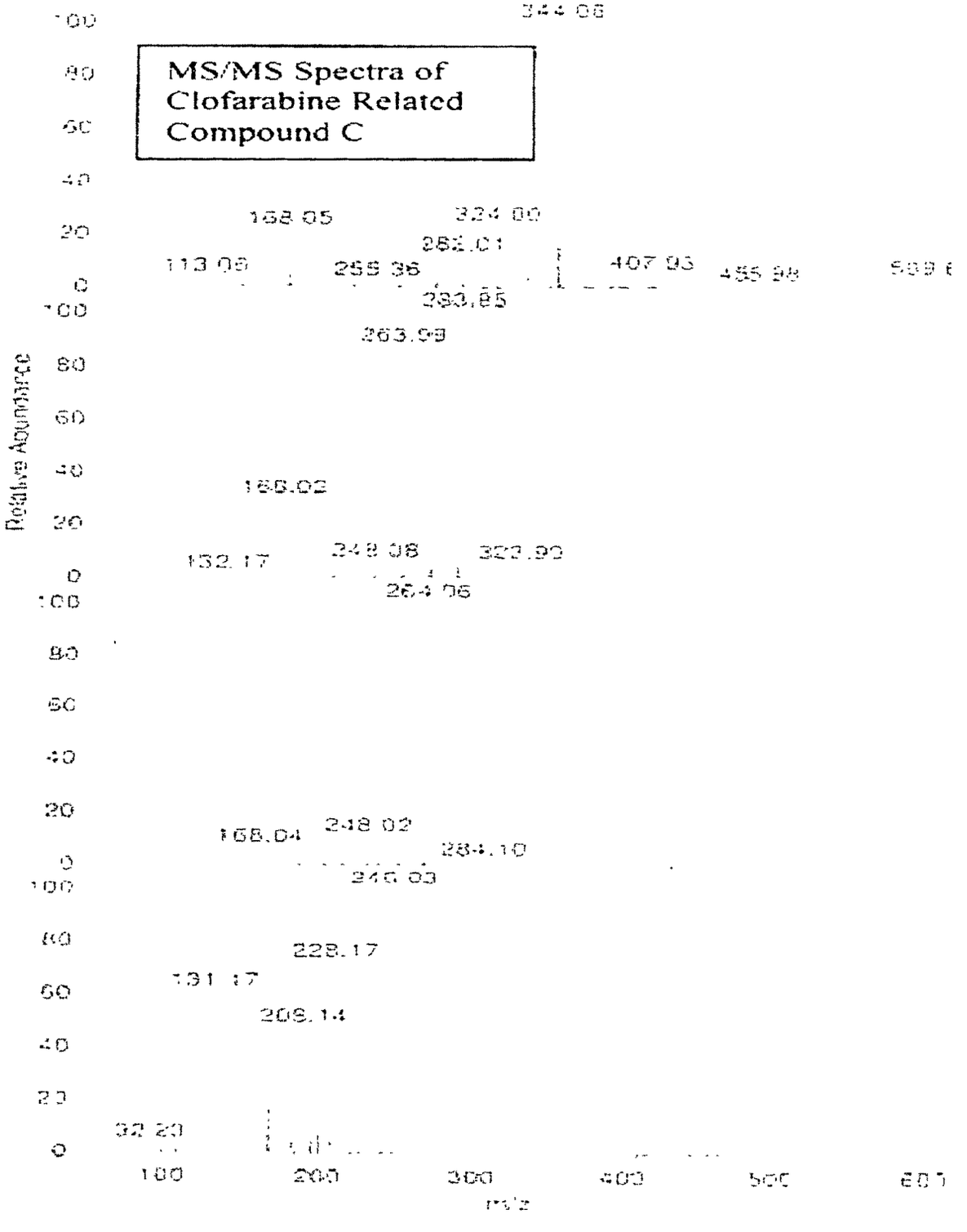
Mass fragmentation Spectra of Clofarabine



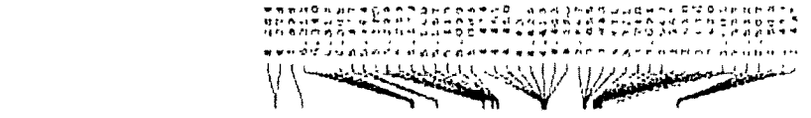

MS/MS Spectra of Clofarabine
224.07
224.03
168.03
78.05
Relative Abundance

Mass Spectra (Molecular ion) of Clofarabine Related compound C



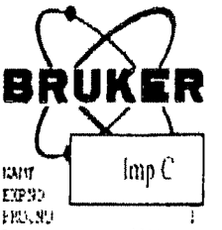

MS/MS Spectra of
Clofarabine Related
Compound C
Relative Abundance
m/z

Proton (^{1}H) NMR of Clofarabine



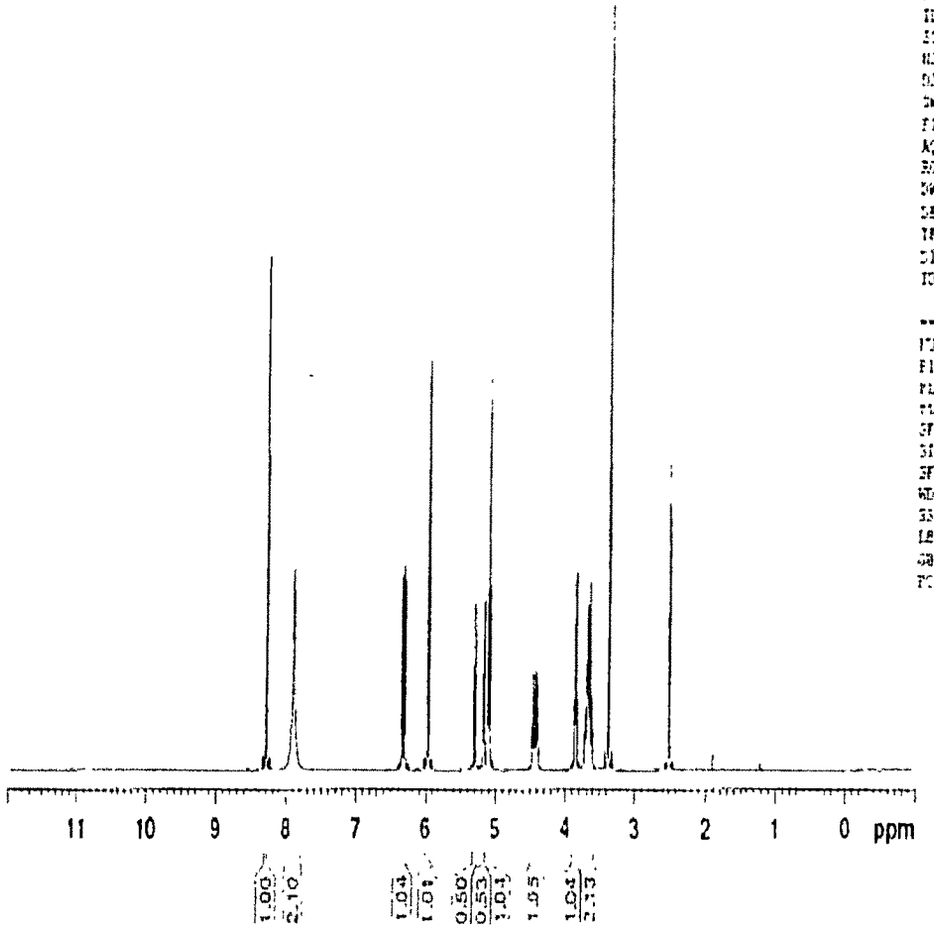
Clofarabine

Proton (^{1}H) NMR

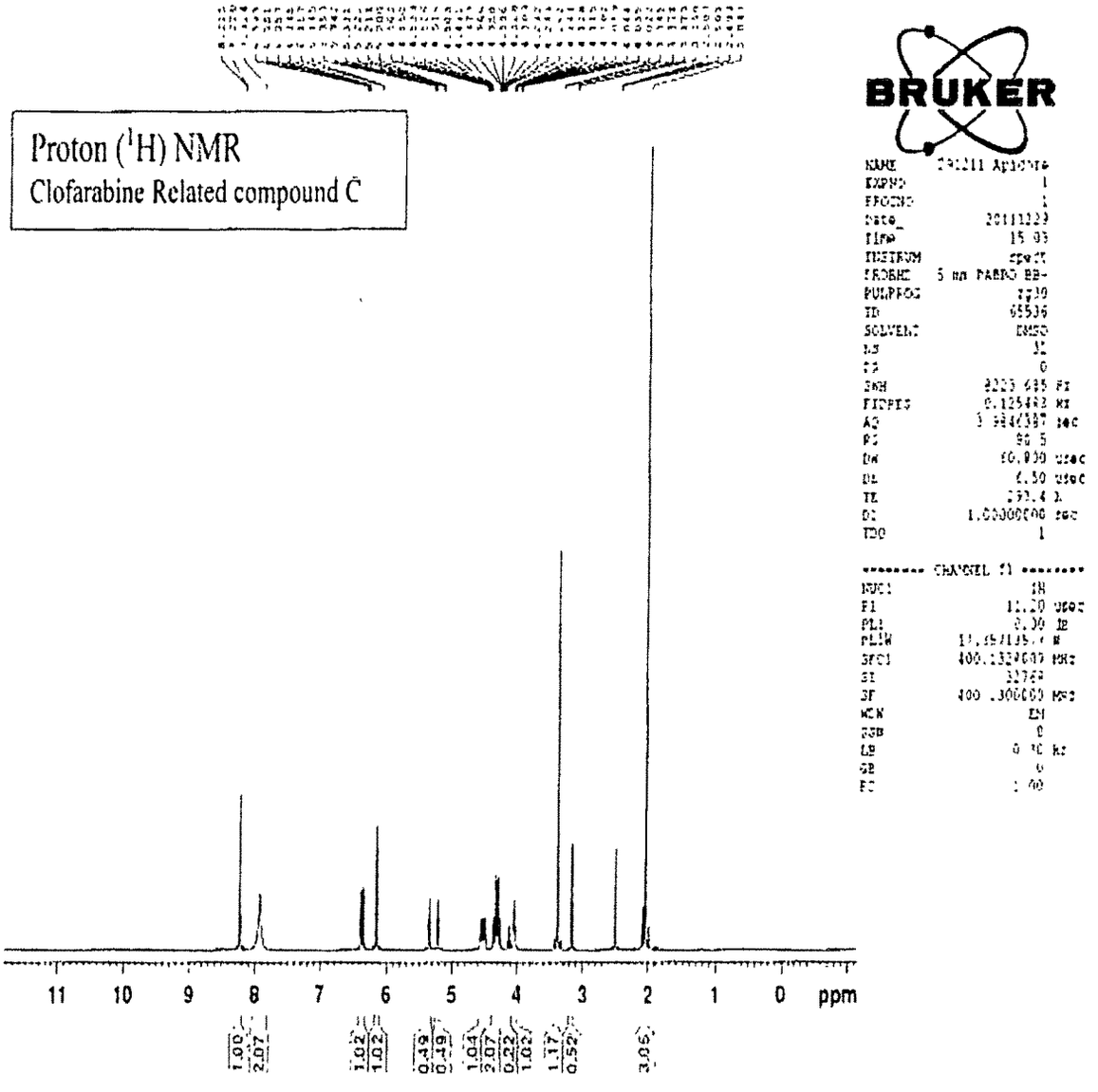


Imp C

NAME	
EXPNO	
PROCNO	1
Date_	20111116
Time	19.00
INSTRUM	spect
PROBHD	5 mm PABBO BB-
PULPROG	zg30
TD	65536
SOLVENT	DMSO
NS	16
DS	0
SWH	8223.685 Hz
FIDRES	0.125483 Hz
AQ	3.9845747 sec
RG	114
DW	60.800 usec
DE	6.50 usec
TE	294.2 K
D1	1.00000000 sec
D0	1
======== CHANNEL f1 ========	
NUC1	1H
P1	11.20 usec
PL1	0.00 dB
PL1W	23.35761452 W
SFO1	400.1320003 MHz
SI	32768
SF	400.1300000 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

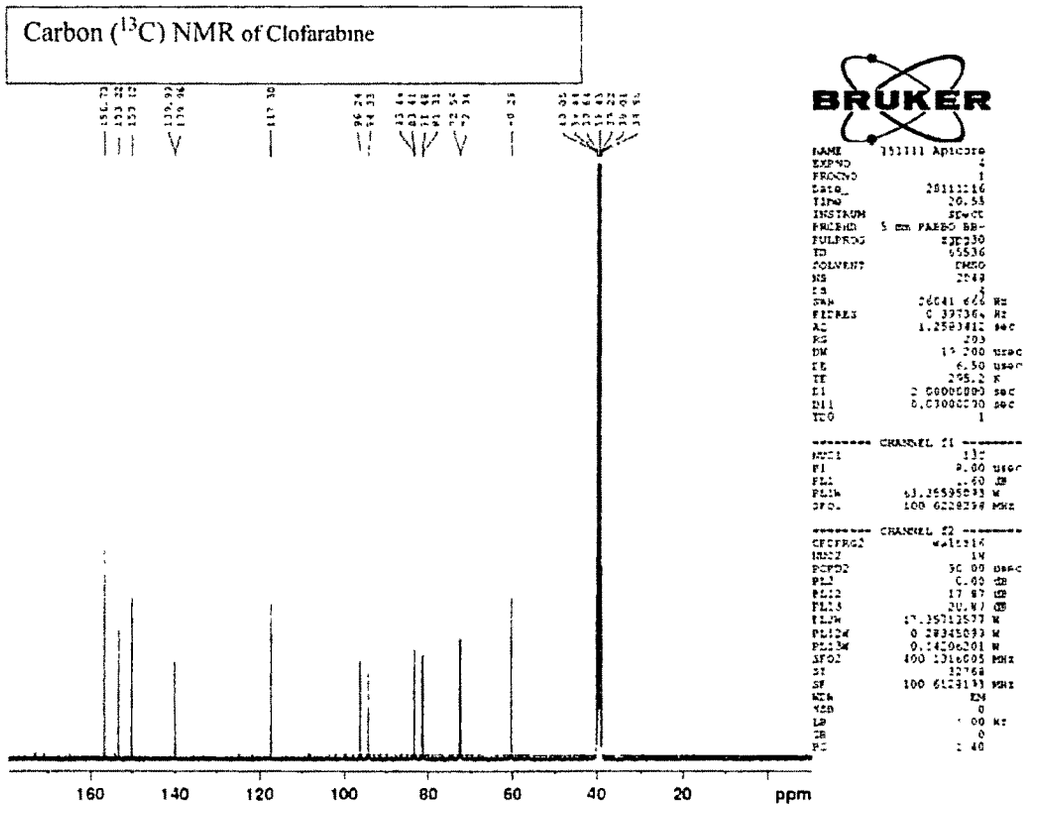


Proton (^{1}H) NMR of Clofarabine related compound C

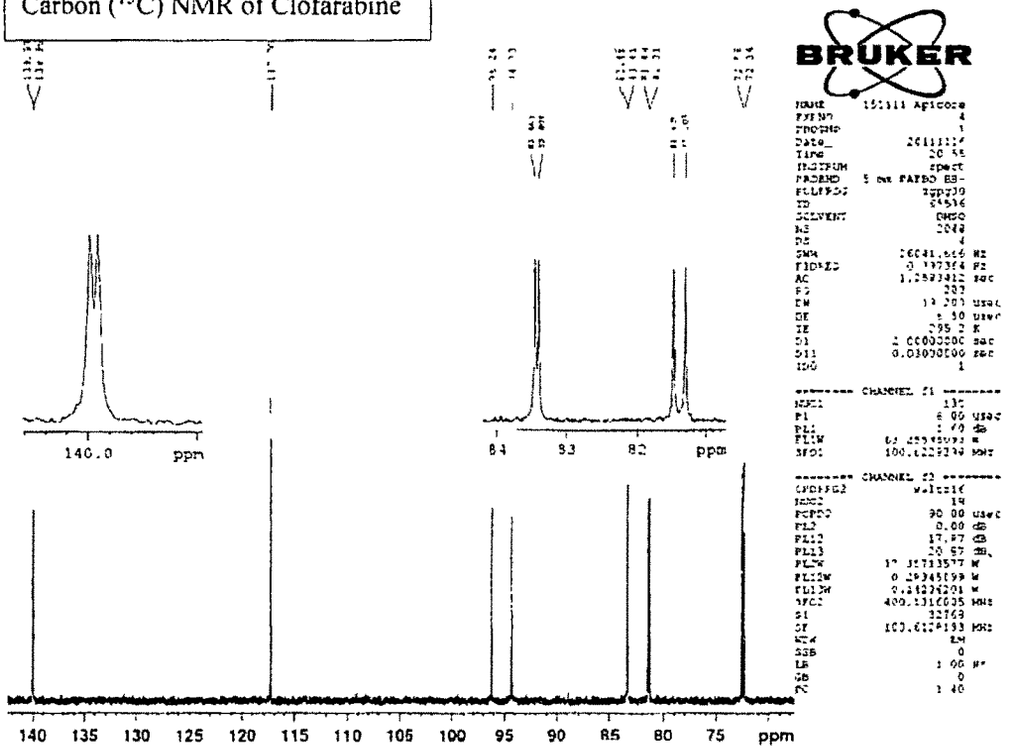


The Impurity acetyl clofarabine values from the above NMR is 8.22(d,1H), 7.92(br.S,2H,NH2),6.35(dd,H1),6.15(d,1H,OH3),5.34,5.22(1H,H2).**5.08(t,1H,OH5) (MISSING),** 4.52(dm,1H,H3),4.36(m,2H,H5),4.0(m,1H4) ,3.38(1.17-OH of DMSO containing water) and 2.04(s,3H,acetyl)

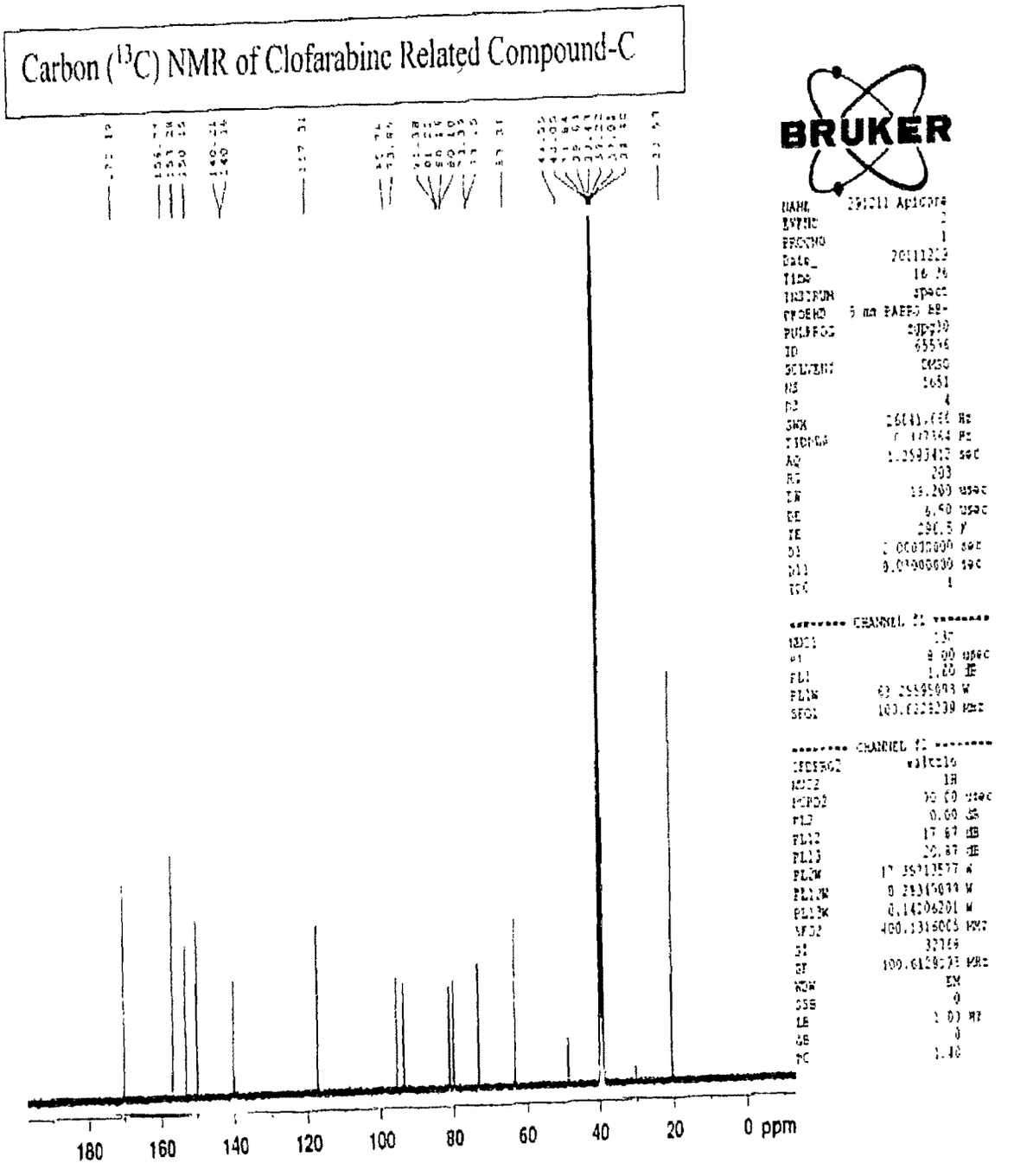
Carbon (^{13}C) NMR of Clofarabine



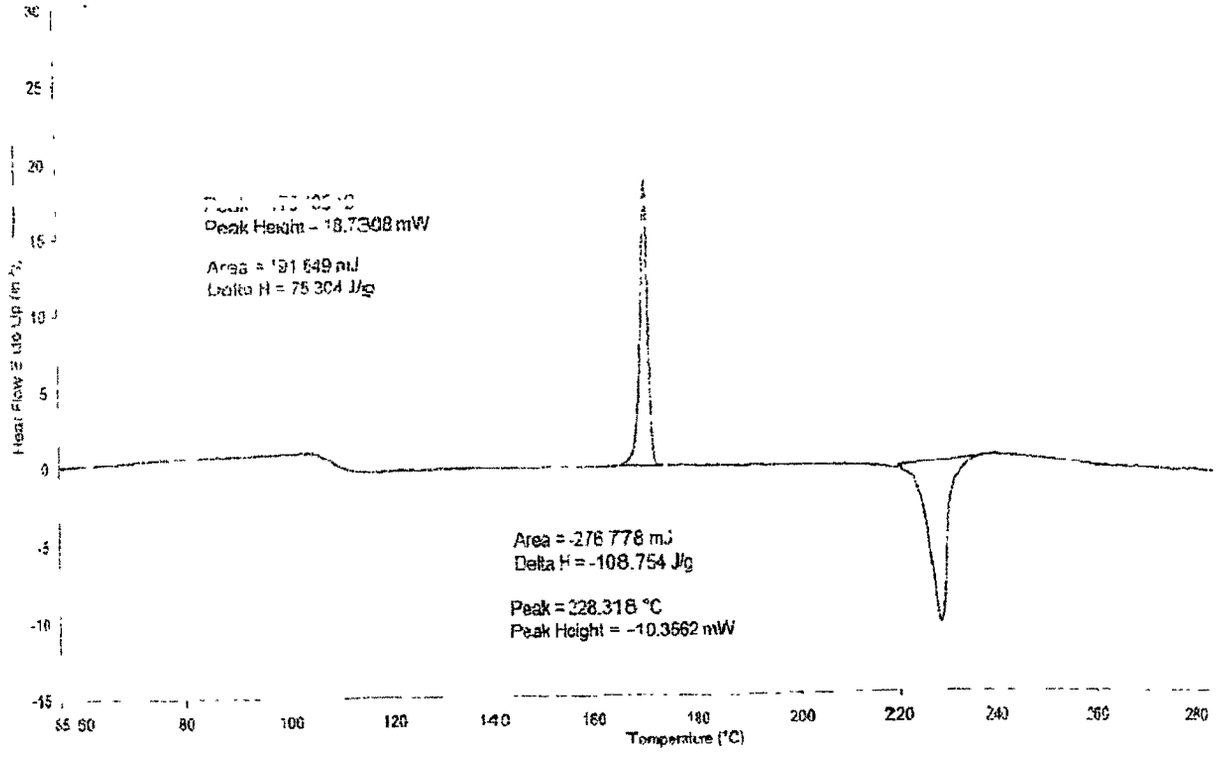
Carbon (^{13}C) NMR of Clofarabine



Carbon (13C) NMR of Clofarabine Related Compound-C

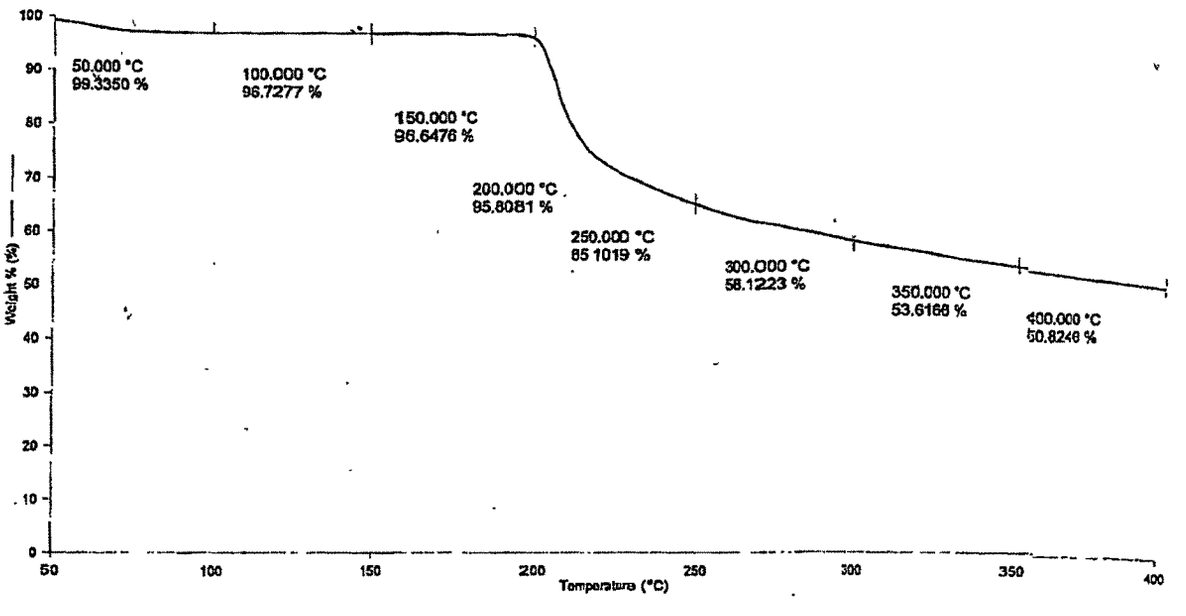


DSC of Clofarabine Related Compound-C



1) Hold for 1.0 min at 50.00°C 2) Heat from 50.00°C to 300.00°C at 10.00°C/min

TGA of Clofarabine Related Compound-C



1) Heat from 50.00°C to 410.00°C at 10.00°C/min

PHYSICO-CHEMICAL CHARACTERIZATION OF CLOFARABINE RELATED COMPOUND-D

The Physico-chemical characterization of Clofarabine Related Compound-D was established by analytical techniques such as FT-IR, HPLC, MS/MS fragmentation, DSC, TGA, ^{1}H & ^{13}C NMR analysis. The chromatographic purity of Clofarabine Related Compound-D was determined by HPLC.

1.0 PHYSICAL PROPERTIES

2.0 APPEARANCE

Table-1 Appearance of Clofarabine Related Compound-D

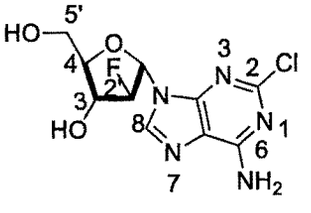
Name	Appearance
Clofarabine Related Compound D	White powder

3.0 CHEMICAL PROPERTIES

3.1 Chemical Name: 6-Amino-2-chloro-9-(2'-deoxy-2-fluoro-α-D-arabinofuranosyl)-9H-purine

3.2 Common Name: Clofarabine Related Compound-D

3.3 Structural Formula



Clofarabine Impurity D

3.4 Molecular Formula: $C_{10}H_{11}ClFN_5O_3$

3.5 Molecular Weight: 303.68

3.6 Melting range: 225.1 °C to 226.5°C

4.0 CHROMATOGRAPHIC PURITY BY HPLC

The Chromatographic purity of Clofarabine Related Compound-D was determined by High performance liquid chromatograph (HPLC) using developed test procedure. The results are depicted in **Table-2.** The chromatograms are depicted in **Exhibits below.**

Table-2 Chromatography purity

Name	% Chromatography purity by HPLC (average)
Clofarabine related compound D	98.73 %

The percentage chromatographic purity by HPLC of Clofarabine Related Compound-D is 98.73% and remaining all the other peaks are unknown.

5.0 CHARACTERIZATION BY FT-IR

The Infrared spectra of Clofarabine Related Compound-D was obtained using FT-IR.
The FT-IR spectra of Clofarabine Related Compound-D and Clofarabine are depicted in Table-3 and **Exhibits below.**

Table-3 FT-IR Frequency table

Bond	Approx. Frequency (cm^{-1})	Intensity
-C-C-	1463.97, 1512.19 & 11597.06	Medium
Halogen	1386.82	Medium
C-O-C	1074.35	Medium

Conclusion: The IR frequencies are in line with the functional groups of literature reported alpha anomer and the prepared Clofarabine Related Compound-D.

6.0 CHARACTERIZATION BY MASS SPECTROMETRY

The Clofarabine Related Compound-D and Clofarabine drug substance was analyzed for the parent ion scan by infusion-MS on Quattro-LC mass spectrometer. The mass data presented in following **Table-4**. The mass spectrum is presented in **Exhibits below.**

Table-4 Molecular ion data for Clofarabine Related Compound-D by infusion-MS Technique

Name	$(M-H)^-$	Molecular weight of Clofarabine Related Compound-D
Clofarabine Related compound-D	301.99	303.68

Impurity fragments and reference

Organic Process Research & Development 2004, 8, Pages 889 to 896

Clofarabine

$(M-H)^-$ Clofarabine: 302.07

$(M\text{-Sugar Moiety})^-$ → 2-Chloro adenine: 168.03

Clofarabine Related Compound-D

$((M-H)^-$ Clofarabine: 301.99

$(M\text{-Sugar Moiety})^-$ → 2-Chloro adenine: 168.02

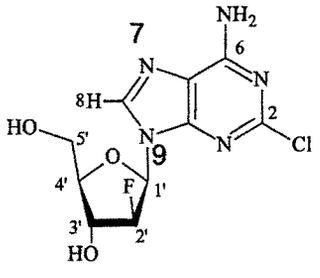
Conclusion

Molecular mass and Mass fragmentation data confirms the basic molecular mass of Clofarabine Related Compound-D.

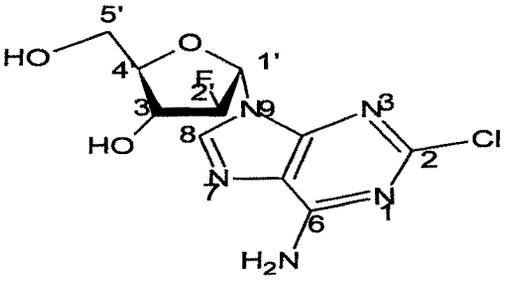
Mass fragmentation data confirms that m/z of 168.02 daltons in negative mode is the fragmentation of the sugar moiety of the nucleoside and the chloro adenine moiety. Based on the fragmentation it can be concluded that the sugar moiety and the chloroadenine heterocycle are attached each other but, the orientation of the attachment cannot be concluded with mass and fragmentation alone. The NMR data below supports the alpha configuration of the clofarabine related compound D.

7.0 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETER- 1H and C13

The Clofarabine Related Compound-D and Clofarabine drug substance was analyzed on 400 MHz Nuclear magnetic resonance spectrometer (NMR) by preparing sample in DMSO. The lot was analyzed for the proton (^{1}H) NMR and carbon (^{13}C) NMR. The NMR spectra for Proton scan and carbon scans are depicted in **Exhibits below.**



Clofarabine



clofarabine Impurity D

1H NMR

Proton Position	Chemical Shift (ppm)	
	Clofarabine	Clofarabine Related Compound-D
H8**	8.27 (d)	8.46 (S)
NH_2	7.89 (br,s)	7.89 (br,s)
H1	6.32 (dd)	5.65-5.53 (d)
OH3'	5.98 (d)	
H2'	5.29 (dt)	5.54-5.52 (m)
OH5'	5.16 (t)	
H3'	4.43 (dm)	5.28-5.17 (dm)
H4'	3.85 (m)	5.09-5.08 (m)
H5'	3.6-3.72 (m)	4.0-4.1 (m)

13C NMR

Carbon Position	Chemical Shift (ppm)	
	Clofarabine	Clofarabine Related Compound-D
C5'	60.28	69.22
C3'	72.56	71.21
C1'	81.31	80.48
C4'	83.46	89.00
C2'	96.24	90.44
C5	117.30	117.68
C8	139.99	139.87
C6	150.12	150.59
C4	153.22	153.47
C2	156.73	156.85

**** The distinguishing proton frequencies between the drug and the Clofarabine Related Compound-D.**

7.1 Structural confirmation

The Clofarabine related compound-D has been identified as 6-Amino-2-chloro-9-(2'-deoxy-2-fluoro-α-D-arabinofuranosyl)-9H-purine based on the characterization data below. The mass data below shows that the mass of the compound is 301.99 in negative ionization and it accounts for clofarabine mass. The alpha configuration has been established by the ^{1}H and ^{13}C NMR picture which shows that the H8 proton of the imidazolyl portion of the purine heterocycle as a singlet and whereas it is doublet in case of beta anomer

(The ^{1}H NMR and ^{13}C NMR values of alpha clofarabine namely 6-Amino-2-chloro-9-(2'-deoxy-2-fluoro-α-D-arabinofuranosyl)-9H-purine is reported in literature -Organic Process Research & Development 2004, 8, Pages 889 to 896.)

The Reported ^{1}HNMR of alpha anomer of clofarabine is 8.32(s,1H,$_{H8}$), 7.90(br,S,2H,NH2) 6.20(dd,$_{H1}$), 6.02(d,1H,$_{OH3'}$),5.62(dt,1H,$_{H2'}$), 5.01(t,1H,$_{OH5'}$), 4.36(dm,1H,$_{H3'}$), 4.20-4.27(m,2H,$_{H4}$) 3.48-3.63(m,2H,$_{H5'}$). [ref]

The ^{13}C NMR Values from the Lit 60.6(C5') 73.3(C3'), 85.9(C1'), 86.21(C4'), 99.41(C2'), 118.08(C5), 139.87(C8), 150.03(C6)153.22(C4), 156.84(C2) [ref]

The ^{1}H NMR values of API-173/11/10/01 are 8.27(d,1H,$_{H8}$), 7.89(br,S,2H,NH_2) 6.32(dd,$_{H1}$),5.98(d,1H,$_{OH3'}$),5.29(dt,1H,$_{H2'}$),5.16(t,1H,$_{OH5'}$),4.43(dm,1H,$_{H3'}$), 3.85(m,1H,$_{H4'}$) 3.6-3.72(m,2H,$_{H5'}$).

The ^{13}C NMR Values from of API-173/11/101/01 60.28($C_{5'}$) 72.56($C_{3'}$), 81.31($C_{1'}$), 83.46($C_{4'}$), 96.24($C_{2'}$), 117.30(C_5), 139.99(C_8), 150.12(C_6)153.22(C_4), 156.73(C_2)

The alpha clofarabine (A-RS-002-063) values from the ^{1}H NMR is 8.46(S,1H,H8),7.89 (br,S,2H,NH_2),5.65-5.53(d,2H,H_1·OH_3),5.54-5.52(1H,H_2,OH_5),5.28-5.17(dm,1H,$H_{3'}$), 5.09-5.08 (m,2H,$H_{4'}$),4.0-4.1(m,,2H,1$H_{5'}$),3.38(OH of DMSO containing water).

The alpha clofarabine (A-RS-002-063) values from the below ^{13}C NMR 69.22($C_{5'}$) 71.21($C_{3'}$), 80.48($C_{1'}$), 89.00($C_{4'}$), 90.44($C_{2'}$), 117.68(C_5), 139.87(C_8), 150.59(C_6)153.472(C_4), 156.85(C_2)

From ^1H NMR splitting, it is clear that the clofarabine is in alpha configuration.
The imidazolyl (H8) proton of the purine moiety is a singlet and while it is a doublet for beta anomer and this confirms the alpha configuration of the moiety. So, the Clofarabine Related Compound-D is identified and characterized as 6-Amino-2-chloro-9-(2'-deoxy-2-fluoro-α-D-arabinofuranosyl)-9H-purine.

Conclusion

The Imidazolyl proton of the purine moiety is singlet peak which confirms to the alpha configuration of the Clofarabine Related Compound-D.

Proton (^1H) and carbon (^{13}C) NMR confirms the structure and alpha configuration of Clofarabine Related Compound-D.

8.0 CHARACTERIZATION BY THERMO GRAVIMETRIC ANALYSIS [TGA]

The TGA of Clofarabine Related Compound-D was performed. The TGA data presented in **Exhibits** depicts only residual moisture present in the sample.

There is no hydrate form observed as there is no bound water found in the sample. Also the Clofarabine Related Compound-D sample does not exist as any solvate either.

9.0 CHARACTERIZATION BY DIFFENTIAL SCANNING CALOREMETER [DSC]

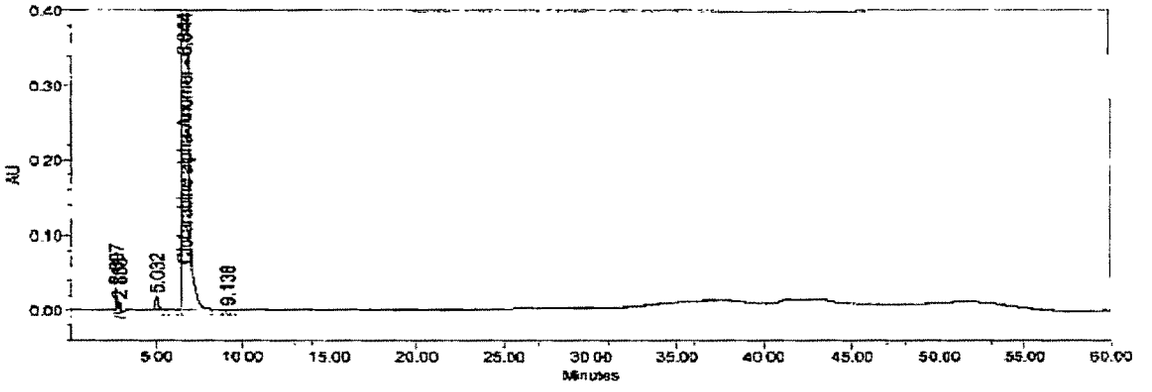
DSC of Clofarabine Related Compound-D was performed. The DSC data presented in **Exhibits** supports the melting range of the compound.

10.0 STORAGE CONDITION

Store in a well closed container at room temperature

EXHIBITS

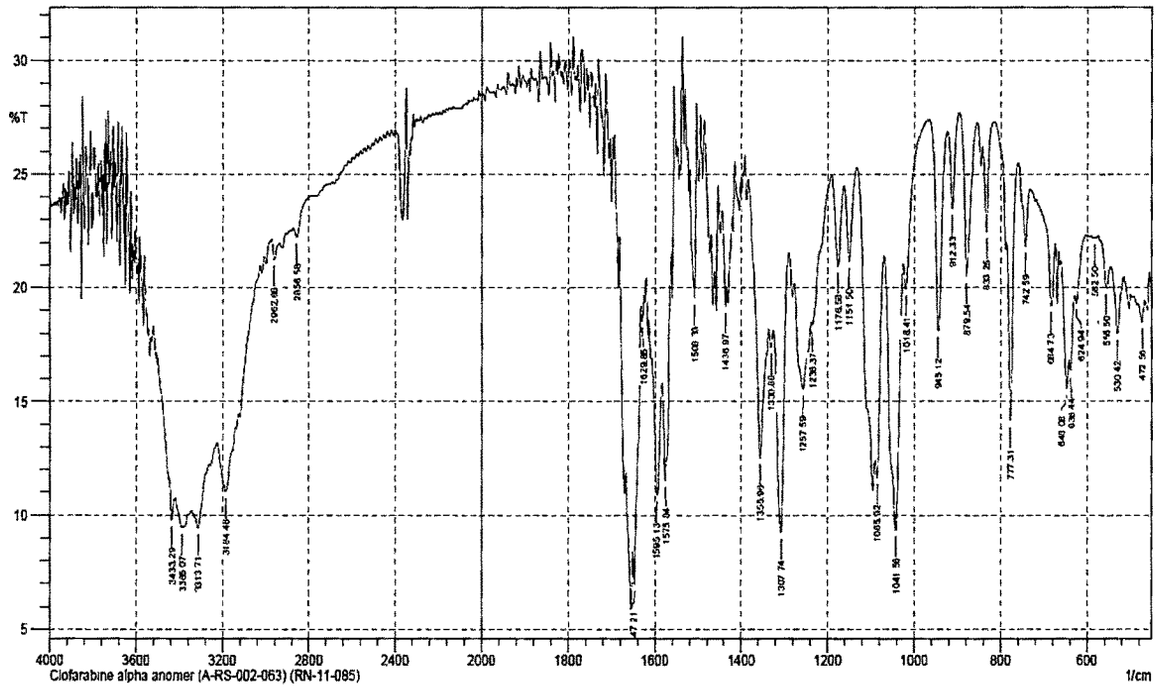
HPLC Purity Chromatogram of Clofarabine Related Compound-D



Peak Table

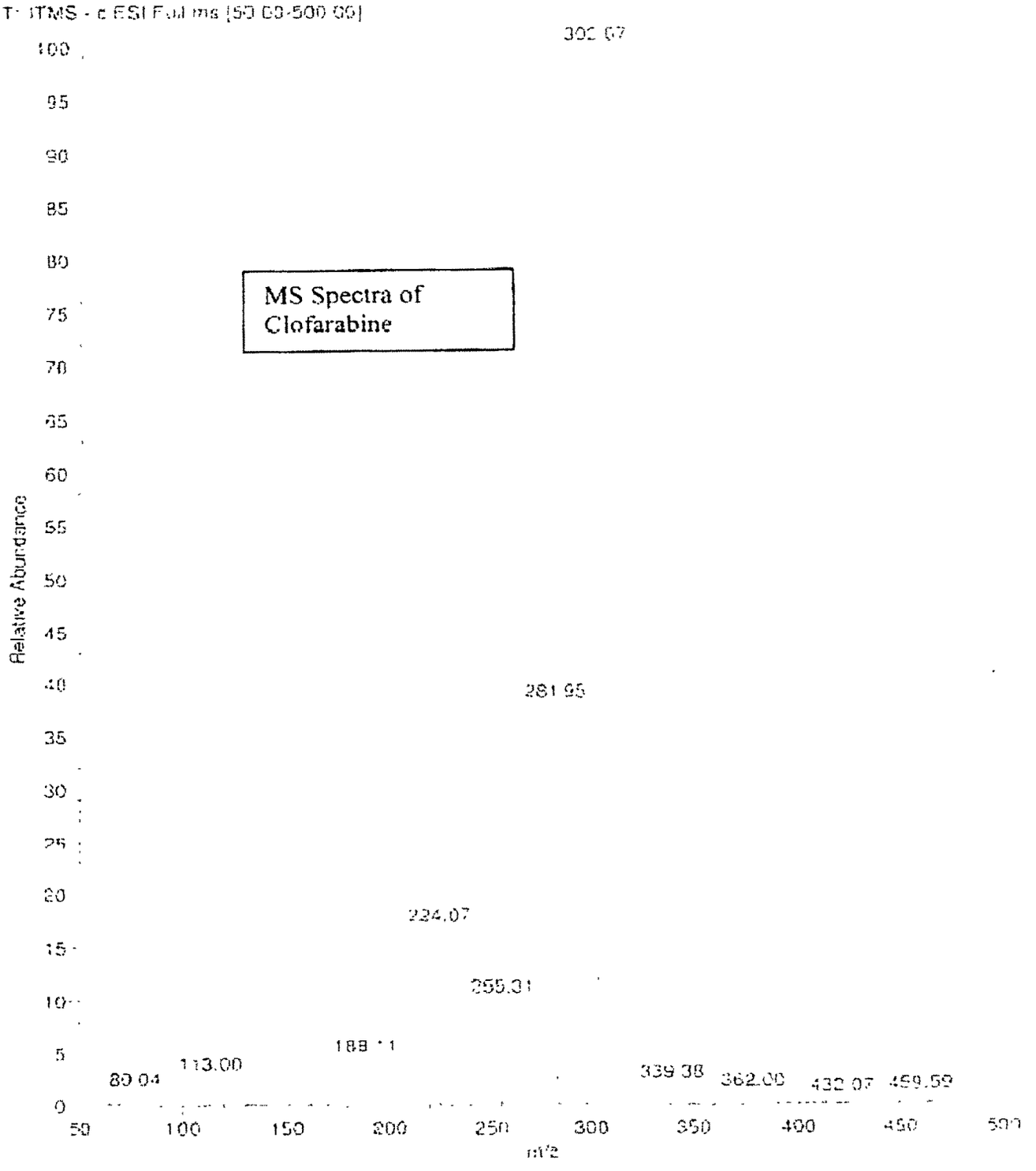
	Peak Name	RT	Area	% Area
1		2.70	121672	0.38
2		2.86	44255	0.14
3		5.03	232092	0.72
4	Clofarabine alpha-Anomer	6.64	31990543	98.74
5		9.14	10254	0.03
Sum			32398816	100.00

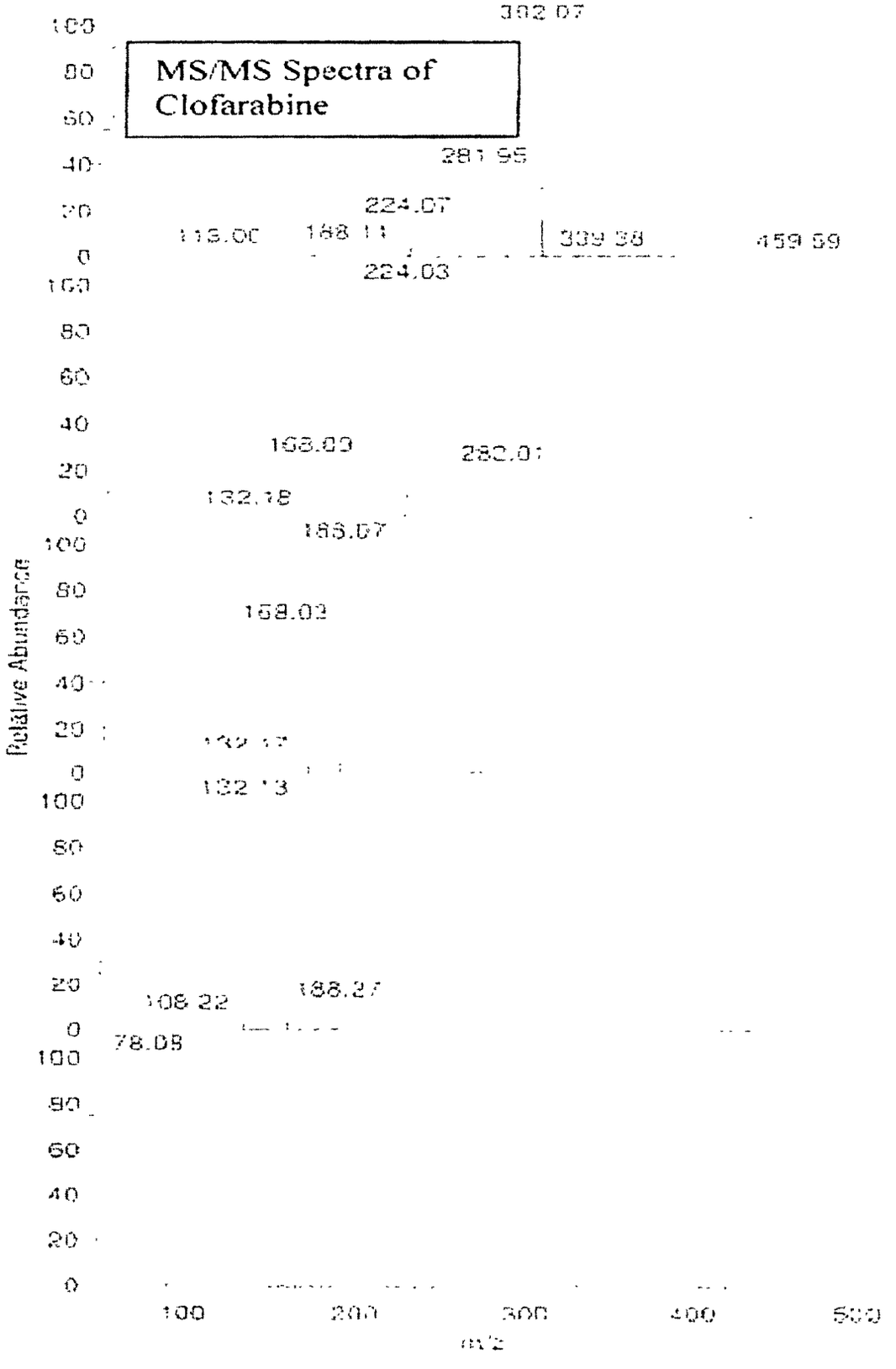
Infrared Spectrum of Clofarabine Related Compound-D



Sample Name : Clofarabine alpha anomer (A-RS-002-063) (RN-11-085)

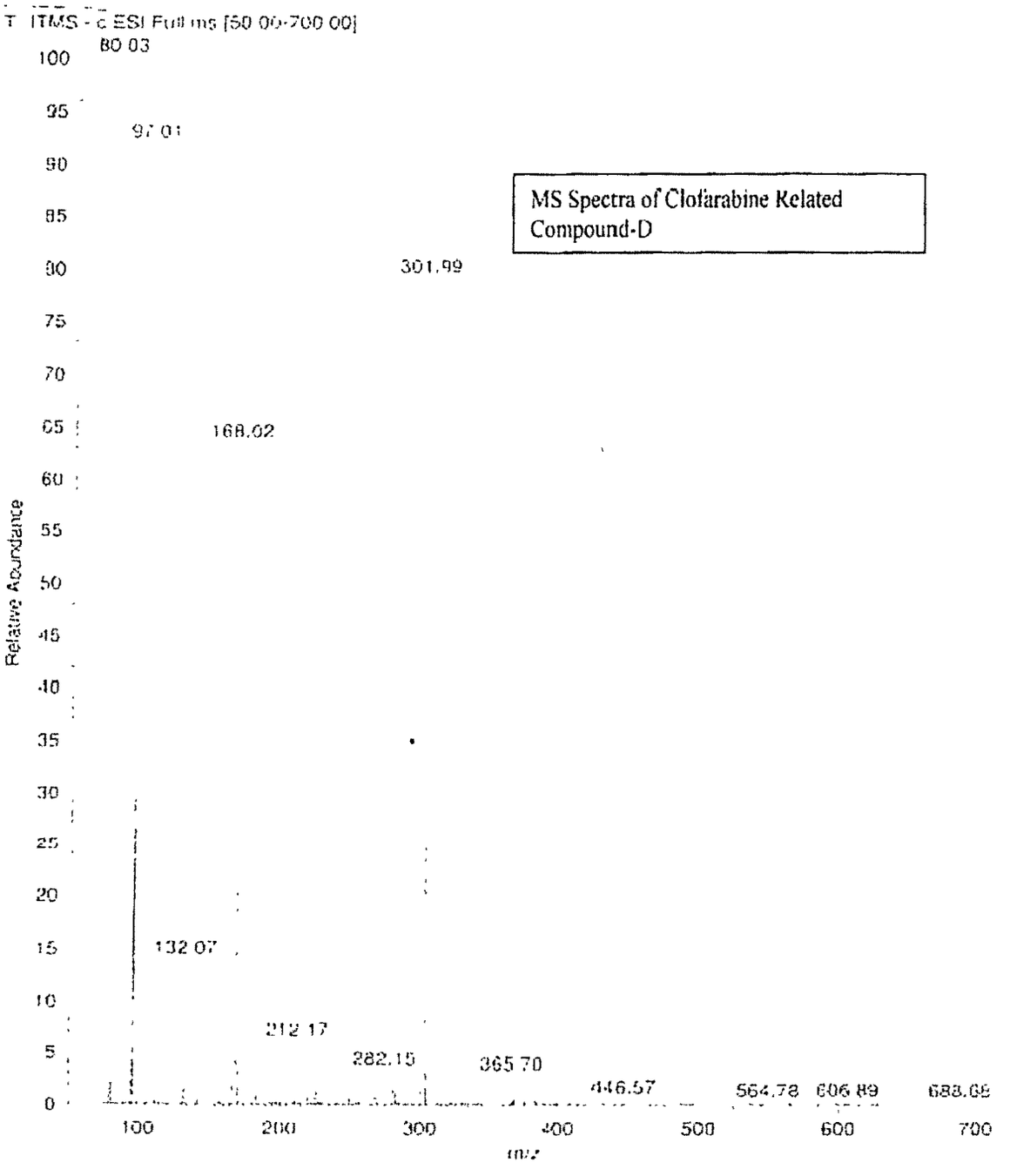
Mass Spectra (Molecular ion) & Fragmentation pattern of (Molecular ion) of Clofarabine



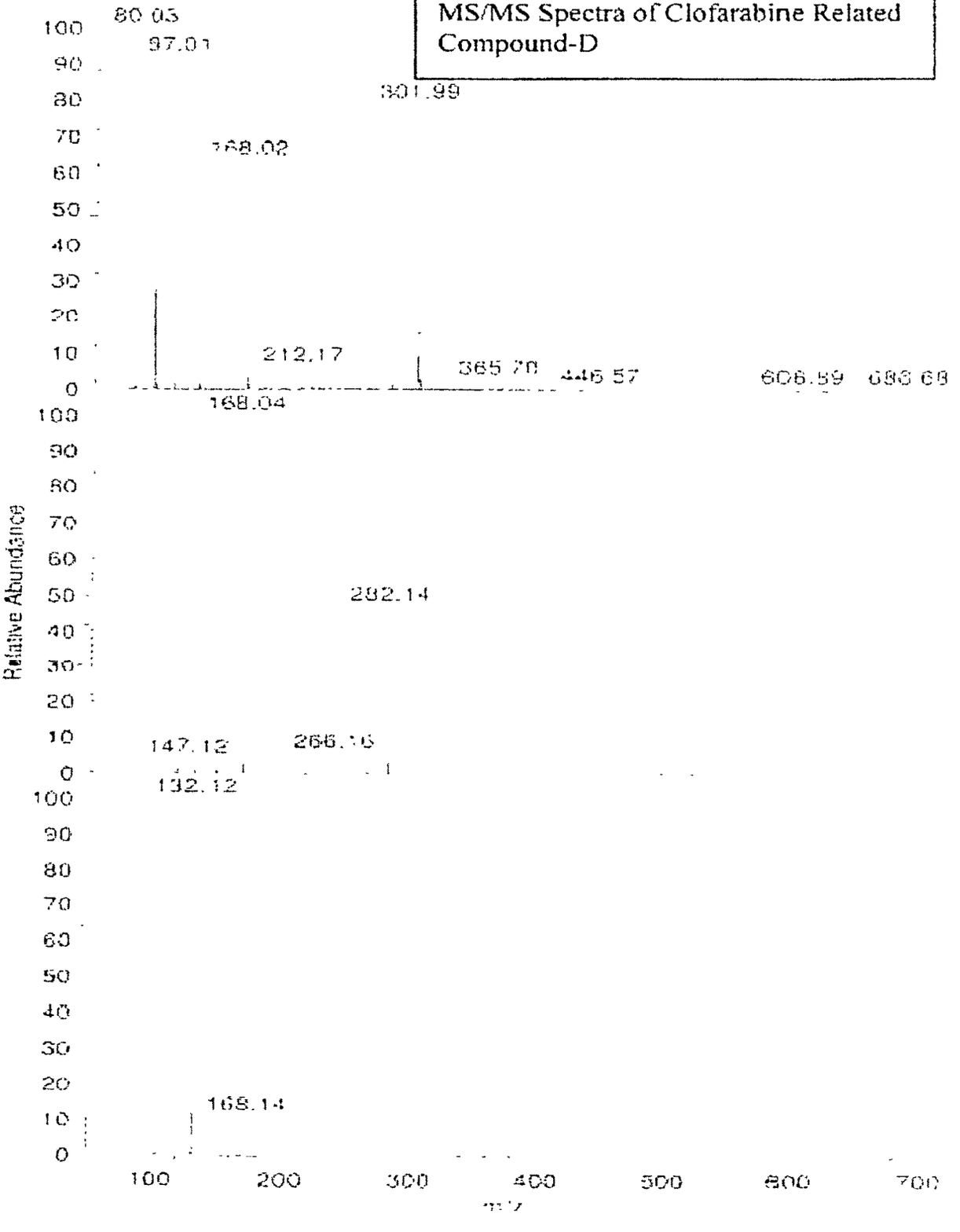

MS/MS Spectra of Clofarabine
Relative Abundance
m/z
382.07
281.95
224.07
224.03
339.38
459.59
168.03
280.01
132.18
185.07
158.02
132.13
108.22
188.27
78.08
100
200
300
400
500

Mass Spectra (Molecular ion) & Fragmentation pattern of (Molecular ion)

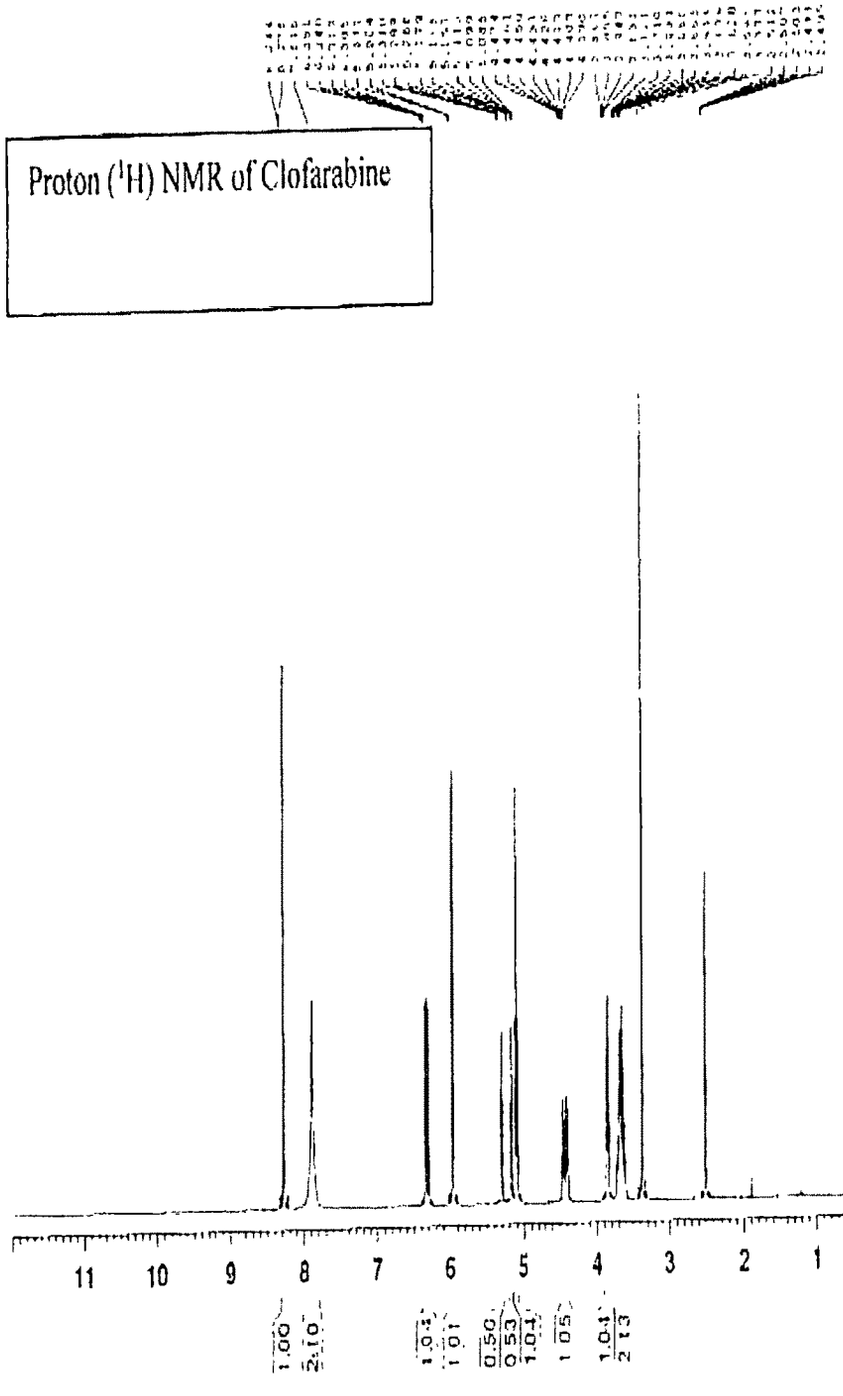
Clofarabine Related Compound-D



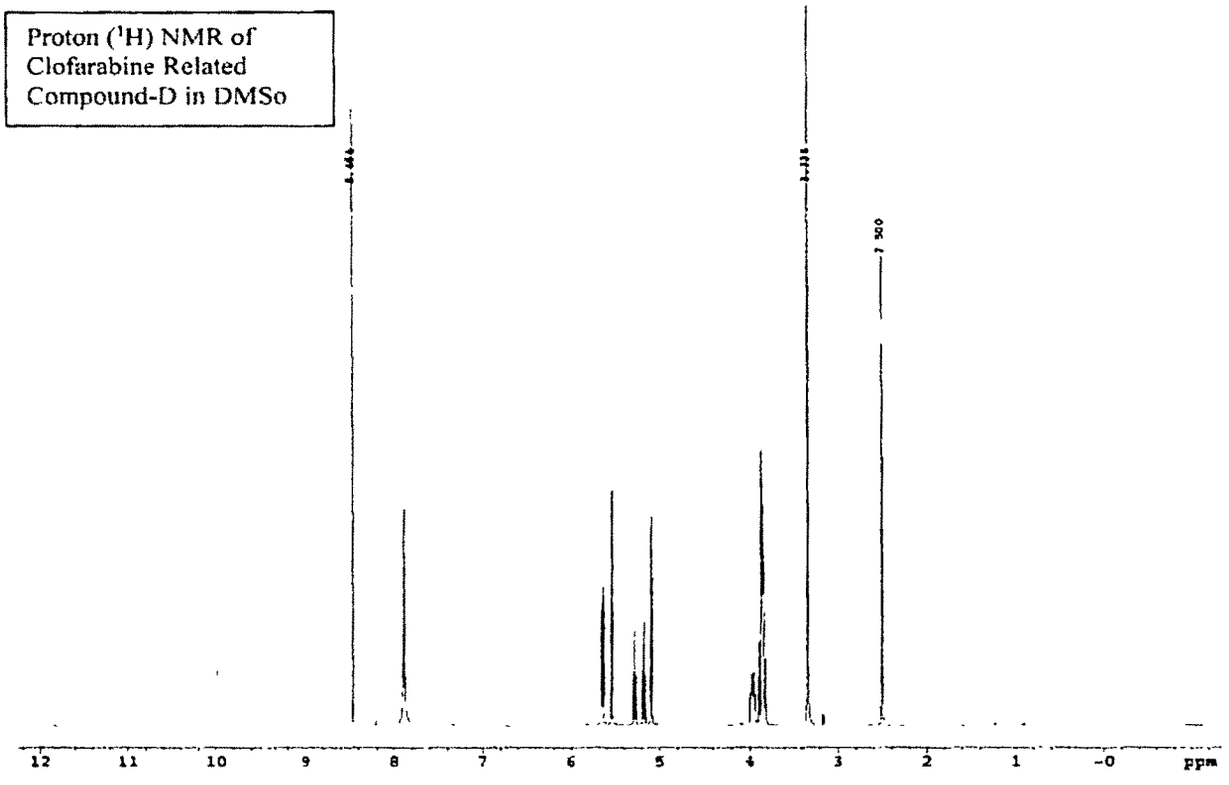
MS/MS Spectra of Clofarabine Related Compound-D

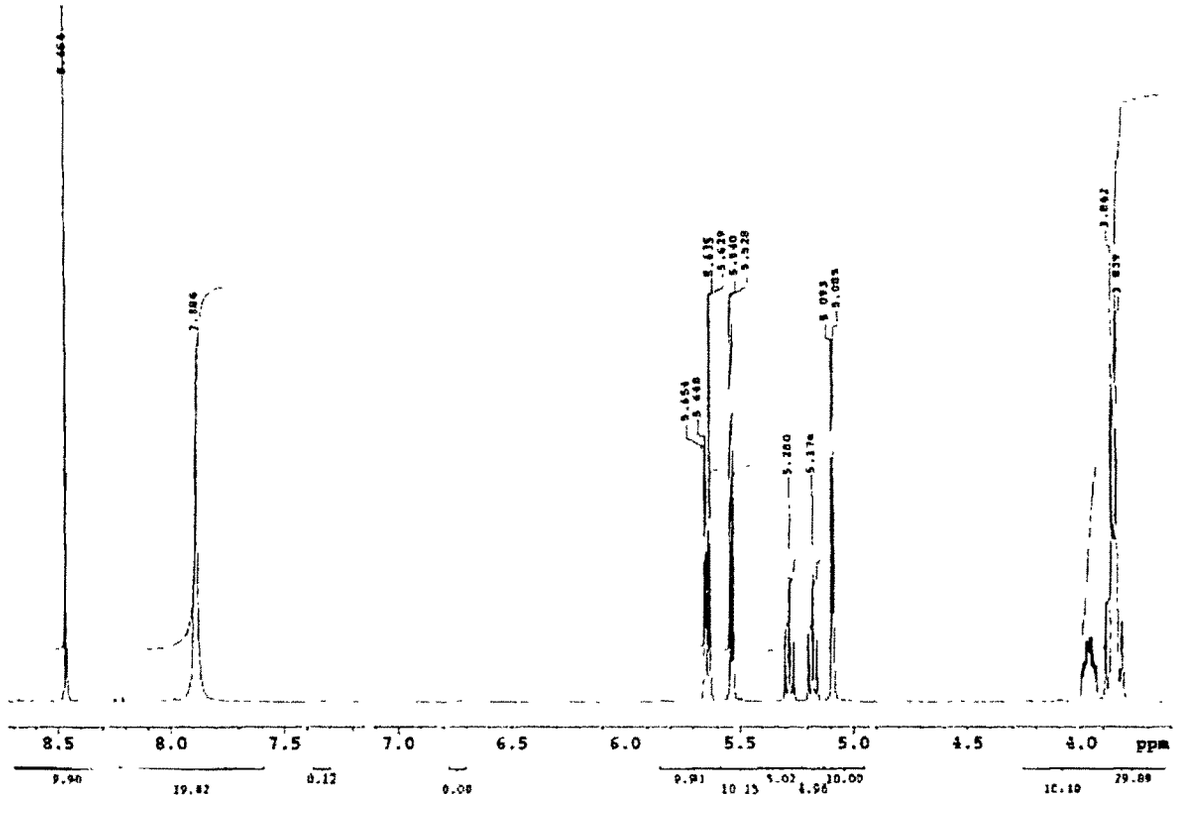


Proton (^{1}H) NMR of Clofarabine

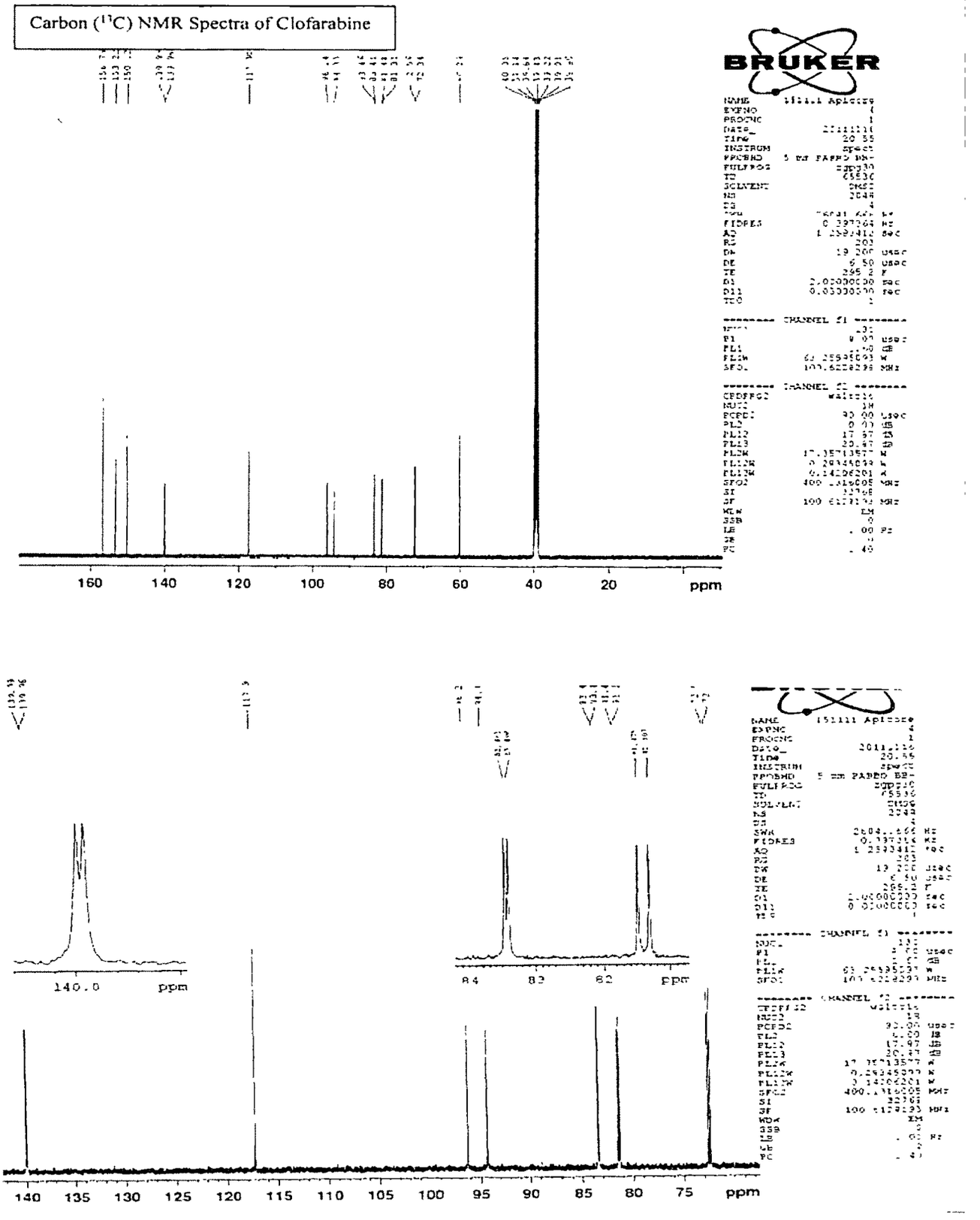


Proton (^{1}H) NMR of Clofarabine Related Compound-D

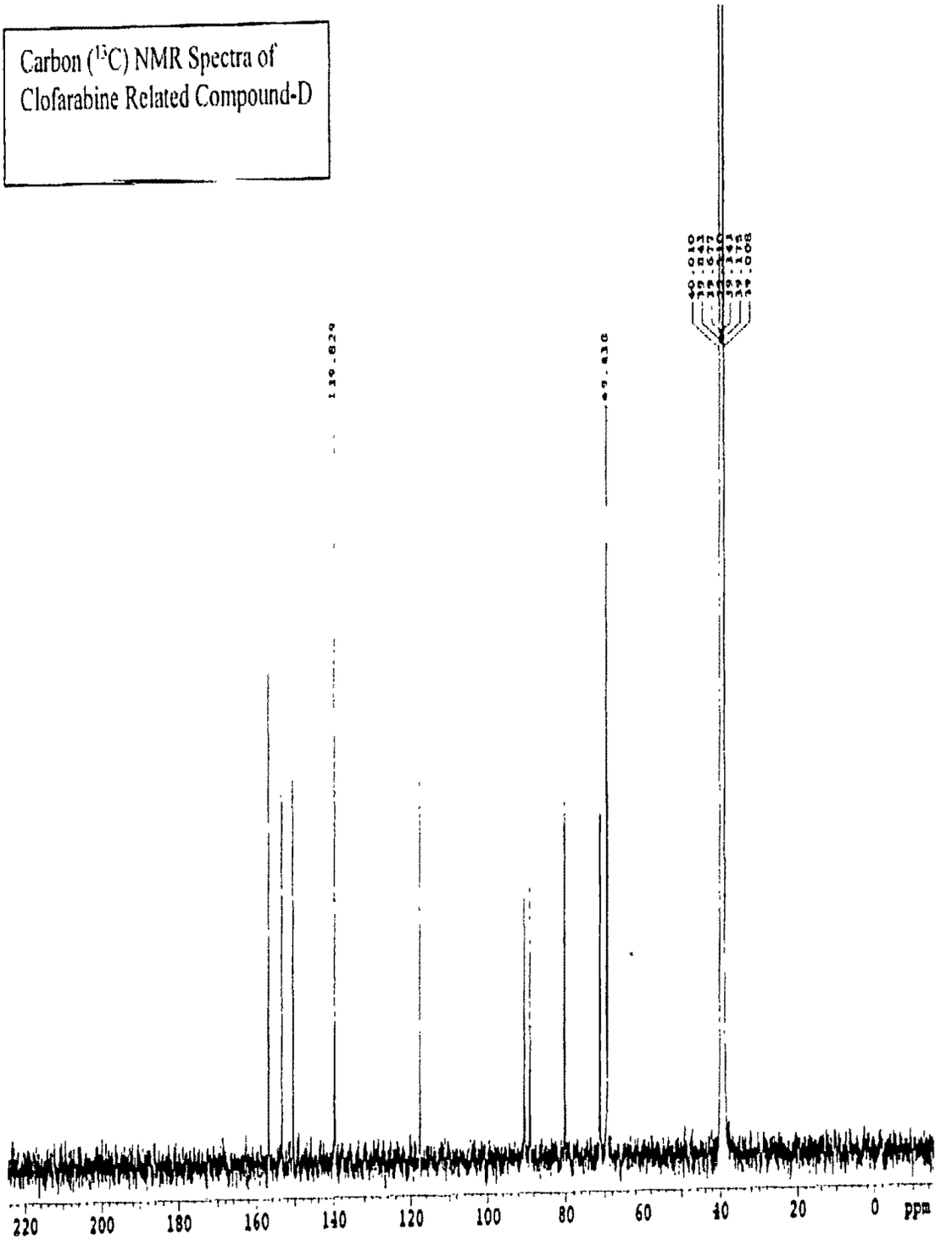




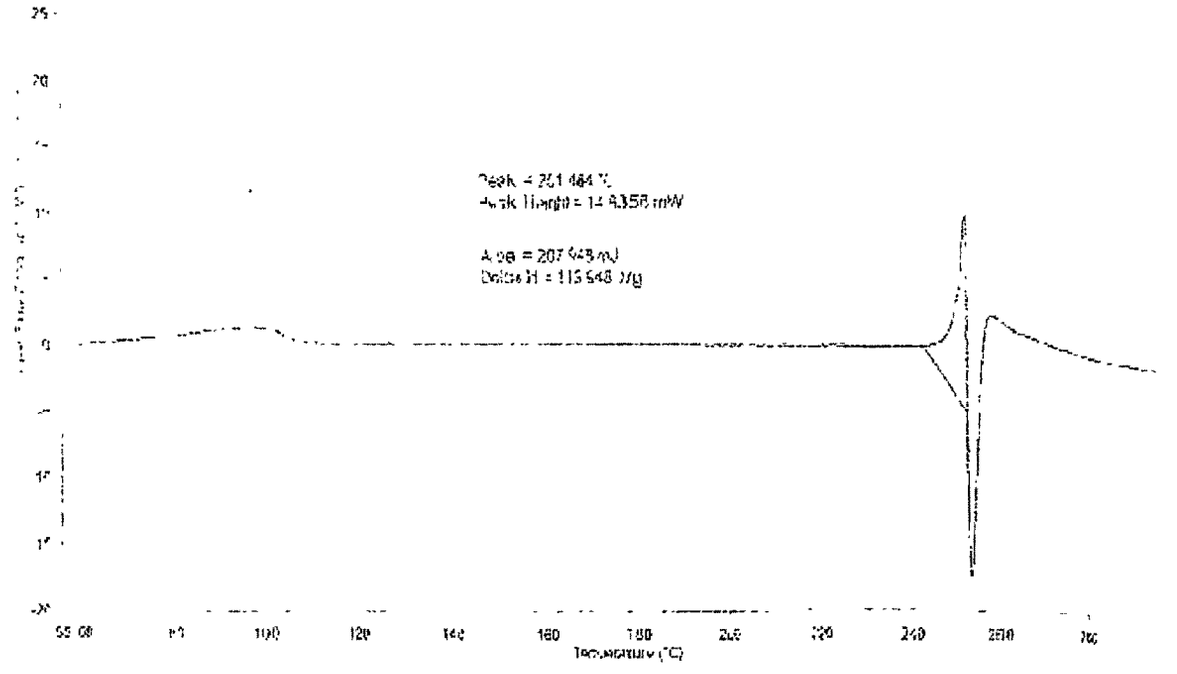
Carbon (^{13}C) NMR of Clofarabine



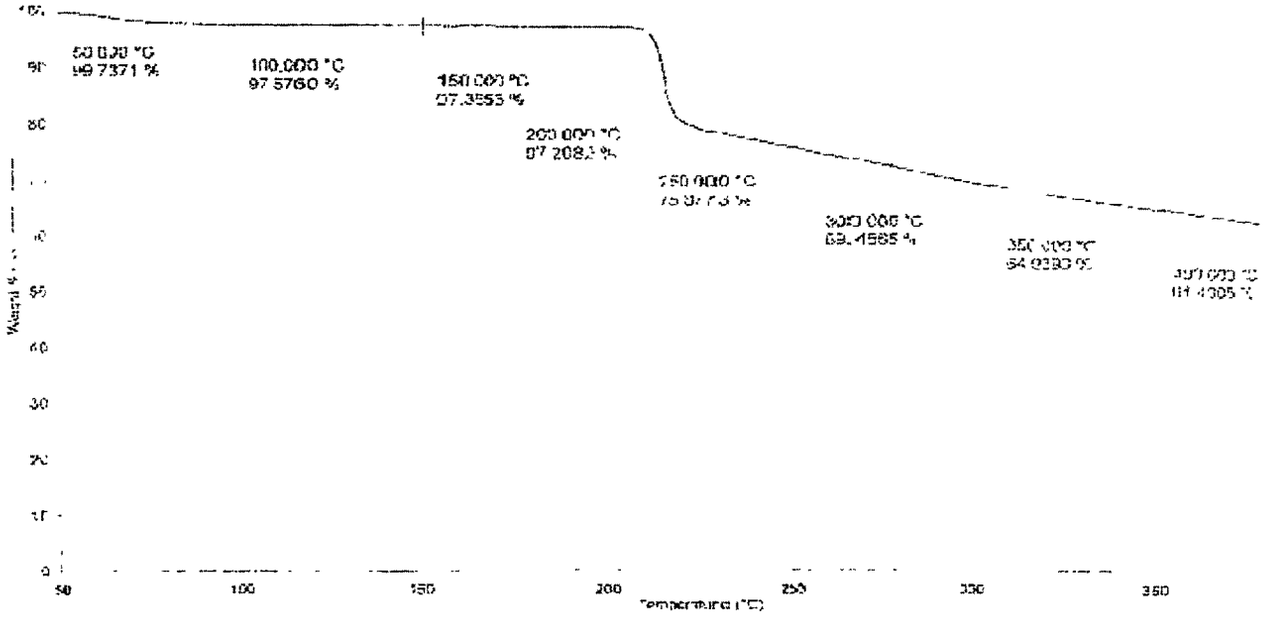
Carbon (^{13}C) NMR of Clofarabine Related Compound-D



DSC of Clofarabine Related Compound D



TGA of Clofarabine Related Compound D



CONCLUSION

Based on the studies performed to study extensively the various degradation pathways we have observed that different impurities can be formed due to the stress effects on the Clofarabine drug substance. Not all stress conditions are generating the same impurities, however the impurities formation mechanism is pretty similar to other nucleoside compounds which are approved in the market.

When the exercise was initiated on drug product also, we have observed the similar trend in the impurity profiles of the Clofarabine since the drug product is the solution of Clofarabine in sterile vial with buffers, therefore exactly similar impurity profiles will be observed.

The impurity isolations and characterizations of the same, led to develop a better HPLC method which is a stability indicating method in order to monitor the related substances present in the drug substances and drug products. This method can be used at Quality Control laboratories which are required to test and release the Clofarabine drug substance and drug products for human use.

The knowledge of the possible degradation pathways achieved in this project prompted us to understand the impurities behavior in the Humans when administered either orally or systemic, and to get the toxicology information of the same. After thorough literature search, we have found that the impurities found via forced degradation studies were partially similar to the metabolites found in human patients and animal studies performed on Clofarabine as a part of clinical studies. These metabolites are already studied for its safety since Clofarabine dosed to human patients has seen extensive metabolism to give rise to the metabolites which are similar to our degradation impurities. Other process and degradation impurities should be controlled by Pharmaceutical companies in order to get the drug approvals for marketing.

SAXAGLIPTIN

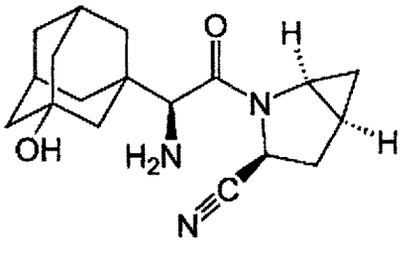
Saxagliptin

Saxagliptin (rINN), previously identified as **BMS-477118**, is a new oral hypoglycemic (anti-diabetic drug) of the new dipeptidyl peptidase-4 (DPP-4) inhibitor class of drugs.[1] Early development was solely by Bristol-Myers Squibb; in 2007 AstraZeneca joined with Bristol-Myers Squibb to co-develop the final compound and collaborate on the marketing of the drug. In June 2008, it was announced that **Onglyza** would be the trade name under which saxagliptin will be marketed.[2]

While used for type 2 diabetes the evidence does not show a change in hard end points such as the risk of heart attacks or strokes

Saxagliptin is used along with diet and exercise to lower blood sugar levels in patients with type 2 diabetes (condition in which blood sugar is too high because the body does not produce or use insulin normally). Saxagliptin is in a class of medications called dipeptidyl peptidase-4 (DPP-4) inhibitors. It works by increasing the amount of insulin produced by the body after meals when blood sugar is high. Saxagliptin is not used to treat type 1 diabetes (condition in which the body does not produce insulin and, therefore, cannot control the amount of sugar in the blood) or diabetic ketoacidosis (a serious condition that may develop if high blood sugar is not treated).

Structure

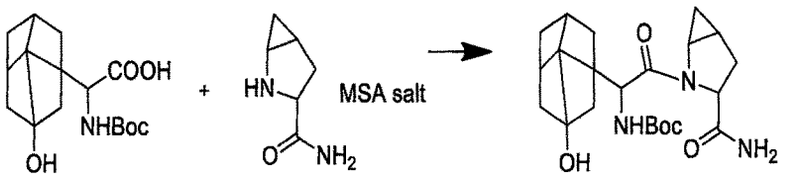


Systematic (IUPAC) name
(1*S*,3*S*,5*S*)-2-[(2*S*)-2-amino-2-(3-hydroxy-1-adamantyl) acetyl]-2-azabicyclo[3.1.0]hexane-3-carbonitrile

Saxagliptin monohydrate is a white to light yellow or light brown, non-hygroscopic, crystalline powder. It is sparingly soluble in water at 24°C ± 3°C, slightly soluble in ethyl acetate, and soluble in methanol, ethanol, isopropyl alcohol, acetonitrile, acetone, and polyethylene glycol 400 (PEG 400).

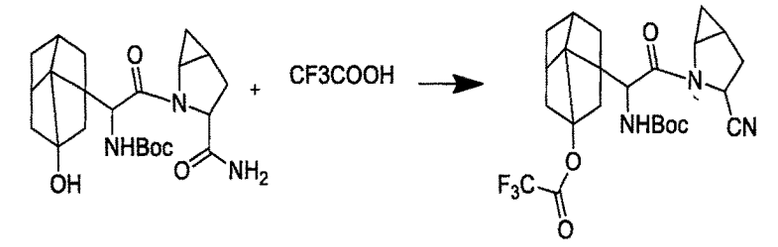
Each film-coated tablet of ONGLYZA for oral use contains either 2.79 mg saxagliptin hydrochloride (anhydrous) equivalent to 2.5 mg saxagliptin or 5.58 mg saxagliptin hydrochloride (anhydrous) equivalent to 5 mg saxagliptin and the following inactive ingredients: lactose monohydrate, microcrystalline cellulose, croscarmellose sodium, and magnesium stearate. In addition, the film coating contains the following inactive ingredients: polyvinyl alcohol, polyethylene glycol, titanium dioxide, talc, and iron oxides.

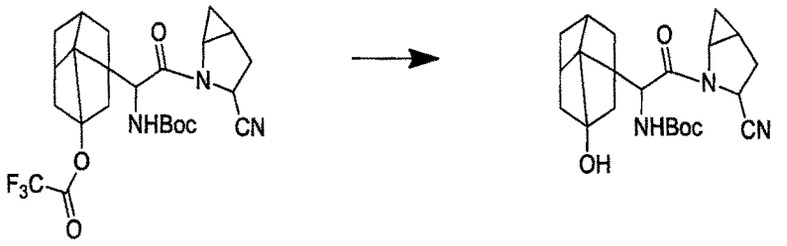
ROUTE OF SYNTHESIS OF SAXAGLIPTIN

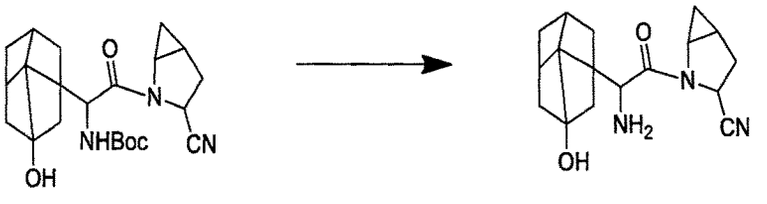


2-azabicyclo[3.1.0]hexane-3-carboxamide

2-((*tert*-butoxycarbonyl)amino)-2-(3-hydroxytetracyclo[5.1.1.0^{3,8}.0^{5,8}]nonan-1-yl)acetic acid







ANALYTICAL METHOD DEVELOPED

1.0 ASSAY AND RELATED SUBSTANCE METHOD BY HPLC (ON ANHYDROUS BASIS)

1.1 Instrumentation

- A High Performance Liquid Chromatograph (HPLC)
  (Shimadzu LC 2010 system or waters 2690 or equivalent)
- **Data handling system**
  LC Solution Software or Empower-2 Software or equivalent chromatographic software
- **Column**
  A stainless steel column of length 250 mm, internal diameter 4.6 mm and filled with Octyl Silane chemically bonded to porous silica particles of 5µ diameter.(Use Luna 5 µ C8(2) 100A(250 x 4.6 mm x 5µ) or Symmetry C8 (250 x 4.6 mm), 5µ or equivalent).

1.2 . Reagents

1) Distilled Water
2) Potassium Dihydrogen Phosphate (KH_2PO_4)
3) Acetonitrile (HPLC grade)
4) Orthophosphoric acid

1.3 Preparation of buffer (Mobile phase A)

6.8 g Potassium Dihydrogen Phosphate (KH_2PO_4) was weighed and dissolved in 1000 mL distilled water. 5.0 mL of Triethyl amine was added and mixed well. The pH of solution was adjusted to 7.0 with diluted ortho-phosphoric acid and mixed well. The solution was filtered through 0.45µ or finer porosity membrane filter.

1.4 Preparation of Mobile phase B

Used filtered and degassed Acetonitrile.

1.5 Elution Program

Time in minutes	% Mobile Phase A	%Mobile Phase B
0.01	80	20
10.00	80	20
30.00	35	65
50.00	35	65
55.00	80	20
65.00	80	20

1.6 Chromatographic parameters

Flow rate	:	1.0 mL/minute
Detection	:	UV at 220 nm
Injection Volume	:	20 µL
Column Oven Temperature	:	40°C
Run time	:	Not less than 60 minutes

1.7 Preparation of diluent

Water: Acetonitrile:: 1:1

1.8 Preparation of Saxagliptin monohydrate Standard solution

50 mg of Saxagliptin Monohydrate standard was accurately weighed and transferred into a 50 mL volumetric flask. 20 mL of diluent was added and sonicated for 5 minutes to dissolve the solid with occasional shaking. It is diluted to volume with diluent and mixed well. (1000 µg/mL Saxagliptin Monohydrate). This is stock standard solution-S.

5.0 mL of above stock solution-S was accurately transferred in to 50 mL volumetric flask, and diluted to volume with diluent and mixed. (100 µg/mL Saxagliptin monohydrate). This is diluted standard solution-S_1.

1.9 Preparation of Sample solution

50 mg of Saxagliptin hydrochloride sample was accurately weighed and transferred in duplicate into individual 50 mL volumetric flask. 20 mL of diluent was added into each volumetric flask and sonicated for 5 minutes to dissolve the solid with occasional shaking. It is diluted to volume with diluent and mixed well. (1000 µg/mL Saxagliptin hydrochloride). This is stock sample solution-A and B.

5.0 mL of each stock sample solution-A and B was accurately transferred into individual 50 mL volumetric flask and diluted to volume with diluent and mixed well. (100 µg/mL Saxagliptin hydrochloride). This is diluted sample solution-A_1 and B_1.

1.10 Preparation of Sample solution for related substance

50 mg of Saxagliptin hydrochloride sample was accurately weighed and transferred into 50 mL volumetric flask. 20 mL of diluent was accurately transferred into each volumetric flask and sonicated to dissolve the solid with occasional shaking. It is diluted to volume with diluent and mixed well.

1.11 Procedure

The diluent, standard preparation and sample preparation were separately injected into the chromatograph and chromatograms were recorded. The peak responses only for the major peak were measured in the chromatogram of standard and sample for assay. The peak responses of all eluting peaks in the chromatogram of the sample solution were determined for related substances. The diluent chromatogram were examined for any extraneous peaks, and disregarded the corresponding peaks observed in the chromatogram of the sample solution for related substances. The sequence of injections is as below:

Sr. no.	Sample	No. of injections
1	Diluent	1
2	Diluted Standard S_1 (system suitability)	6
3	Sample preparation for Assay – 1	1
4	Sample preparation for Assay – 2	1
5	Sample preparation for related sustenance	1

The relative retention time (RRT) of Saxagliptin and related impurities with respect to Saxagliptin is given in following table;

Name of compound	Approximate Retention time in minutes	Relative retention time (RRT)
Saxagliptin	11.7	1.0
Saxagliptin related impurity-1	22.2	1.9
Specified unknown impurity at RRT - 0.3	3.7	0.3
Specified unknown impurity at RRT - 0.6	6.8	0.6

1.12 System Suitability Parameter

Sr. No.	Parameter of system suitability	Specification
1	% Relative Standard deviation (RSD) for area of Saxagliptin	Not more than 2.0 %
2	Theoretical plates (Column efficiency)	Not less than 3000
3	Tailing factor (Asymmetry)	Not more than 2.0

1.13 Calculation

Calculated Assay (% w/w, on anhydrous basis) for both samples as per formula given below:

1.13.1 **Assay of Saxagliptin hydrochloride** (% w/w, on anhydrous basis)

$$= \frac{At}{As} \times \frac{\text{Std. wt.}}{50} \times \frac{5}{50} \times \frac{50}{\text{Sample wt.}} \times \frac{100}{5} \times \frac{351.88}{(100-Z)} \times \frac{}{315.42} \times P$$

where,

At : Peak area of Saxagliptin in the chromatogram of sample preparation

As : Average peak area of Saxagliptin in the chromatogram of standard preparation

Std. wt. : Weight of Saxagliptin standard in mg

Sample wt. : Weight of Saxagliptin sample preparation in mg

P : % Potency /Assay of Saxagliptin standard (As is basis)

Z : % Water content

1.13.2 Percentage individual known/unknown impurity (on anhydrous basis):

$$= \frac{At}{As} \times \frac{\text{Std. wt.}}{50} \times \frac{5}{50} \times \frac{50}{\text{Sample wt.}} \times \frac{100}{(100-Z)} \times \frac{351.88}{315.42} \times P$$

Where,

At : Peak area of known/unknown impurity in the chromatogram of sample preparation

As : Average peak area of Saxagliptin in the chromatogram of standard preparation

Std. wt.	:	Weight of Saxagliptin standard in mg
Sample wt.	:	Weight of Saxagliptin sample preparation in mg
P	:	% Potency /Assay of Saxagliptin standard (As is basis)
Z	:	% Water content

1.14 Specification

Assay:

Not less than 98.0 % and not more than 102.0 % w/w (on Anhydrous basis)

Related impurities:

Saxagliptin related impurity-1	:	Not more than 0.15 %
Specified unknown impurity at RRT - 0.3	:	Not more than 0.15 %
Specified unknown impurity at RRT - 0.6	:	Not more than 0.15 %
Unspecified unknown impurity	:	Not more than 0.10 %
Total impurities	:	Not more than 0.50 %

where:

Saxagliptin related impurity-1: (S)-N-BOC-3-hydroxyadamantyl glycine-L-Cis-4, 5-methanoproline Nitrile **(BOC – Nitrile Compound)**

Forced Degradation

Degradation is done as per below stress conditions:

Sample Preparation	Blank Preparation	Remarks
10 mg of sample was weighed accurately and transferred into a 10 mL volumetric flask, 2.0 mL of 5 N HCl, was added and heated the flask on a water bath at 80°C for about an hour. The solution was cooled to room temperature, neutralized with 5 N NaOH and added diluent to dissolve the solid. Further it is diluted to volume with diluent and mixed.	Prepared a blank solution excluding sample	Hydrolytic degradation with acid
10 mg of sample was weighed accurately and transferred into a 10 mL volumetric flask, 2.0 mL of 5 N NaOH was added and heated the flask on a water bath at 80°C for about an hour. The solution was cooled to room temperature and neutralized with 5 N HCl. It is further diluted to volume with diluent and mixed.	Prepared a blank solution excluding sample	Hydrolytic degradation with base
10 mg of sample was weighed accurately and transferred into a 10 mL volumetric flask, 2.0 mL of 50 % Hydrogen peroxide solution was added to the flask and heated the flask on a water bath at 80°C for about an hour. The solution was cooled to room temperature and diluent was added to dissolve the solid. It is further diluted to volume with diluent and mixed.	Prepared a blank solution excluding sample	Oxidative degradation with Hydrogen peroxide (H_2O_2)

1.1 Sample preparation and degradation conditions in detail are given below:

1.2 STRESS STUDIES (FORCED DEGRADATION / INDUCED DEGRADATION)

1.2.1 Preparation of control sample solution for related substances (RS)

10 mg of Saxagliptin HCl sample was accurately weighed and transferred into a 10 mL volumetric flask. 4 mL of diluent was added and sonicated to dissolve the solid and diluted to volume with diluent and mixed well. (Stock sample solution for related substances)

1.2.2 Preparation of sample solution for Hydrolytic degradation with acid (For Related Substances)

10 mg of Saxagliptin HCl sample was accurately weighed and transferred into a 10 mL volumetric flask. 2.0 mL of 5 N Hydrochloric acid was added to the flask. The solution was heated at 80°C for 1 hour. The solution was cooled to room temperature and neutralized with 5 N sodium hydroxide. The diluent was added and mixed well. It is further diluted to volume with diluent and mixed well. (Stock acid degradation sample solution for Related Substances)

1.2.3 Preparation of blank solution for Hydrolytic degradation with acid (For Related Substances)

A blank solution for acid hydrolysis of Related Substances was prepared by following the same procedure given under section **1.2.2** omitting the sample.

1.2.4 Preparation of sample solution for Hydrolytic degradation with base (For Related Substances)

10 mg of Saxagliptin HCl sample was accurately weighed and transferred into a 10 mL volumetric flask. 2.0 mL of 5 N sodium hydroxide was added to the flask and the solution was heated at 80°C for 1 hour. The solution was cooled to room temperature and neutralized with 5 N Hydrochloric acid. The diluent was added and mixed well. It is further diluted to volume with diluent and mixed well. (Stock base degradation sample solution for Related Substances)

1.2.5 Preparation of blank solution for Hydrolytic degradation with base (For Related Substances)

A blank solution for base hydrolysis of Related Substances was prepared by following the same procedure given under section **1.2.4** omitting the sample.

1.2.6 Preparation of sample solution for Oxidative degradation with Hydrogen peroxide (For Related Substances)

10 mg of Saxagliptin HCl sample was accurately weighed and transferred into a 10 mL volumetric flask. 2.0 mL of 50% Hydrogen peroxide was added and mixed well. The solution was treated at 80°C for 1 hour and then cooled to room temperature. The diluent was added and mixed well, it is further diluted to volume with diluent and mixed well. (Stock Oxidative degradation sample solution for Related Substances)

1.2.7 Preparation of blank solution for Oxidative degradation with Hydrogen peroxide (For Related Substances)

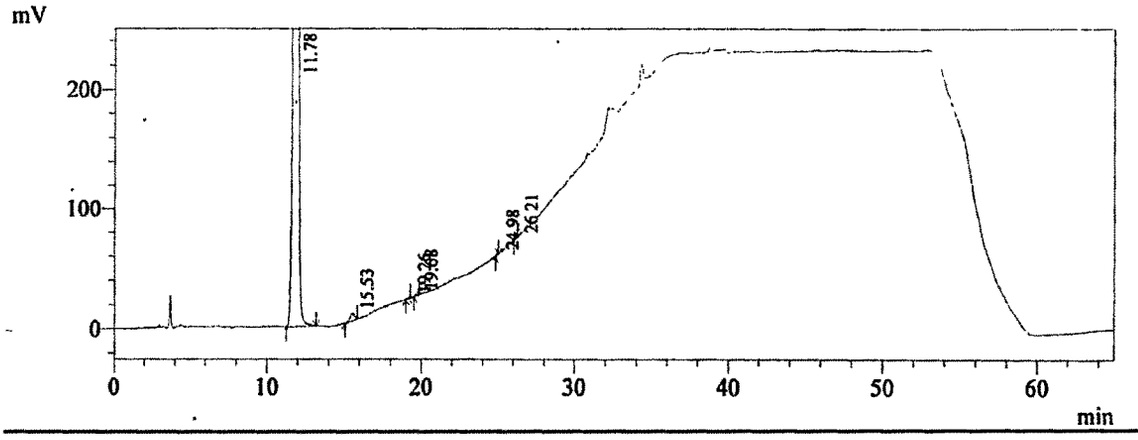
A blank solution for oxidation degradation of Related Substances was prepared by following the same procedure given under section **1.2.6** omitting the sample.

Table: % Purity in Forced degradation samples for Related Substances

Mode of Degradation	Condition	% Purity of Saxagliptin HCl
Control sample	- Drug	**99.32**
Hydrolytic degradation with acid	- Drug + 2 mL 5 N Hydrochloric acid, heated the solution for 1 hour at 80° C temperature on water bath	**93.26**
Hydrolytic degradation with base	- Drug + 2 mL 5 N Sodium hydroxide, heated the solution for 1 hour at 80° C temperature on water bath	**0.36**
Oxidative degradation with Hydrogen peroxide	- Drug + 2 mL 50 % Hydrogen peroxide, heated the solution for 1 hour at 80° C temperature on water bath	**3.86**

EXHIBITS

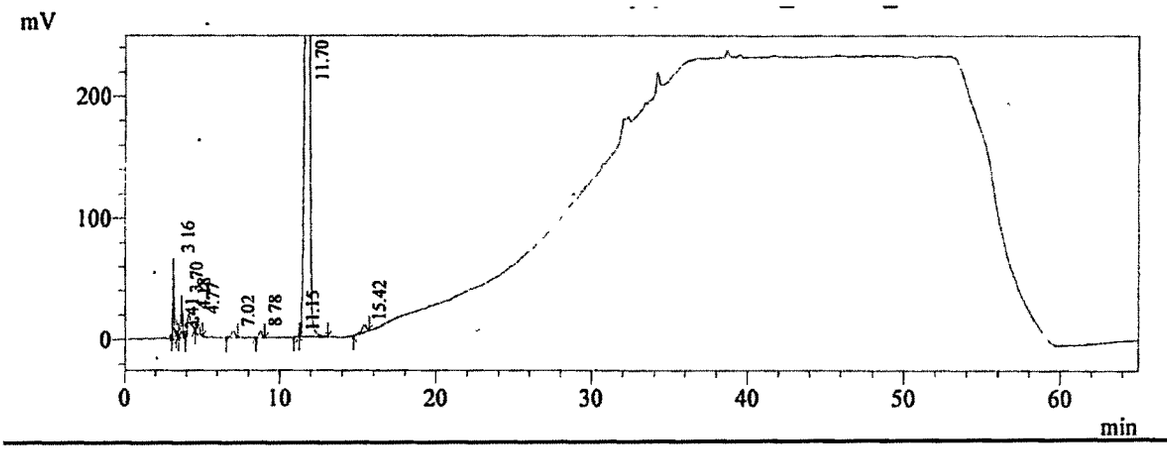
Chromatogram of Control Sample



Detector A Ch1 220nm

Peak#	Ret. Time	Area	Area %	Name
1	11.78	18927804	99.32	Saxa API
2	15.53	104403	0.55	Unk
3	19.26	4641	0.02	Unk
4	19.68	8856	0.05	Unk
5	24.98	7111	0.04	Unk
6	26.21	5247	0.03	Unk
Total		19058061	100.00	

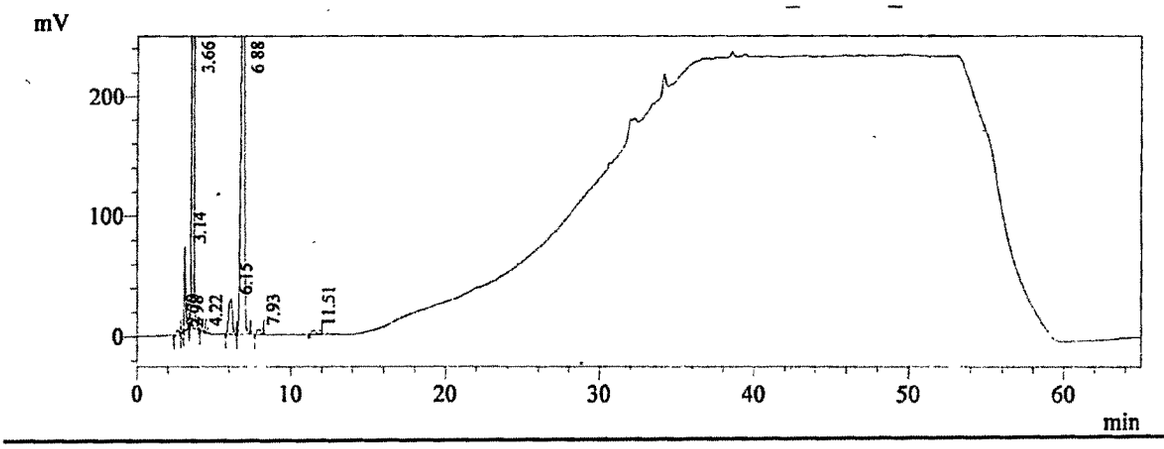
Chromatogram of Acid degradation sample



Detector A Ch1 220nm

Peak#	Ret. Time	Area	Area %	Name
1	3.16	339446	1.82	Unk
2	3.41	5645	0.03	Unk
3	3.70	208329	1.12	Unk
4	4.18	299460	1.61	Unk
5	4.77	133525	0.72	Unk
6	7.02	89091	0.48	Unk
7	8.78	73630	0.40	Unk
8	11.15	7964	0.04	Unk
9	11.70	17372542	93.26	Saxa API
10	15.42	99004	0.53	Unk
Total		18628637	100.00	

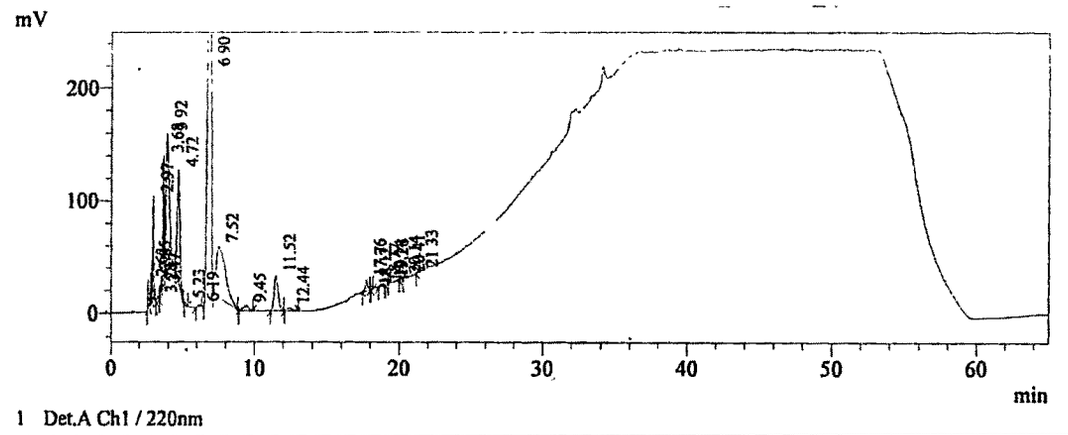
Chromatogram of Base degradation sample



Detector A Ch1 220nm

Peak#	Ret. Time	Area	Area %	Name
1	2.70	44904	0.26	Unk
2	2.98	6465	0.04	Unk
3	3.14	448137	2.64	Unk
4	3.66	10381564	61.13	Unk
5	4.22	14271	0.08	Unk
6	6.15	495418	2.92	Unk
7	6.88	5471742	32.22	Unk
8	7.93	60204	0.35	Unk
9	11.51	60718	0.36	Saxa API
Total		16983423	100.00	

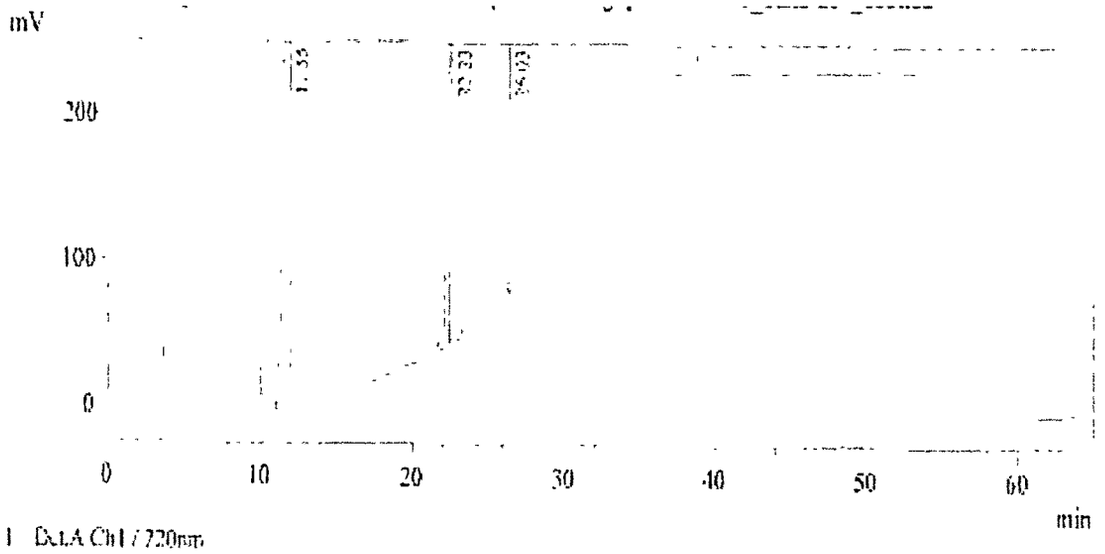
Chromatogram of Oxidative degradation sample



Detector A Ch1 220nm

Peak#	Ret. Time	Area	Area %	Name
1	2.63	145889	1.00	Unk
2	2.85	66418	0.45	Unk
3	2.97	412689	2.82	Unk
4	3.28	29627	0.20	Unk
5	3.47	24971	0.17	Unk
6	3.68	431842	2.96	Unk
7	3.92	1999624	13.69	Unk
8	4.72	1251456	8.56	Unk
9	5.23	18786	0.13	Unk
10	6.19	18456	0.13	Unk
11	6.90	7259371	49.68	Unk
12	7.52	2070004	14.17	Unk
13	9.45	106223	0.73	Unk
14	11.52	564282	3.86	Saxa API
15	12.44	53778	0.37	Unk
16	17.76	97007	0.66	Unk
17	18.12	2786	0.02	Unk
18	18.77	8992	0.06	Unk
19	19.14	11208	0.08	Unk
20	19.28	8365	0.06	Unk
21	20.14	10474	0.07	Unk
22	20.41	15215	0.10	Unk
23	21.33	3994	0.03	Unk
Total		14611458	100.00	

Chromatogram of Saxagliptin with two synthesized impurities

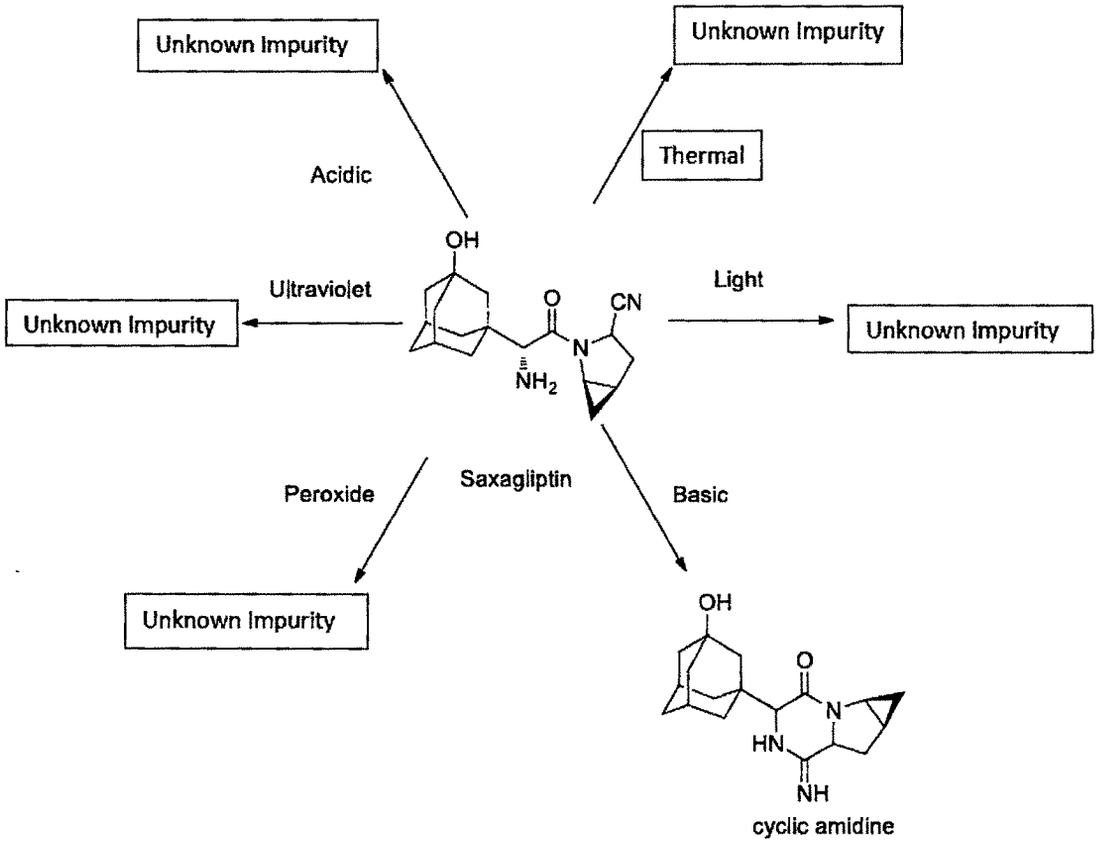


1 DAD A Ch1 / 220nm

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Area %	Name
1	11.55	18690269	54.78	Saxa API
2	22.23	3441821	10.09	Amide Coupling
3	26.03	11984196	35.13	BOC Prot. Saxa
Total		34116286	100.00	

Saxagliptin hydrochloride



CONCLUSION

Based on the studies performed to study extensively the various degradation pathways we have observed that different impurities can be formed due to the stress effects on the Saxagliptin drug substance. When the exercise was initiated on drug product also, we have observed the similar trend in the impurity profiles of the Saxagliptin drug substance and Saxagliptin drug product as in Onglyza.

Not all stress conditions are generating the same impurities. Majority of the stress conditions were not degrading the drug Saxagliptin under the conditions tried except for the basic degradation. The impurity isolations were attempted which were formed through the basic degradation in order to characterize the same, but the impurities of cyclic amidine was found not stable if isolated either from the degradation mix after the stress conditions were applied on the drug substance or if tried to synthesize the same. However the degradation samples in solution were enough to develop a better HPLC method with good resolution of these basic impurities from the drug substance Saxagliptin, which is a stability indicating method in order to monitor the related substances present in the drug substances. This method might be used at Quality Control laboratories which are required to test and release the Saxagliptin drug substance and drug products for human use.

The knowledge of the possible degradation pathways achieved in this project prompted us to understand the impurities behavior in humans when administered either orally or systemic, and to get the toxicology information of the same. Some of the data is published and some other impurities were not studied for further toxicology so minimizing the impurities to the level of acceptable unknown levels in the drug substance and drug product are to be considered.

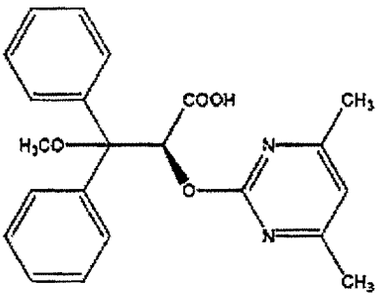
Other process and degradation impurities should be controlled by pharmaceutical companies in order to get the drug approvals for marketing. The main form of degradation takes place in the basic pH, so the development of the drug products or drug substance should consider controlling the pH of the samples during the manufacturing, shipment and storage of the drug substances and drug products.

AMBRISENTAN

AMBRISENTAN

Letairis is the brand name for **Ambrisentan,** an endothelia receptor antagonist that is selective for the endothelia type-A (ET_A) receptor. The chemical name of ambrisentan is (+)-(2S)-2-[(4,6-dimethylpyrimidin-2-yl)oxy]-3-methoxy-3,3-diphenylpropanoic acid. It has a molecular formula of $C_{22}H_{22}N_2O_4$ and a molecular weight of 378.42. It contains a single chiral center determined to be the (S) configuration and has the following structural formula:

Structural Formula

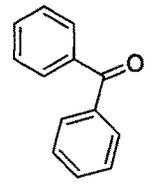


Ambrisentan is a white to off-white, crystalline solid. It is a carboxylic acid with a pKa of 4.0. Ambrisentan is practically insoluble in water and in aqueous solutions at low pH. Solubility increases in aqueous solutions at higher pH. In the solid state ambrisentan is very stable, is not hygroscopic, and is not light sensitive.

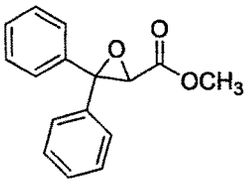
Letairis is available as 5 mg and 10 mg film-coated tablets for once daily oral administration. The tablets include the following inactive ingredients: croscarmellose sodium, lactose monohydrate, magnesium stearate and microcrystalline cellulose. The tablets are film-coated with a coating material containing FD&C Red #40 Aluminum Lake, lecithin, polyethylene glycol, polyvinyl alcohol, talc, and titanium dioxide. Each square, pale pink Letairis tablet contains 5 mg of ambrisentan. Each oval, deep pink Letairis tablet contains 10 mg of ambrisentan. Letairis tablets are unscored.

SYNTHESIS

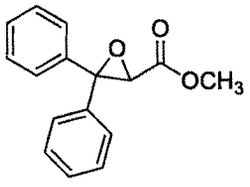
ROUTE OF SYNTHESIS OF AMBRISENTAN

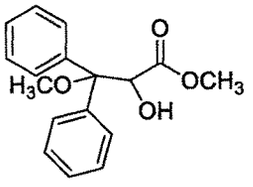


benzophenone

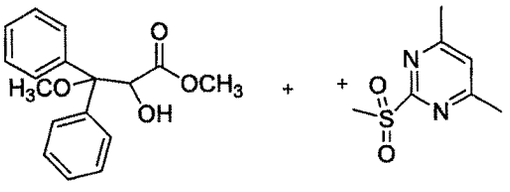


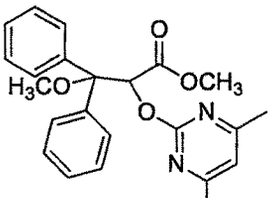
methyl 3,3-diphenyloxirane-2-carboxylate



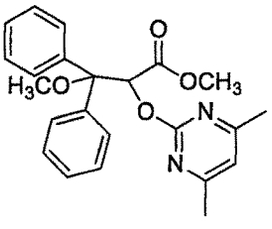


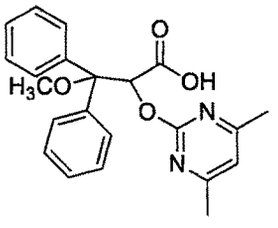
methyl 2-hydroxy-3-methoxy-3,3-diphenylpropanoate





methyl 2-((4,6-dimethylpyrimidin-2-yl)oxy)-3-methoxy-3,3-diphenylpropanoate





2-((4,6-dimethylpyrimidin-2-yl)oxy)-3-methoxy-3,3-diphenylpropanoic acid

Ambrisentan

1.1 Sample degradation procedure

1.2 STRESS STUDIES (FORCED DEGRADATION / INDUCED DEGRADATION)

1.2.1 Preparation of Control sample solution for related substances (RS)

50 mg of Ambrisentan sample was accurately weighed and transferred in to a 50 mL volumetric flask. 20 mL of diluent was added and sonicated to dissolve the solid and further diluted to volume with diluent and mixed well. (Stock sample solution for related substances)

1.2.2 Preparation of sample solution for Hydrolytic degradation with acid (For Related Substances)

50 mg of Ambrisentan sample was accurately weighed and transferred into a 50 mL volumetric flask. 5.0 mL of 1 N Hydrochloric acid was added to the flask. The solution was treated at 80°C for 1 hour and cooled to room temperature followed by neutralization with 1 N Sodium hydroxide. It is diluted to volume with diluent and mixed well. (Stock acid degradation sample solution for Related Substances)

1.2.3 Preparation of blank solution for Hydrolytic degradation with acid (For Related Substances)

A blank solution for acid hydrolysis of Related Substances was prepared by following the same procedure given under section **1.2.2** omitting the sample.

1.2.4 Preparation of sample solution for Hydrolytic degradation with base (For Related Substances)

50 mg of Ambrisentan sample was accurately weighed and transferred into a 50 mL volumetric flask. 5.0 mL of 1 N Sodium hydroxide was added to the flask. The solution was treated at 80°C for 1 hour and cooled to room temperature followed by neutralization with 1 N Hydrochloric acid. It was diluted to volume with diluent and mixed well. (Stock base degradation sample solution for Related Substances)

1.2.5 Preparation of blank solution for Hydrolytic degradation with base (For Related Substances)

A blank solution for base hydrolysis of Related Substances was prepared by following the same procedure given under section **1.2.4** omitting the sample.

1.2.6 Preparation of sample solution for Oxidative degradation with Hydrogen peroxide (For Related Substances)

50 mg of Ambrisentan sample was accurately weighed and transferred into a 50 mL volumetric flask. 5.0 mL of 10% Hydrogen peroxide was added to the flask and mixed well. The solution was treated at 80°C for 1 hour and cooled to room temperature. It is diluted to volume with diluent and mixed well. (Stock Oxidative degradation sample solution for Related Substances)

1.2.7 Preparation of blank solution for Oxidative degradation with Hydrogen peroxide (For Related Substances)

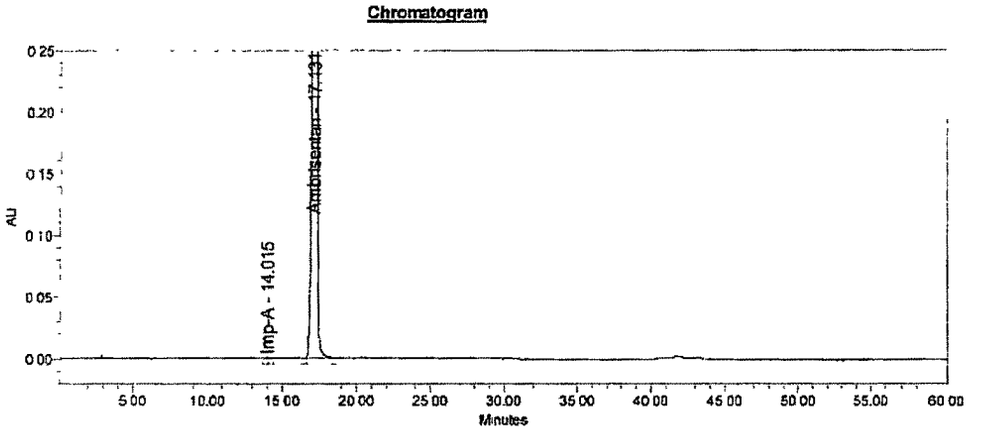
A blank solution for oxidation degradation of Related Substances was prepared by following the same procedure given under section **1.2.6** omitting the sample.

Table: % Purity in Forced degradation samples for Related Substances

Mode of Degradation	**Condition**	**% Purity of Ambrisentan**
Control sample	- Drug	**99.96**
Hydrolytic degradation with acid	- Drug + 5 mL 1 N Hydrochloric acid, heated the solution for 1 hour at 80° C temperature on water bath	**63.10**
Hydrolytic degradation with base	- Drug + 5 mL 1 N Sodium hydroxide, heated the solution for 1 hour at 80° C temperature on water bath	**99.88**
Oxidative degradation with Hydrogen peroxide	- Drug + 5 mL 10 % Hydrogen peroxide, heated the solution for 1 hour at 80° C temperature on water bath	**99.05**

EXHIBITS

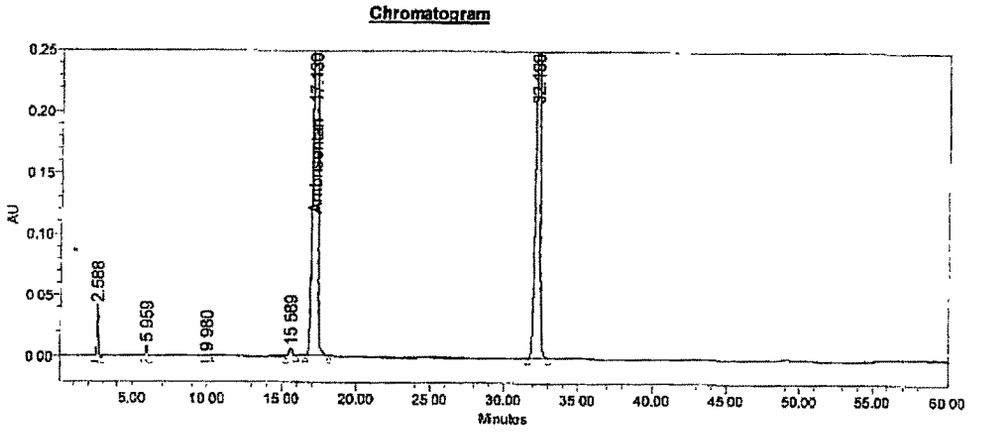
Chromatogram of Control Sample



Peak Table

	Peak Name	RT	Area	% Area
1	Imp-A	14.01	7919	0.04
2	Ambrisentan	17.13	22267425	99 96
Sum			22275344	100 00

Chromatogram of Acid degradation Sample

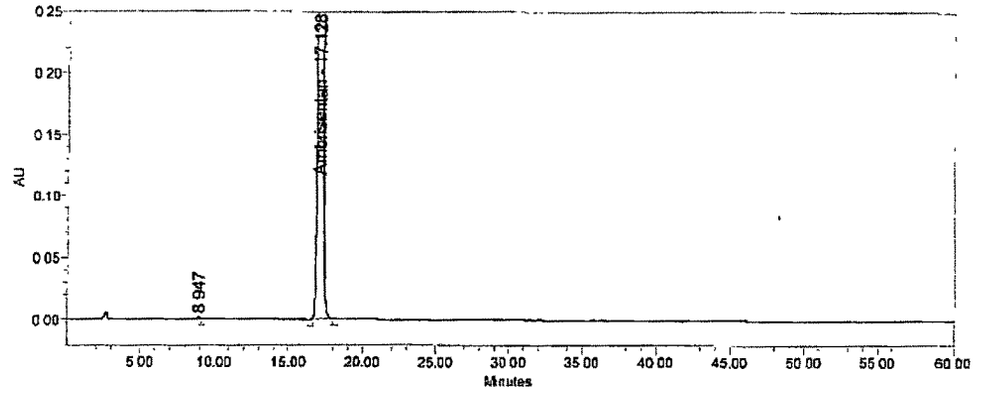


Peak Table

	Peak Name	RT	Area	% Area
1		2.59	249630	1.45
2		5.96	75180	0.44
3		9 98	7466	0.04
4		15 59	94489	0 55
5	Ambrisentan	17.13	10865195	63.10
6		32.20	5928277	34 43
Sum			17220238	100 00

Chromatogram of Base degradation Sample

Chromatogram

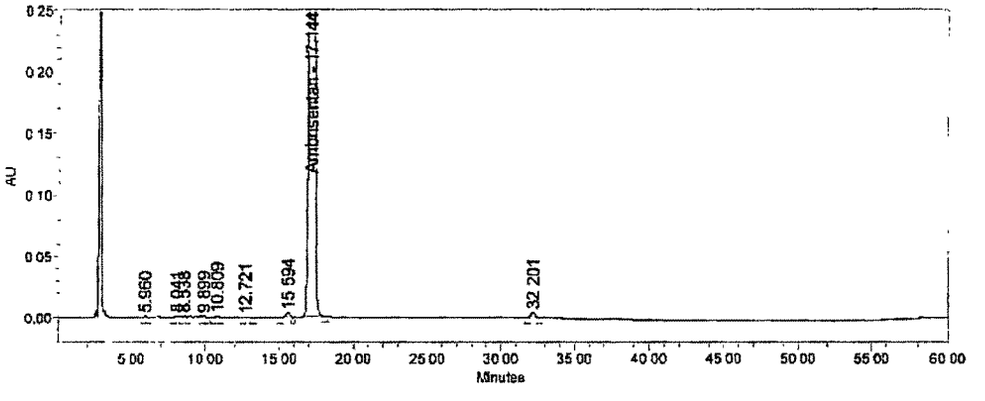


Peak Table

	Peak Name	RT	Area	% Area
1		8.95	23185	0.12
2	Ambrisentan	17.13	18926282	99.88
Sum			18949467	100.00

Chromatogram of Oxidative degradation Sample

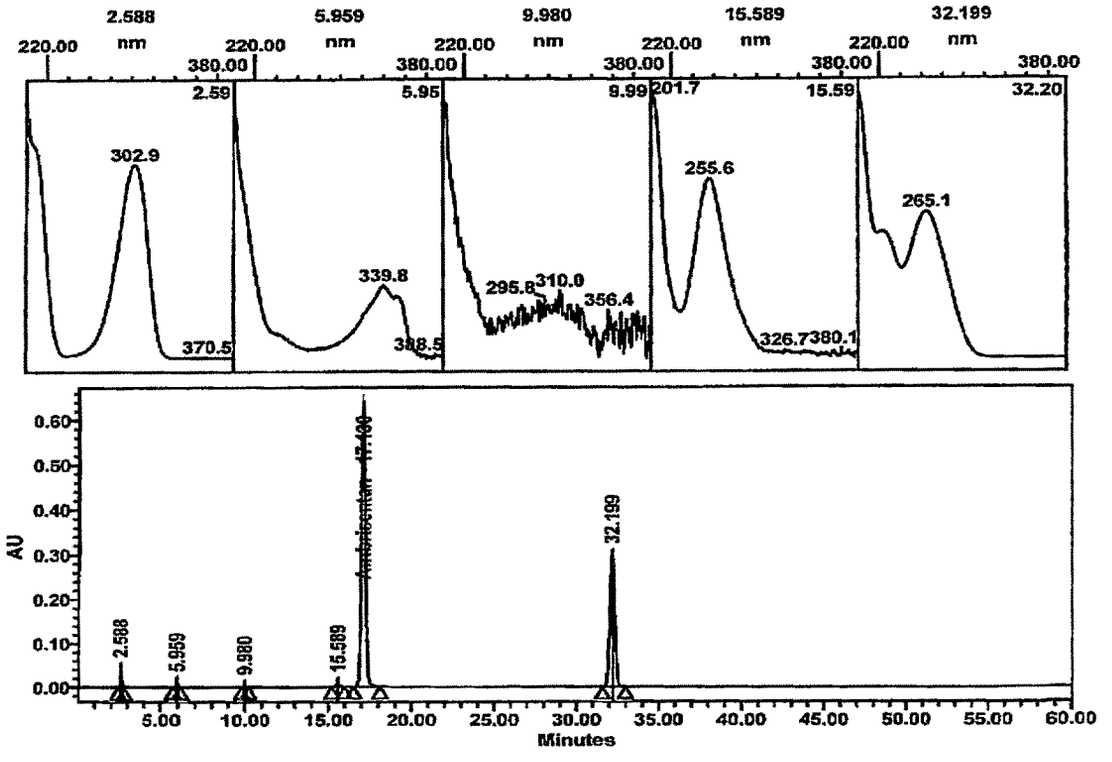
Chromatogram



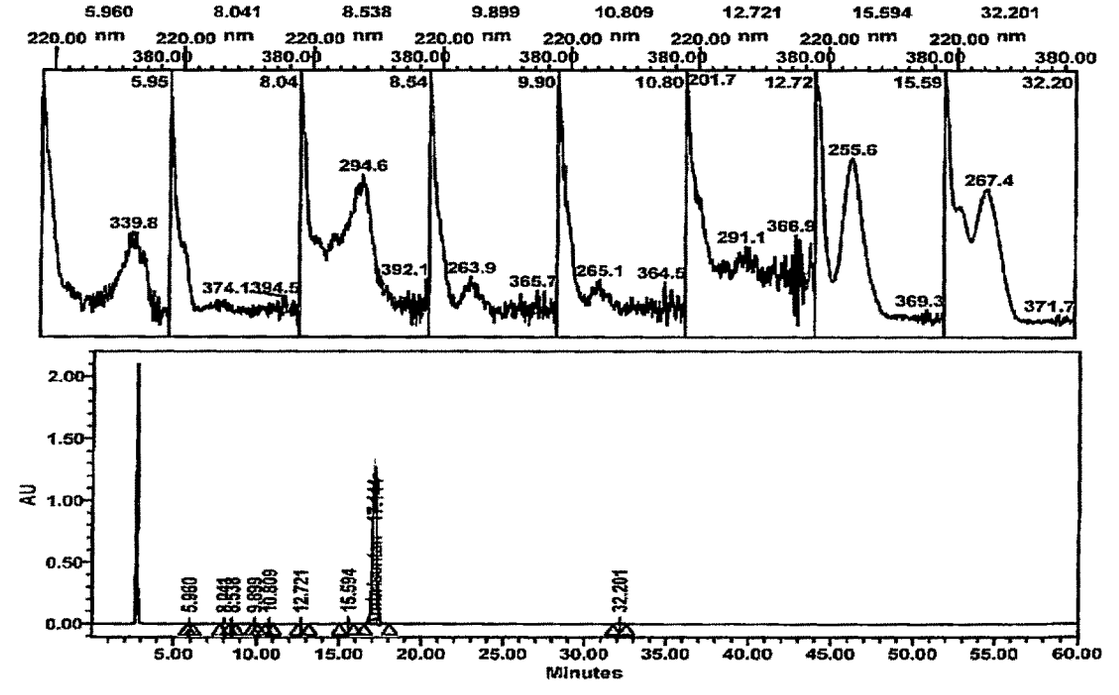
Peak Table

	Peak Name	RT	Area	% Area
1		5.96	7626	0.03
2		8.04	15762	0.07
3		8.54	9499	0.04
4		9.90	9834	0.04
5		10.81	14325	0.06
6		12.72	7994	0.04
7		15.59	57724	0.26
8	Ambrisentan	17.14	22016049	99.05
9		32.20	88545	0.40
Sum			22227358	100.00

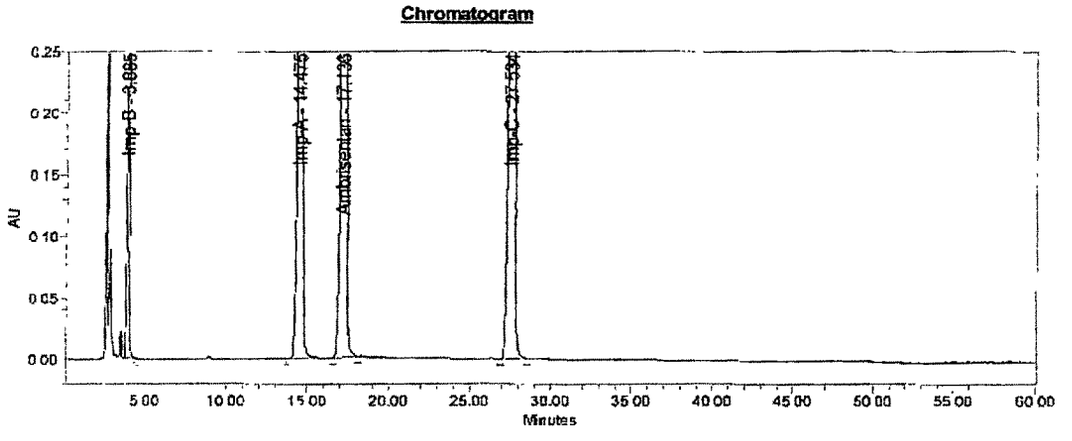
Peak Purity Acid degradation sample



Peak Purity Oxidative degradation sample



Control sample chromatogram spiked with known impurities



Peak Table

	Peak Name	RT	Area	% Area
1	Imp-B	3.88	6469429	9.07
2	Imp-A	14.47	19468149	27.30
3	Ambrisentan	17.14	23045531	32.31
4	Imp-C	27.53	22339261	31.32
Sum			71322369	100.00

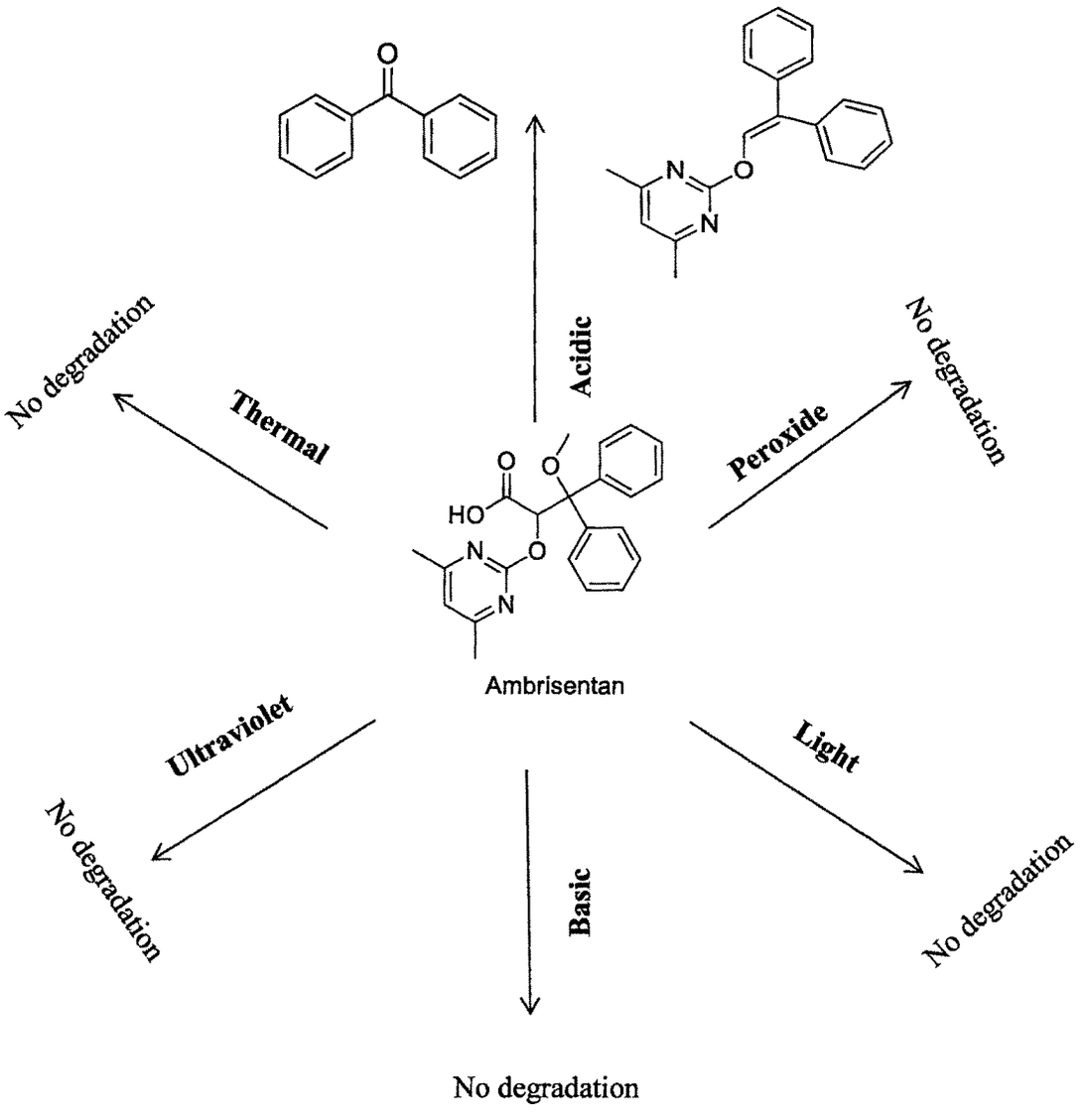
Where

Ambrisentan Impurity – A: Methyl (S)-2-hydroxy-3-methoxy-3, 3-diphenyl propionate:

Ambrisentan Impurity – B: 4, 6-Dimethyl-2-(methyl sulfonyl) pyrimidine:

Ambrisentan Impurity – C: Methyl (2S)-2-[(4, 6-dimethyl pyrimidine-2-yl) oxy] - 3-methoxy 3, 3-diphenyl propionate: **CONDENSED S-ESTER**

DEDRADATION PATHWAYS FOR AMBRISENTAN:



CONCLUSION

Based on the studies performed to study extensively the various degradation pathways we have observed that different impurities can be formed due to the stress effects on the Ambrisentan drug substance. Not all stress conditions are generating the same impurities. The isolation of impurities and their characterizations of the same, led to develop a better HPLC method which is stability indicating in order to monitor the related substances present in the drug substances. This method can be used at Quality Control laboratories which are required to test and release the Ambrisentan drug substance and drug products for human use.

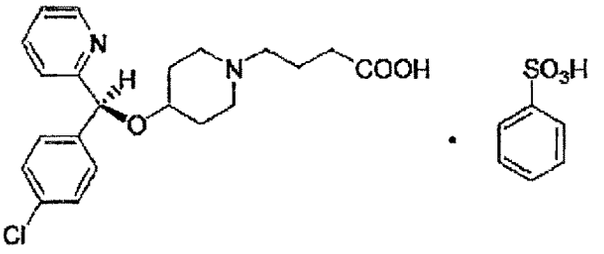
The attempt should be made to minimize the impurities to the level of acceptable unknown levels in the drug substance and drug product. Other process and degradation impurities should be controlled by Pharmaceutical companies in order to get the drug approvals for marketing.

The main form of degradation is at the acidic pH, so the development of the drug products or drug substance should consider controlling the pH of the samples during the manufacturing, shipment and storage of the drug substances and drug products.

BEPOTASTINE BESILATE

BEPOTASTINE BESILATE

BEPREVE (bepotastine besilate ophthalmic solution) 1.5% is a sterile, topically administered drug for ophthalmic use. Each mL of BEPREVE contains 15 mg bepotastine besilate. Bepotastine besilate is designated chemically as (+) -4-[[(S)-p-chloro-alpha -2pyridylbenzyl] oxy]-1-piperidine butyric acid monobenzenesulfonate. The chemical structure for bepotastine besilate is:



Bepotastine besilate is a white or pale yellowish crystalline powder. The molecular weight of bepotastine besilate is 547.06 daltons. BEPREVE ophthalmic solution is supplied as a sterile, aqueous 1.5% solution, with a pH of 6.8.

The osmolality of BEPREVE (bepotastine besilate ophthalmic solution) 1.5% is approximately 290 mOsm/kg.

Each mL of BEPREVE (bepotastine besilate ophthalmic solution) 1.5% contains

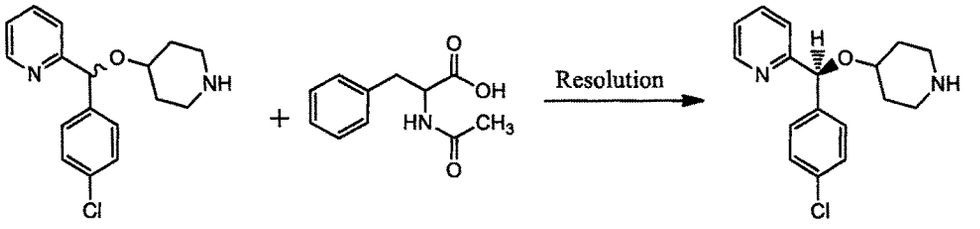
Active: Bepotastine besilate 15 mg (equivalent to 10.7 mg bepotastine)

Preservative: benzalkonium chloride 0.005%

Inactives: monobasic sodium phosphate dihydrate, sodium chloride, sodium hydroxide to adjust pH, and water for injection, USP.

SYNTHESIS

ROUTE OF SYNTHESIS OF BEPOTASTINE BESILATE



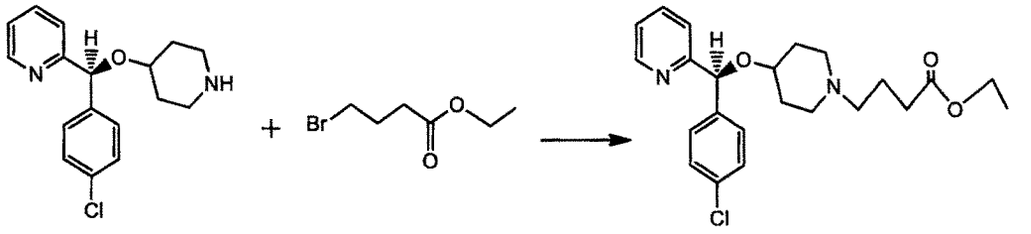
(RS)-4-[(4-Chlorophenyl) (2-pyridyl)methoxy]piperidine

N-Acetyl-L-phenylalanine

(S)-4-[(4-Chlorophenyl) (2-pyridyl)methoxy]piperidine

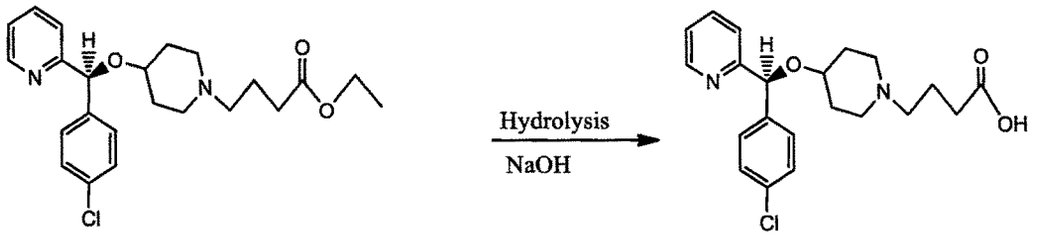
302.80

302.80



(S)-4-[(4-Chlorophenyl) (2-pyridyl)methoxy]piperidine

Ethyl-[4-[(4-Chlorophenyl) (2-pyridyl)methoxy]piperidino]butanoate



Ethyl-[4-[(4-Chlorophenyl) (2-pyridyl)methoxy]piperidino]butanoate

(S)-[4-[(4-Chlorophenyl)(2-pyridyl) methoxy]piperidino]butanoic acid

402.91

388.88

ANALYTICAL METHOD DEVELOPED

ASSAY (% W/W ON DRIED BASIS) AND RELATED SUBSTANCE (% W/W ON AS IS BASIS BY HPLC)

1.1 Instrumentation

- A High Performance Liquid Chromatograph equipped with Gradient elution capability, Ultraviolet Spectrophotometer as detector and an auto sampler.
  (Shimadzu LC 2010 system or Waters 2690 or equivalent was used)

- **Data handling system**
  LC Solution Software, Empower 2 or equivalent chromatographic software

- **Column**
  A stainless steel column of length 150 mm, internal diameter 4.6 mm and filled with Octadecyl Silane chemically bonded to porous silica particles of 5µ diameter or Inertsil ODS-3V (150 x 4.6mm x 5µ) or equivalent.

1.2 Reagents

1. Distilled Water
2. Monobasic potassium phosphate
   (Potassium dihydrogen orthophosphate, KH_2PO_4)
3. Triethyl amine
4. Orthophosphoric acid
5. Tetrahydrofuran

1.3 Preparation of buffer [Mobile phase-A]

1.3 g of Potassium dihydrogen orthophosphate (KH_2PO_4) was weighed and dissolved in 1000 mL distilled water and mixed well. 10 ml of Triethyl amine was added and mixed well. The pH was adjusted to 2.4 ± 0.1 with Ortho-phosphoric acid and filtered through 0.45µ or finer porosity membrane filter.

1.4 Preparation of Mobile phase B

ACN: MeOH: THF: 800:200:1

1.5 Elution Program (Gradient Composition)

Initial program: Mobile Phase A: Mobile phase B: 90: 10

Time in minutes	Mobile phase A (% v/v)	Mobile phase B (% v/v)
0.01	90	10
5.00	90	10
40.00	50	50
45.00	50	50
50.00	90	10
60.00	90	10

1.6 Chromatographic parameters

Flow rate	: 1.0 mL/minute
Detection	: UV at 210 nm
Injection Volume	: 20 µL
Column Oven Temperature	: 40°C
Run time	: Not less than 60 minutes

1.7 Preparation of diluent

Water: Acetonitrile:: 90:10

1.8 Preparation of Bepotastine Besilate Standard solution for assay and RS

50 mg of Bepotastine Besilate standard was weighed accurately and transferred into a 50 mL volumetric flask. 20 mL of diluent was added and sonicated to dissolve the solid with occasional shaking. It is diluted to volume with diluent and mixed well. This is stock standard solution-S

5.0 mL of above stock solution-S was accurately transferred in to 50 mL volumetric flask and diluted to volume with diluent and mixed. This is diluted standard solution-S_1

1.9 Preparation of Benzene sulfonic acid Standard solution

50 mg of Bepotastine Besilate standard was accurately weighed and transferred into a 50 mL volumetric flask. 20 mL of diluent was added and sonicated to dissolve the solid with occasional shaking. It is diluted to volume with diluent and mixed well. This is stock standard solution-S_2

5.0 mL of above stock solution-S was transferred accurately in to 50 mL volumetric flask and diluted to volume with diluent and mixed. This is diluted standard solution-S_3

1.10 Preparation of Sample solution for assay

50 mg of Bepotastine Besilate sample was weighed accurately and transferred in duplicate into individual 50 mL volumetric flask. 20 mL of diluent was added into each volumetric flask and sonicated to dissolve the solid with occasional shaking. It is diluted to volume with acetonitrile and mixed well. This is stock sample solution-A and B

5.0 mL of each stock sample solution-A and B was accurately transferred into individual 50 mL volumetric flask and diluent was added to volume and mixed well. This is diluted sample solution-A_1 and B_1

1.11 Preparation of Sample solution for related substances (RS)

50 mg of Bepotastine Besilate sample was weighed accurately and transferred into 50 mL volumetric flask. 20 mL of diluent was added to the volumetric flask and sonicated to dissolve the solid with occasional shaking. It is diluted to volume with acetonitrile and mixed well. This is sample solution-C for related substances.

1.12 Procedure

The diluent, standard preparation and sample preparationwere separately injected into the chromatograph and the chromatograms were recorded. The peak responses were measured only for the major peak in the chromatogram of standard and sample for assay. The peak responses were determined of all eluting peaks in the chromatogram of the sample solution

for related substances. Diluent chromatogram was examined for any extraneous peaks, and disregarded the corresponding peaks observed in the chromatogram of the sample solution for related substances.

The injection sequence as mentioned below was followed.

Sr. no.	**Sample**	**No. of injections**
1	Diluent	1
2	Diluted Standard S_1 (system suitability)	6
3	Benzene sulfonic acid	1
4	Sample preparation for Assay – 1	1
5	Sample preparation for Assay – 2	1
6	Sample preparation (RS)	1

The table below for retention and relative retention time of known impurities was followed

Name of compound	**Retention time (Minutes)**	**Relative retention time (RRT)**
Besilate	6.2	0.28
Bepotastine	22.2	1.0
Bepotastine Besilate related compound – A	29.6	1.33

1.14 System Suitability Parameter

System suitability Parameters	Specification
% Relative Standard deviation (RSD) for area of Bepotastine and Besilate	Not more than 2.0 %
Theoretical plates (Column efficiency)	Not less than 3000
Tailing factor (Asymmetry)	Not more than 2.0

1.15 Calculation

1.15.1 **Assay of Bepotastine Besilate** (% w/w, on dried Basis) =

$$= \frac{At}{As} \times \frac{\text{Std. wt (mg)}}{50} \times \frac{5}{50} \times \frac{50}{\text{Sample wt (mg)}} \times \frac{50}{5} \times \frac{100}{(100-Z)} \times P$$

Where,

At : Peak area of (Bepotastine + Besilate) in the chromatogram of sample preparation

As : Average peak area of (Bepotastine +Besilate) in the chromatogram of standard preparation

Std. wt. : Weight of Bepotastine Besilate standard in mg

Sample wt. : Weight of Bepotastine Besilate sample preparation in mg

P : % Potency /Assay of Bepotastine Besilate standard (As is basis)

Z : % Loss on drying

1.15.2 **Related substances**

(i) Percentage individual known / unknown impurity

$$= \frac{At}{As} \times \frac{\text{Std. wt (mg)}}{50} \times \frac{5}{50} \times \frac{50}{\text{Sample wt (mg)}} \times P$$

Where,

At : Peak area of known / unknown impurity in sample preparation

As : Average peak area of (Bepotastine + Besilate) in standard preparation

Std. wt : Weight of Bepotastine Besilate standard in mg

Sample wt : Weight of Bepotastine Besilate sample preparation in mg

P : % Potency / Assay of Bepotastine Besilate standard (As is basis)

(ii) Percentage Besilate

$$= \frac{At}{As} \times \frac{\text{Std. wt (mg)}}{50} \times \frac{5}{50} \times \frac{50}{\text{Sample wt (mg)}} \times \frac{50}{5} \times \frac{100}{(100-Z)} \times P$$

Where,

At : Peak area of Besilate in sample preparation

As : Average peak area of Benzene Sulfonic Acid in standard preparation

Std. wt : Weight of Benzene Sulfonic Acid standard in mg

Sample wt : Weight of Bepotastine Besilate sample preparation in mg

P : % Potency / Assay of Benzene Sulfonic Acid standard (As is basis)

1.15.3 Unknown impurity

Report maximum unknown impurity obtained from calculation.

1.15.4 Percentage total Impurities

Report summation of all the impurities

1.16 Report

Report the average assay obtained from both determinations (% w/w, on dried basis)

1.17 Specification

Assay

Not less than 98.0 % w/w & not more than 102.0 % w/w (On dried basis)

Related impurities

Bepotastine Besilate related compound – A	:	Not more than 0.15 %
Any individual unknown impurity	:	Not more than 0.10 %
Total impurities	:	Not more than 0.50 %

Where:

Bepotastine Besilate related compound – A:

Ethyl(s)-4-[(4-chlorophenyl) (2-pyridyl) methoxy piperidine] butanoate

2.1 SAMPLE DEGRADATION PROCEDURE

2.2 STRESS STUDIES (FORCED DEGRADATION / INDUCED DEGRADATION)

2.2.1 Preparation of sample solution for hydrolytic degradation with acid (For Related Substances)

10 mg of Bepotastine Besilate sample was accurately weighed and transferred into a 10 mL volumetric flask. 2.0 mL of 5 N Hydrochloric acid was added. The solution was treated at 80°C for 1 hour and cooled to room temperature. It is neutralized with 5 N Sodium

hydroxide and further diluted to volume with diluent and mixed well. (Stock acid degradation sample solution for Related Substances)

2.2.2 Preparation of blank solution for hydrolytic degradation with acid (For Related Substances)

A blank solution for acid hydrolysis of Related Substances was prepared by following the same procedure given under section **2.2.1** omitting the sample.

2.2.3 Preparation of sample solution for Hydrolytic degradation with base (For Related Substances)

10 mg of Bepotastine Besilate sample was accurately weighed and transferred into a 10 mL volumetric flask. 2.0 mL of 5 N Sodium hydroxide was added. The solution was treated at 80°C for 1 hour and cooled to room temperature. It is neutralized with 5 N Hydrochloric acid and diluent was added and mixed well. It is diluted to volume with diluent and mixed well (Stock base degradation sample solution for Related Substances).

2.2.4 Preparation of blank solution for Hydrolytic degradation with base (For Related Substances)

A blank solution for base hydrolysis of Related Substances was prepared by following the same procedure given under section **2.2.3** omitting the sample.

2.2.5 Preparation of sample solution for Oxidative degradation with Hydrogen peroxide (For Related Substances)

10 mg of Bepotastine Besilate sample was accurately weighed and transferred into a 10 mL volumetric flask. 2.0 mL of 50% Hydrogen peroxide was added and mixed well. The solution was treated at 80°C for 1 hour and cooled the solution to room temperature. It is diluted to volume with diluent and mixed well (Stock Oxidative degradation sample solution for Related Substances).

2.2.6 Preparation of blank solution for Oxidative degradation with Hydrogen peroxide (For Related Substances)

A blank solution for oxidation degradation of Related Substances was prepared by following the same procedure given under section **2.2.5** omitting the sample.

Table: % Purity in Forced degradation samples for Related Substances

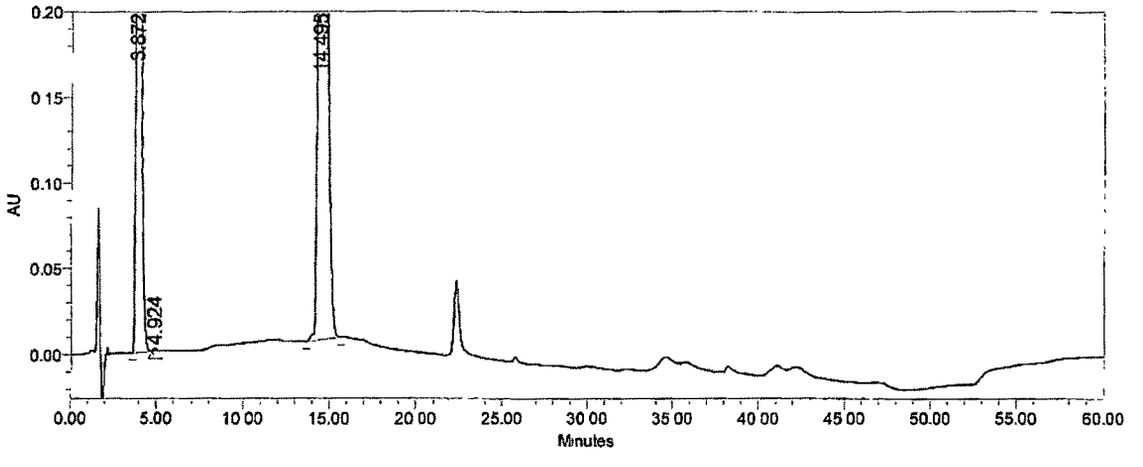
Mode of Degradation	Condition	% Purity of Bepotastine Besilate
Hydrolytic degradation with acid	- Drug + 2 mL 5 N Hydrochloric acid, heated the solution for 1 hour at 80° C temperature in water bath	**39.92+60.04 =99.96**
Hydrolytic degradation with base	- Drug + 2 mL 5 N Sodium hydroxide, heated the solution for 1 hour at 80° C temperature in water bath	**48.98+50.99 =99.97**
Oxidative degradation with Hydrogen peroxide	- Drug + 2 mL 50 % Hydrogen peroxide, heated the solution for 1 hour at 80° C temperature in water bath	**39.72+59.03 =98.75**

Note: % Purity is calculated for both Bepotastine and Besilate separately and the sum is provided in the table.

EXHIBITS

Chromatogram of Acid degradation sample

Chromatogram

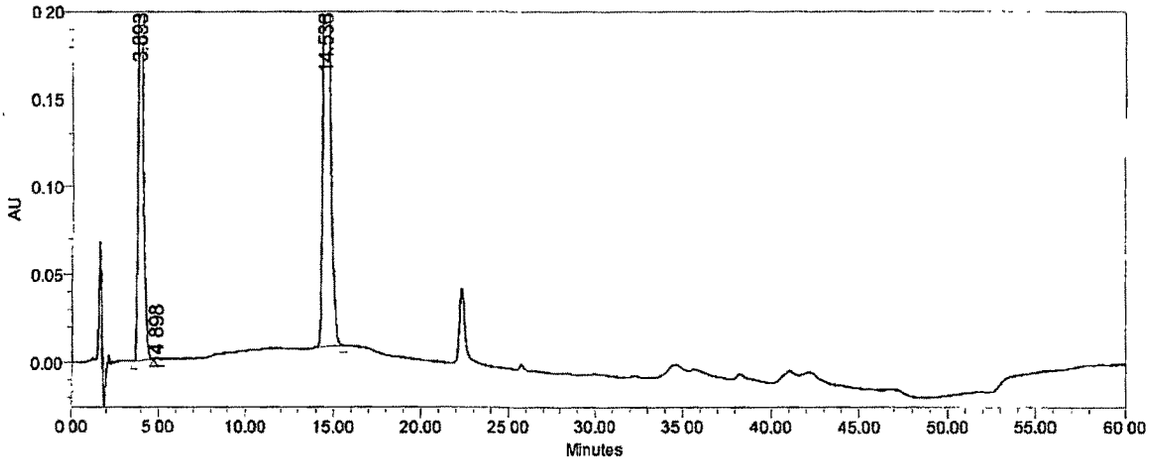


Peak Table

	RT	Area	% Area
1	3.87	15104530	39.92
2	4.92	14987	0.04
3	14.50	22717130	60.04
Sum		37836646	100.00

Chromatogram of Base degradation sample

Chromatogram

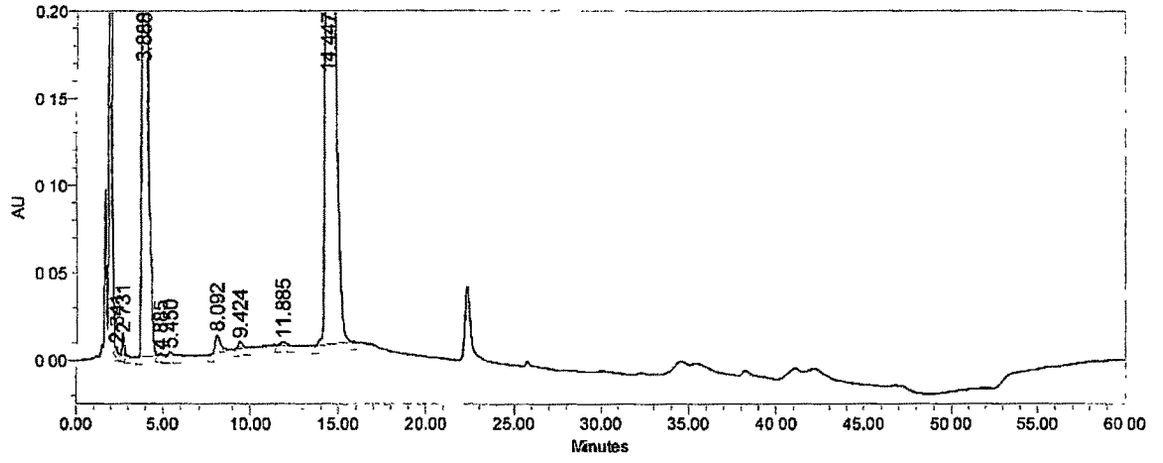


Peak Table

	RT	Area	% Area
1	3.89	8459985	48.98
2	4.90	5673	0.03
3	14.54	8806917	50.99
Sum		17272575	100.00

Chromatogram of Oxidative degradation sample

Chromatogram

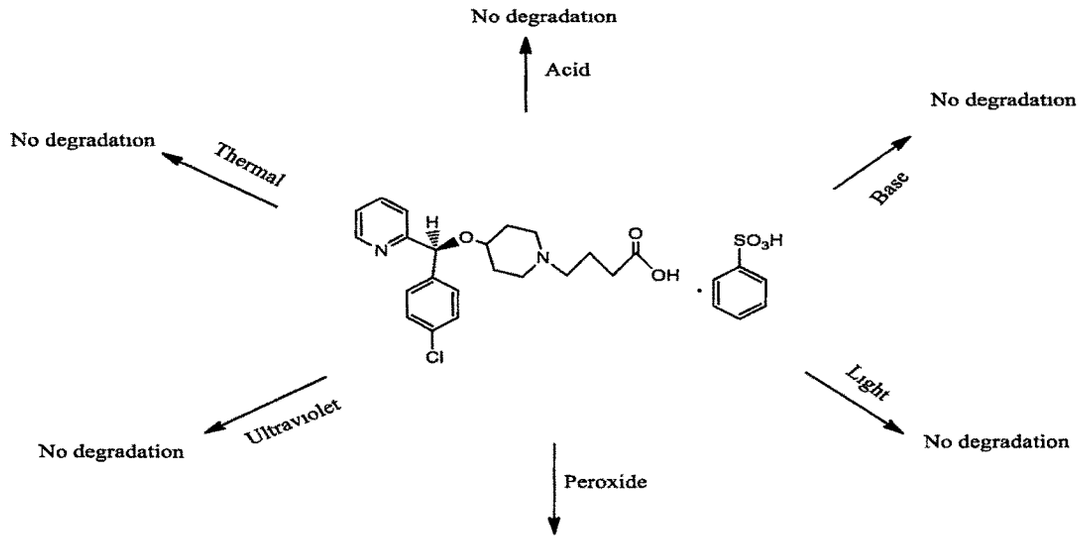


Peak Table

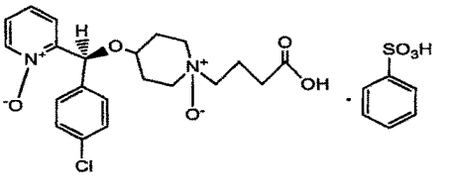
	RT	Area	% Area
1	2.34	8916	0.02
2	2.73	80961	0.22
3	3.89	14686538	39.72
4	4.88	11266	0 03
5	5 45	25299	0.07
6	8.09	240231	0.65
7	9.42	62163	0.17
8	11.89	33632	0.09
9	14.45	21829299	59.03
Sum		36978306	100.00

BEPOTASTINE BESILATE DEGADATION PATHWAYS

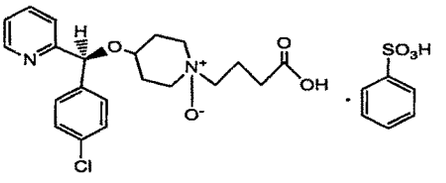
Bepotastine besilate:-



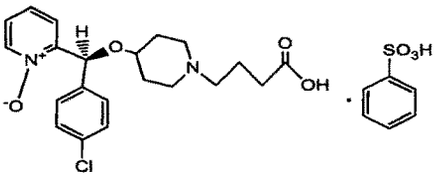
Impurity:-A



Impurity:-B



Impurity:-C



CONCLUSION

Based on the studies performed to study extensively the various degradation pathways we have observed that different impurities can be formed due to the stress effects on the Bepotastine Besilate drug substance. Not all stress conditions are generating the same impurities. Majority of the stress conditions were not degrading the drug Bepotastine Besylate under the conditions tried except for the oxidative degradation. The impurity isolations were attempted which were formed through the oxidative degradation in order to characterize the same, but the impurities were found unstable if isolated either from the degradation mix after the stress conditions were applied on the drug substance or synthesis of drug substance. However the degradation samples in solution were enough to develop a better HPLC method with good resolution of these oxidative impurities from the drug substance Bepotastine Besilate, which is a stability indicating method in order to monitor the related substances present in the drug substances. This method might be used at quality control laboratories which are required to test and release the Bepotastine Besilate drug substance and drug products for human use.

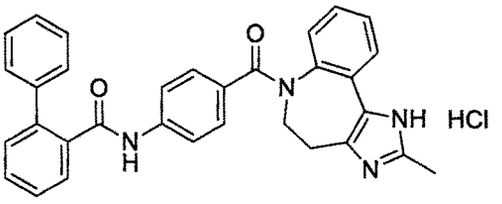
The attempt should be made to minimize the impurities to the level of acceptable unknown levels in the drug substance and drug product. Other process and degradation impurities should be controlled by pharmaceutical companies in order to get the drug approvals for marketing.

The main form of degradation is happening due to oxidative degradation, so the development of the drug products or drug substance should consider controlling the oxygen getting in contact with the drug either in the form of primary packaging or secondary packaging. Use of desiccant bags to absorb the atmospheric moisture should be considered in this drug packaging and handling.

CONIVAPTAN HCl

CONIVAPTAN HCl

Conivaptan hydrochloride is chemically [1,1'-biphenyl]-2-carboxamide, N-[4-[(4,5-dihydro-2methylimidazo[4,5-d][1]benzazepin-6(1H)-yl)carbonyl]phenyl]-, monohydrochloride, having a molecular formula $C_{32}H_{26}N_4O_2$•HCl and molecular weight of 535.04. The structure of conivaptan hydrochloride is:



Conivaptan hydrochloride is a white to off-white or pale orange-white powder that is very slightly soluble in water (0.15 mg/mL at 23° C). Conivaptan hydrochloride injection is supplied as a sterile premixed solution with dextrose in a flexible plastic container.

Each container contains a clear, colorless, sterile, non-pyrogenic solution of conivaptan hydrochloride in dextrose. Each 100 mL, single-use premixed INTRAVIA container contains 20 mg of conivaptan hydrochloride and 5 g of Dextrose Hydrous, USP. Lactic Acid, USP is added for pH adjustment to pH 3.4 to 3.8. The flexible plastic container is fabricated from a specially designed multilayer plastic (PL 2408). Solutions in contact with the plastic container leach out certain of the chemical components from the plastic in very small amounts; however, biological testing was supportive of the safety of the plastic container materials. The flexible container has a foil overwrap. Water can permeate the plastic into the overwrap, but the amount is insufficient to significantly affect the premixed solution.

Indications and Usage

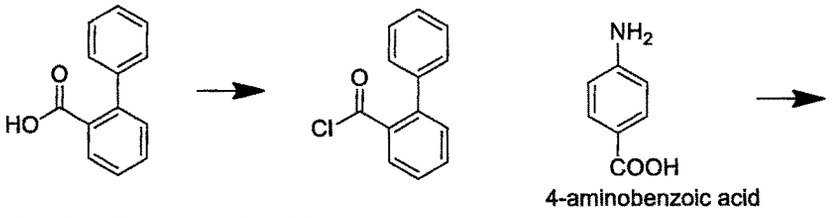
To raise serum sodium in hospitalized patients with euvolemic and hypervolemic hyponatremia.

Contraindications

Anuric patients; coadministration with potent CYP3A4 inhibitors (eg, clarithromycin, indinavir, itraconazole, ketoconazole, ritonavir); hypersensitivity to corn, corn products, or any component of the product; hypovolemic hyponatremia.

SYNTHESIS

ROUTE OF SYNTHESIS OF CONIVAPTAN

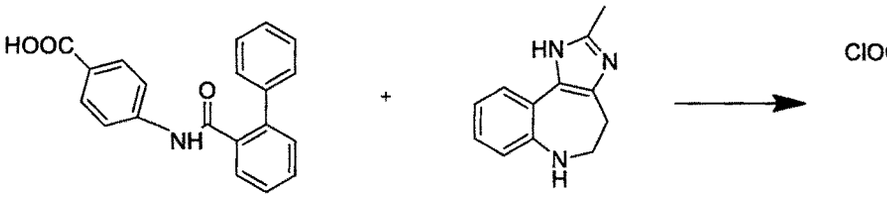


[1,1'-biphenyl]-2-carboxylic acid

[1,1'-biphenyl]-2-carbonyl chloride

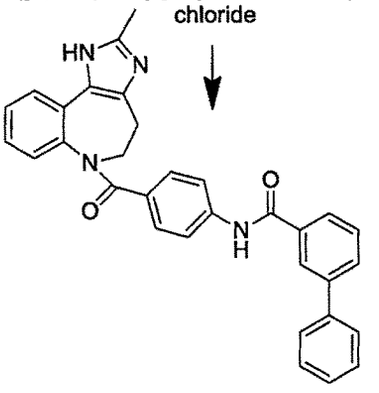
4-aminobenzoic acid

4-([1,1'-biphenyl]-2-ylcarboxamido)benzoic acid



2-methyl-1,4,5,6-tetrahydrobenzo[*b*]imidazo[4,5-*d*]azepine

4-([1,1'-biphenyl]-2-ylcarboxamido)benzoyl chloride



N-(4-(2-methyl-1,4,5,6-tetrahydrobenzo[*b*]imidazo[4,5-*d*]azepine-6-carbonyl)phenyl)-[1,1'-biphenyl]-3-carboxamide

Conivaptan

ANALYTICAL METHOD DEVELOPED

ASSAY (ON ANHYDROUS BASIS) AND RELATED SUBSTANCES (RS) (BY HPLC)

1.1 Instrument

- A High Performance Liquid Chromatograph (HPLC) or Shimadzu LC 2010 system or equivalent
- **Data handling system**

  LC Solution Software or equivalent chromatographic software
- **Column**

  A stainless steel column of length 150 mm, internal diameter 4.6 mm and filled With Octadecyl Silane chemically bonded to porous silica particles of 5µ diameter or Symmetry C-18 (150 x 4.6mm x 5µ) or equivalent.

1.2 Reagents

1) Distilled water
2) Triethyl amine
3) Ortho phosphoric acid
4) Acetonitrile

1.3 Preparation of Mobile Phase

1.3.1 Preparation of Mobile phase-A (Buffer)

Transferred 1 mL of Triethyl amine into 1000 mL of distilled water and mixed well. The pH was adjusted to 6.0 ± 0.05 using ortho phosphoric acid and mixed well. The solution was filtered through 0.45µ filter.

1.3.2 Preparation of mobile Phase-B

Used filtered (through 0.45µ filter) acetonitrile

1.4 Preparation of Mobile phase

650 mL of buffer (Mobile Phase- A) solution and 350 mL of Mobile phase-B was transferred in to a suitable container, mixed well and degassed by sonication.

OR

Binary Elution Program

Degas Mobile phase A and Mobile phase B by sonication.

Mobile phase A (% v/v)	:	Mobile phase B (% v/v)
65		35

1.5 Preparation of Diluent

Prepared filtered and degassed mixture of distilled water and Mobile phase B (Acetonitrile) in 50:50 ratio

1.6 Chromatographic parameters

Flow rate	:	1.0 mL/min.
Detection wavelength	:	210 nm
Injection Volume	:	5µL
Column oven temperature	:	30°C
Runtime	:	Not less than 30 min. for assay and Not less than 60 min for related substances (RS)

1.7 Preparation of Conivaptan hydrochloride stock Standard solution for assay

50 mg of Conivaptan hydrochloride standard was accurately weighed and transferred into a 50 mL volumetric flask. 20 mL of diluent was added and sonicated to dissolve the solid with occasional shaking. It is diluted to volume with diluent and mixed well. (1000 µg/mL Conivaptan hydrochloride. This is stock standard solution-S

1.8 Preparation of Conivaptan hydrochloride diluted standard solution for assay

5 mL of above stock solution-S was added in to 50 mL volumetric flask, and diluted to volume with diluent and mixed. (100 µg/mL Conivaptan hydrochloride). This is diluted standard solution-S_1

1.9 Preparation of Sample solution for assay

50 mg of Conivaptan hydrochloride sample was accurately weighed and transferred in duplicate into individual 50 mL volumetric flasks. 20 mL of diluent was added into each volumetric flask and sonicated to dissolve the solid with occasional shaking. It is diluted to volume with diluent and mixed well and contains (1000 µg/mL Conivaptan hydrochloride). This is stock sample solution-A and B

5.0 mL of each stock sample solution-A and B was transferred into individual 50 mL volumetric flask and diluted to volume with diluent and mixed well. (100 µg/mL Conivaptan hydrochloride). This is diluted sample solution-A_1 and B_1.

1.10 Preparation of Sample solution for related substances (RS)

50 mg of Conivaptan hydrochloride sample was accurately weighed and transferred into 50 mL volumetric flask. 20 mL of diluent was added and sonicated to dissolve the solid with occasional shaking. It is diluted to volume with diluent and mixed well. (1000 µg/mL Conivaptan hydrochloride). This is sample solution-C for related substances.

1.11 Procedure

The diluent, standard preparation and sample preparation were separately injected into the chromatograph. Recorded the chromatograms. The peak responses were measured only for the major peak in the chromatogram of standard and sample for assay. The peak responses were determined for all eluting peaks in the chromatogram of the sample solution for related substances. The diluent chromatogram was examined for any extraneous peaks, and the corresponding peaks observed in the chromatogram of the sample solution for related substances were disregarded. The injection sequence as mentioned below is followed.

Sr. no.	Sample	No. of injections
1	Diluent	1
2	Diluted Standard S_1 (system suitability)	6
3	Sample preparation – 1	1
4	Sample preparation – 2	1
5	Sample preparation (RS)	1
6	Bracketing standard (Diluted standard preparation)	1

1.12 System Suitability Parameter

- **% Relative Standard deviation (RSD) for area of Conivaptan hydrochloride**

  Not more than 2.0 %

- **Theoretical plates (Column efficiency)**

  Not less than 3000

- **Tailing factor (Asymmetry)**

  Not more than 2.0

1.13 Calculation

Calculated Assay (% w/w, on anhydrous basis) for both sample as per formula given below:

1.13.1 Assay

- Assay of Conivaptan hydrochloride
  (% w/w, on anhydrous basis) =

$$= \frac{At}{As} \times \frac{\text{Std. wt (mg)}}{50} \times \frac{5}{50} \times \frac{50}{\text{Sample wt (mg)}} \times \frac{50}{5} \times \frac{100}{(100-Z)} \times P$$

Where,

At	=	Peak area of Conivaptan Hydrochloride in sample preparation
As	=	Average peak area of Conivaptan Hydrochloride in standard preparation
Std. wt.	=	Weight of Conivaptan Hydrochloride standard in mg
Sample wt.	=	Weight of Conivaptan Hydrochloride sample preparation in mg
P	=	Potency (As is basis) or Assay (As is basis) of Conivaptan Hydrochloride standard
Z	=	% Water content of sample

- Report

The average of the results obtained from the analysis of sample preparation in duplicate as assay (% w/w, on anhydrous basis)

1.13.2 Related substances

- % Unknown impurity =

$$= \frac{At}{As} \times \frac{\text{Std. wt (mg)}}{50} \times \frac{5}{50} \times \frac{50}{\text{Sample wt (mg)}} \times P$$

Where,

At	=	Peak area of Unknown impurity in sample preparation
As	=	Average peak area of Conivaptan hydrochloride in standard preparation
Std. wt.	=	Weight of Conivaptan hydrochloride standard in mg
Sample wt.	=	Weight of Conivaptan hydrochloride sample preparation in mg
P	=	Potency (As is basis) or Assay (As is basis) of Conivaptan hydrochloride standard

- Any individual unknown impurity

Report maximum individual unknown impurity among all individual unknown impurity as calculated above

- Percentage total Impurity

  = Report summation of all impurities

The approximate retention time of Conivaptan hydrochloride is as under:

Compound Name	RT in minutes (Approx.)
Conivaptan hydrochloride	16.50

1.1 SAMPLE DEGRADATION PROCEDURE

1.2 STRESS STUDIES (FORCED DEGRADATION / INDUCED DEGRADATION)

1.2.1 Preparation of Control sample solution for related substances (RS)

10 mg of Conivaptan sample was accurately weighed and transferred in to a 10 mL volumetric flask. 4 mL of diluent was added and sonicated to dissolve the solid and diluted to volume with diluent and mixed well (Stock sample solution for related substances).

1.2.2 Preparation of sample solution for Hydrolytic degradation with acid (For Related Substances)

10 mg of Conivaptan sample was accurately weighed and transferred into a 10 mL volumetric flask. 2.0 mL of 1 N Hydrochloric acid was added. The solution was treated at 80°C for 1 hour and cooled to room temperature. It is neutralized with 1 N Sodium hydroxide and diluted to volume with diluent and mixed well (Stock acid degradation sample solution for Related Substances).

1.2.3 Preparation of blank solution for hydrolytic degradation with acid (For Related Substances)

A blank solution for acid hydrolysis of Related Substances was prepared by following the same procedure given under section **1.2.2** omitting the sample.

1.2.4 Preparation of sample solution for Hydrolytic degradation with base (For Related Substances)

10 mg of Conivaptan sample was accurately weighed and transferred into a 10 mL volumetric flask. 2.0 mL of 1 N Sodium hydroxide was added. The solution was treated at 80°C for 1 hour and cooled the solution to room temperature. It is neutralized with 1 N Hydrochloric acid and the diluent was added and mixed well. It is further diluted to volume with diluent and mixed well (Stock base degradation sample solution for Related Substances).

1.2.5 Preparation of blank solution for hydrolytic degradation with base (For Related Substances)

A blank solution for base hydrolysis of Related Substances was prepared by following the same procedure given under section **1.2.4** omitting the sample.

1.2.6 Preparation of sample solution for oxidative degradation with Hydrogen peroxide (For Related Substances)

10 mg of Conivaptan sample was accurately weighed and transferred into a 10 mL volumetric flask. 2.0 mL of 50% Hydrogen peroxide was added and mixed well. The solution was treated at 80°C for 1 hour and cooled to room temperature. It is diluted to volume with diluent and mixed well (Stock Oxidative degradation sample solution for Related Substances).

1.2.7 Preparation of blank solution for oxidative degradation with Hydrogen peroxide (For Related Substances)

A blank solution for oxidation degradation of Related Substances was prepared by following the same procedure given under section **1.2.6** omitting the sample.

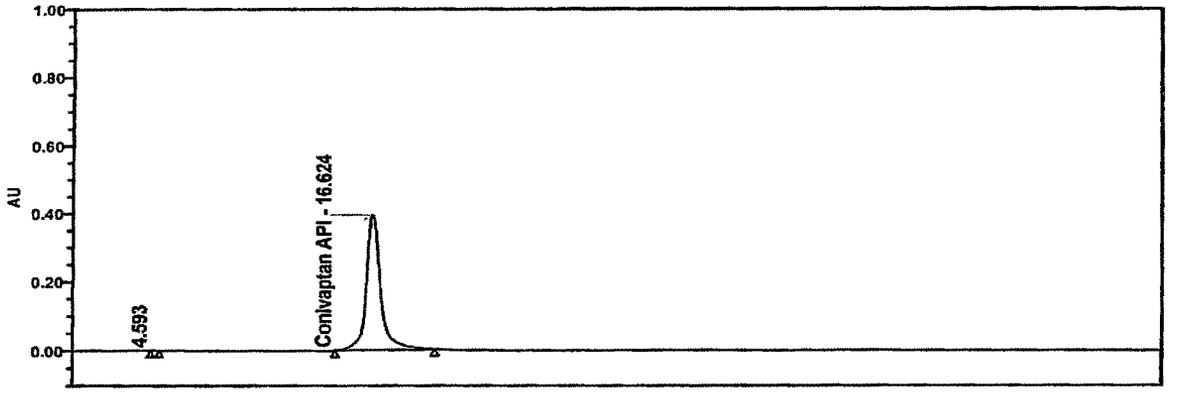
Table: % Purity in Forced degradation samples for Related Substances

Mode of Degradation	Condition	% Purity of Conivaptan
Control sample	- Drug	**99.91**
Hydrolytic degradation with acid	- Drug + 2 mL 1 N Hydrochloric acid, heated the solution for 1 hour at 80° C temperature on water bath	**99.74**
Hydrolytic degradation with base	- Drug + 2 mL 1 N Sodium hydroxide, heated the solution for 1 hour at 80° C temperature on water bath	**99.72**
Oxidative degradation with Hydrogen peroxide	- Drug + 2 mL 50 % Hydrogen peroxide, heated the solution for 1 hour at 80° C temperature on water bath	**97.67**

EXHIBITS

Chromatogram of control sample

Chromatogram

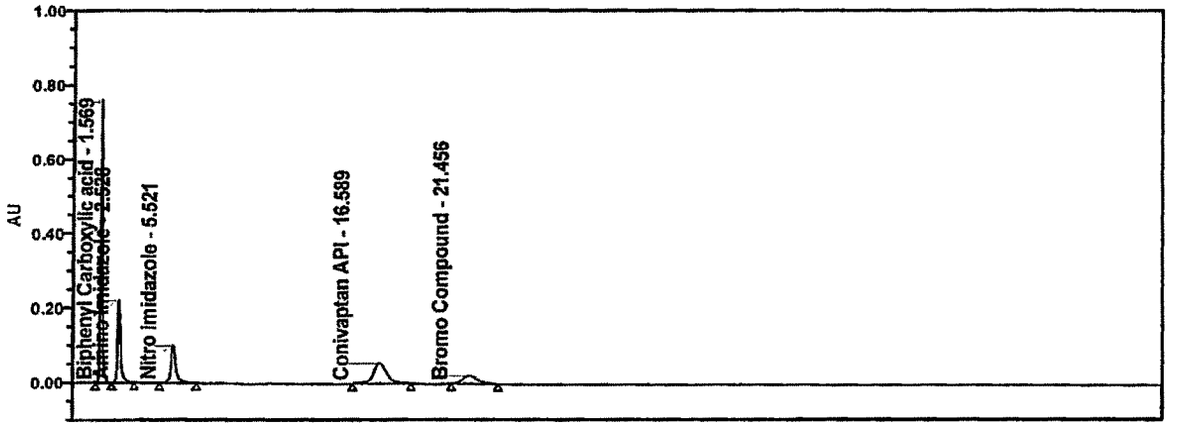


Peak Table

	Peak Name	RT	Area	% Area
1		4.59	18893	0.09
2	Conivaptan API	16.62	21549537	99.91
Sum			21568430	100.00

Chromatogram of control sample spiked with Synthesized impurities

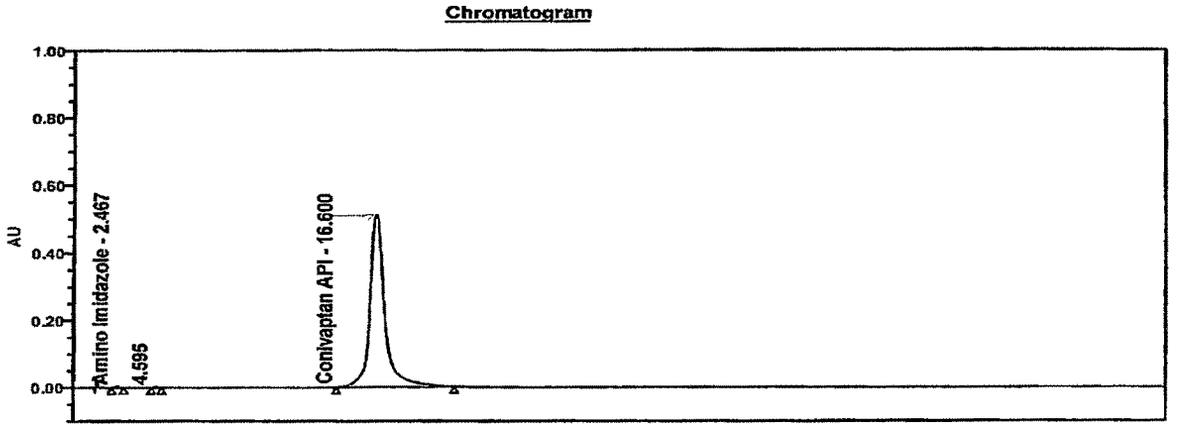
Chromatogram



Peak Table

	Peak Name	RT	Area	% Area	RT Ratio
1	Biphenyl Carboxylic acid	1.57	4794002	37.28	0.09
2	Amino Imidazole	2.53	2321837	18.05	0.15
3	Nitro imidazole	5.52	1898545	14.76	0.33
4	Conivaptan API	16.59	2704012	21.03	
5	Bromo Compound	21.46	1141914	8.88	1.29
Sum			12860310	100.00	

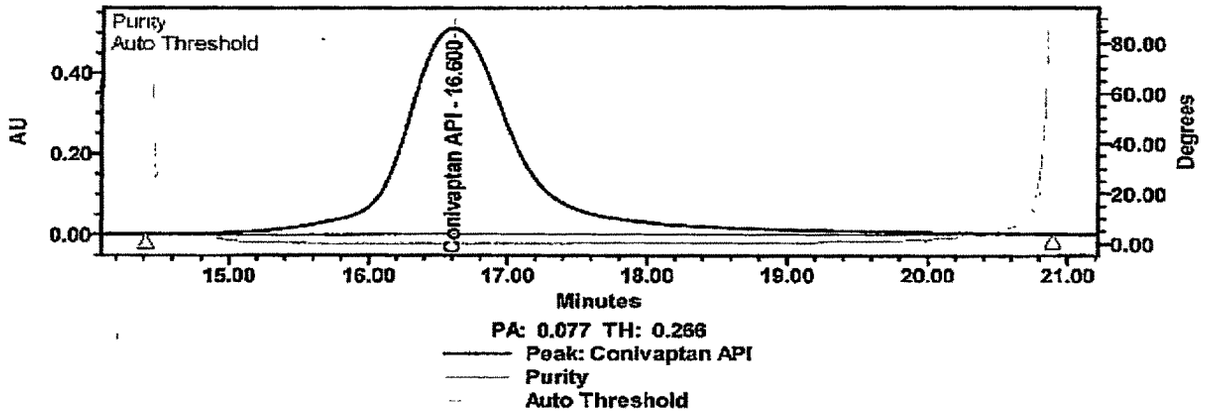
Chromatogram of Acid degradation sample

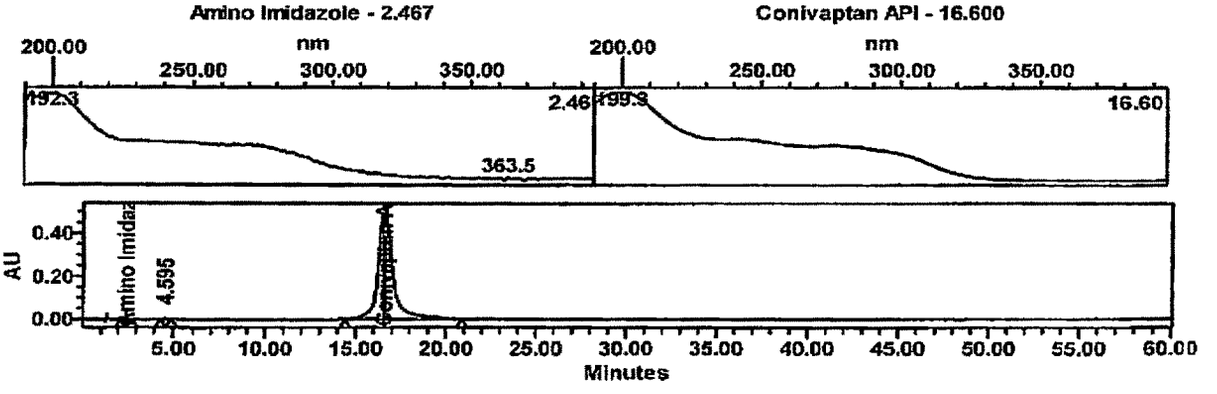


Peak Table

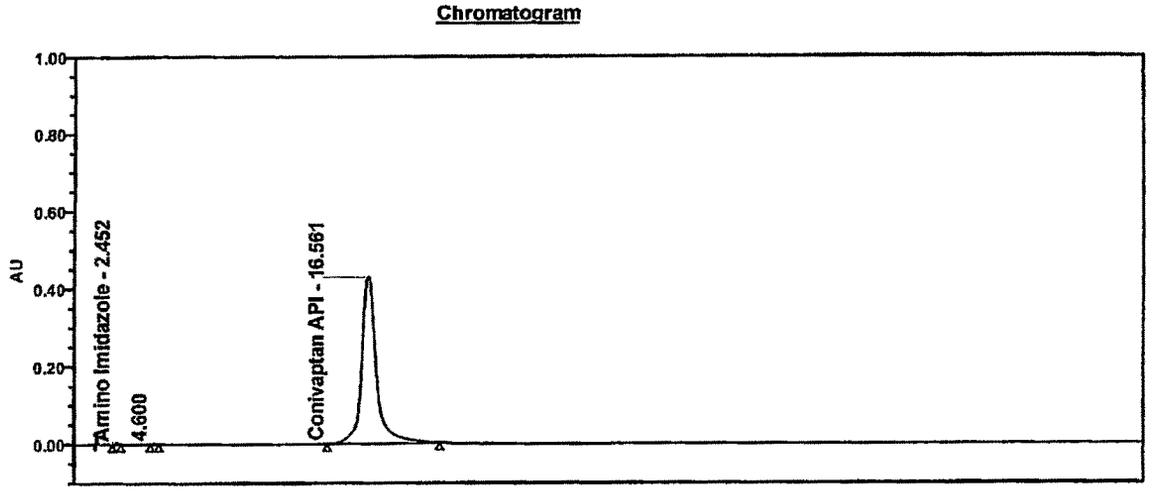
	Peak Name	RT	Area	% Area	RT Ratio
1	Amino Imidazole	2.47	48521	0.17	0.15
2		4.59	27411	0.09	
3	Conivaptan API	16.60	28903883	99.74	
Sum			28979815	100.00	

Peak Purity of Acid degradation sample





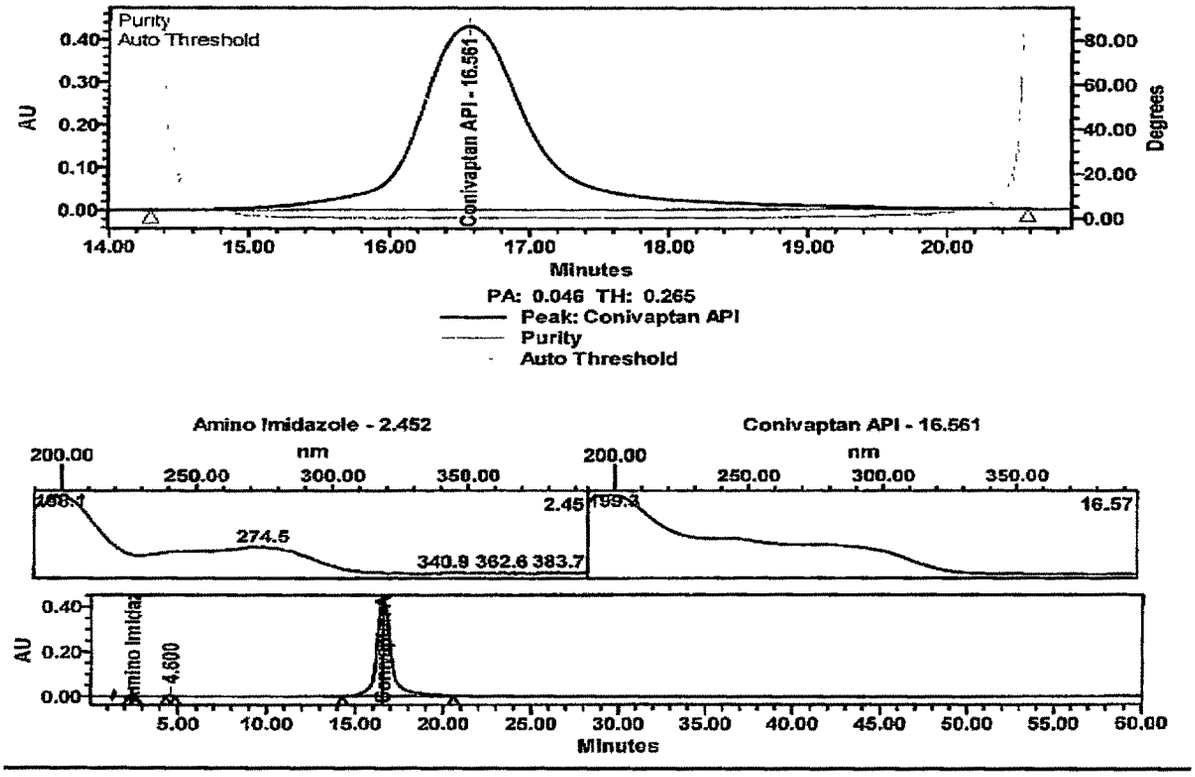
Chromatogram of Base degradation sample



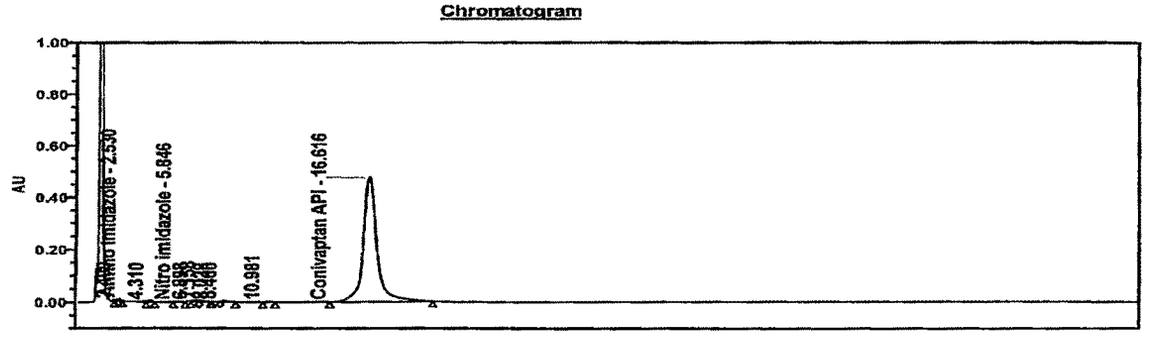
Peak Table

	Peak Name	RT	Area	% Area	RT Ratio
1	Amino Imidazole	2.45	42916	0.18	0.15
2		4.60	26035	0.11	
3	Conivaptan API	16.56	24374742	99.72	
Sum			24443693	100.00	

Peak purity of Base degradation sample



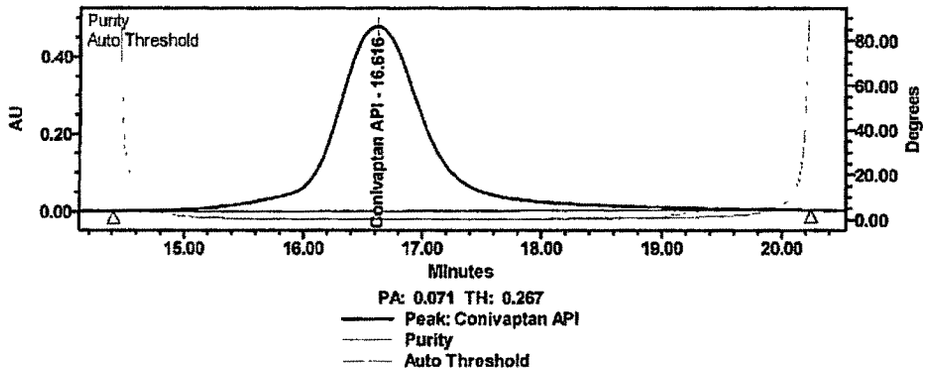
Chromatogram of Oxidative degradation sample

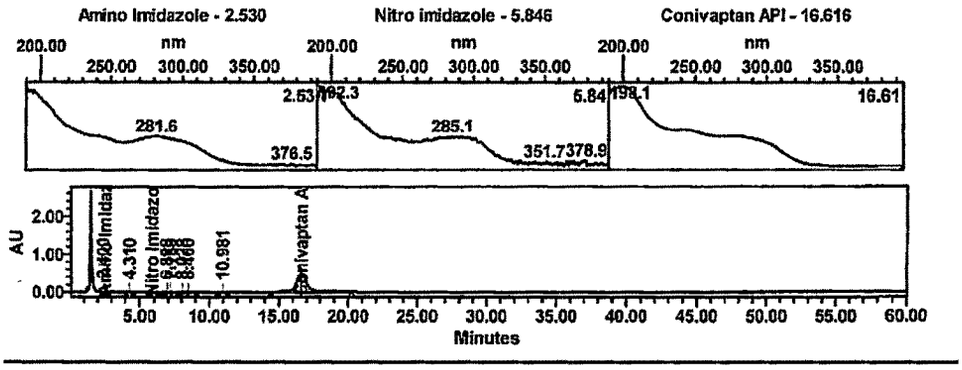


Peak Table

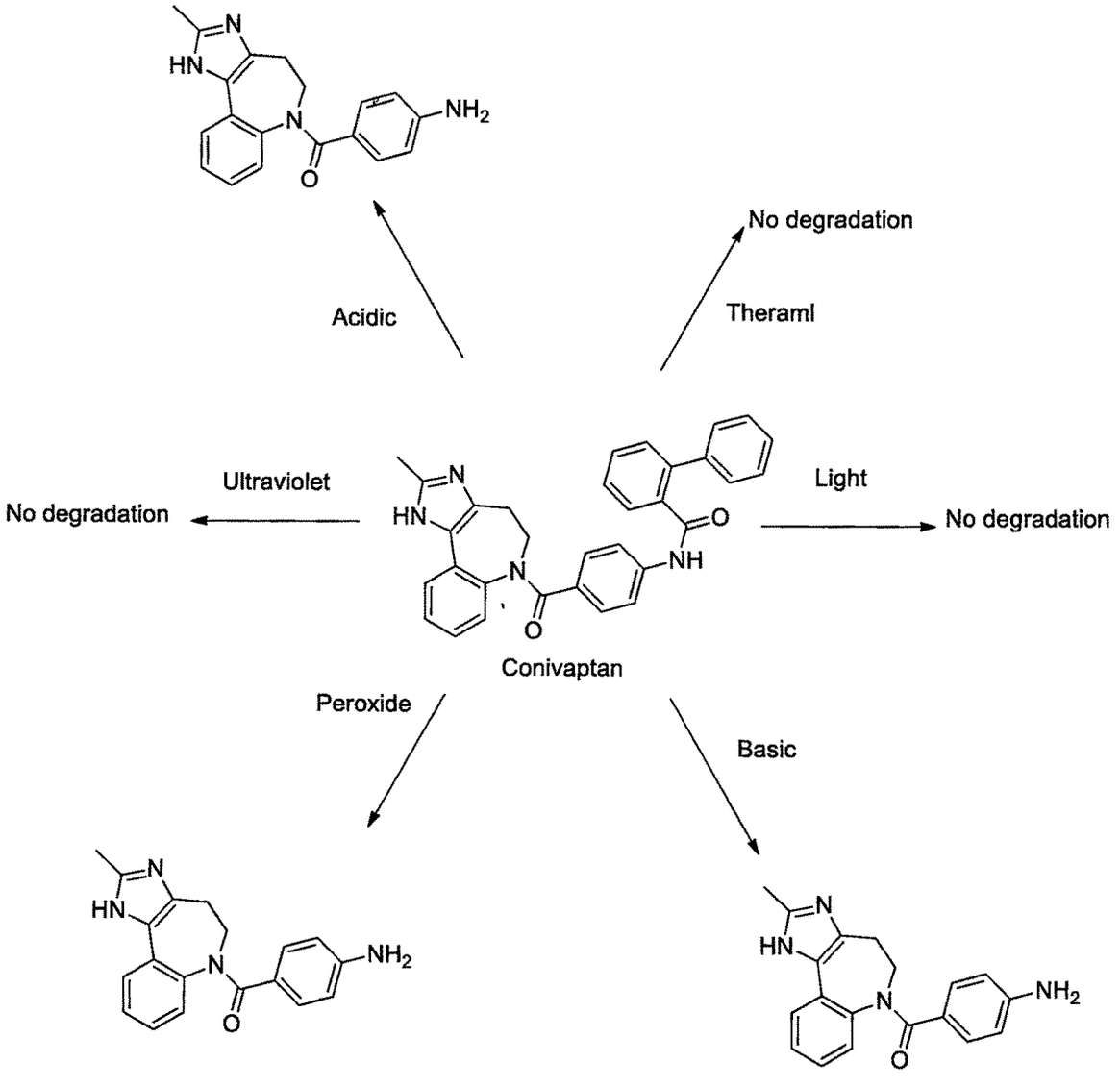
	Peak Name	RT	Area	% Area	RT Ratio
1		2.40	43572	0.16	
2	Amino Imidazole	2.53	48798	0.18	0.15
3		4.31	73372	0.28	
4	Nitro Imidazole	5.85	39722	0.15	0.35
5		6.90	128354	0.48	
6		7.16	119681	0.45	
7		8.03	36110	0.14	
8		8.46	107442	0.41	
9		10.98	20516	0.08	
10	Conivaptan API	16.62	25884224	97.67	
Sum			26501792	100.00	

Peak purity of Oxidative degradation sample





Conivaptan hydrochloride

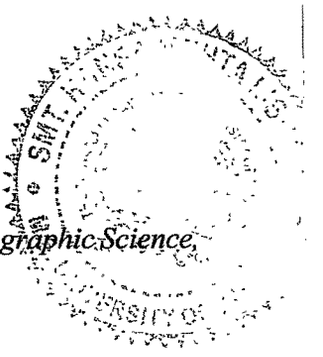


CONCLUSION

Based on the studies performed to study extensively the various degradation pathways we have observed that different impurities can be formed due to the stress effects on the Conivaptan HCl drug substance. All the stress conditions are generating the same impurities. The impurity isolations and characterizations of the same is not required in this product since the degradation impurities were nothing but the intermediates, which were available from the process of synthesizing the same and by using them in the spiking study, led to develop a better HPLC method which is a stability indicating method in order to monitor the related substances present in the drug substances. This method can be used at Quality Control laboratories which are required to test and release the Conivaptan HCl drug substance and drug products for human use.

The attempt should be made to minimize the impurities to the level of acceptable unknown levels in the drug substance and drug product. Other process and degradation impurities should be controlled by Pharmaceutical companies in order to get the drug approvals for marketing.

The main form of degradation is happening in the Acidic pH, basic pH and also under Oxidation conditions, so the development of the drug products or drug substance should consider controlling the pH of the samples during the manufacturing, shipment and storage of the drug substances and drug products and also control the pH of the solutions during manufacturing operations as a part of finished product manufacturing.



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