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A Commodious Synthesis, Modelling and Docking Studies of Strontium ranelate for the treatment of Osteoporosis

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Abstract: Post-menopausal Osteoporosis is the most common form of osteoporosis and usually affects women over the age of 60. It leads to bone loss as well as brittle bones that lead to breakage. Owing to this happening not only the quality of life is affected but also it causes the alarming rate of deaths. In other words, it paves a path for inevitable. If we ascertain the probable causes of such phenomena the following are, if the female have menopause; they have an eating disorder severe enough to stop their periods; they lost their height in the past 10 years; they used steroids more than 6 months; there was a family history of osteoporosis etc., this disease was discovered in time the condition can be treated with a variety of different drugs that are in the market, otherwise it is unreliable to cure the disease.

Keywords:
Post-menopausal Osteoporosis, Commodious Synthesis, Modeling, Docking, Strontium ranelate, Osteoporosis.

INTRODUCTION

1. Osteoporosis

1.1 Definition:

Osteoporosis is a condition where the bones become thin and weak, and break easily. It frequently goes undiagnosed until a fracture occurs, as there are no warning signs. The spine, wrist and hips are particularly vulnerable to fracture [1].

1.2 Symptoms

Occasionally a person develops a dowager hump or kyphosis at the top of their spine due to collapse of the vertebrae, and notices they have lost height. But mostly the thinning bones remain hidden away inside the body, with no symptoms or signs to alert someone to the fact that they have osteoporosis, until a minor bump or fall causes a bone fracture such as a broken hip or crushed vertebrae.

The break that results can cause pain, disability and loss of independence, or even prove fatal, especially when it leaves an older person immobilized. Only one in three people return to their level of function after breaking a hip and one in five will require long term nursing care.

1.3 Causes and risk factors

The bones are a living tissue, just like the rest of the body. They consist of cells which both build and break down bone, within a surrounding substance known as the extra cellular matrix which is composed of proteins and mineralized components (it's rather like scaffolding made of millions of tiny struts). The composition of the extra cellular matrix determines how strong the bone is, and higher the concentration of calcium, the greater the strength. Bone is constantly being broken down and rebuilt (this is known as remodeling, a process which maintains bone strength).

Bone reaches a peak of being most dense and strong around the third decade of life. From this point, bone mass slowly decreases. The greater the peak bone strength the longer it takes for bone mass to fall to dangerous levels. So it's vital that by early adult life a person has done everything possible to build their bones up with good nutrition and regular physical activity. Genetics also play a large part in determining an individual's peak bone strength.

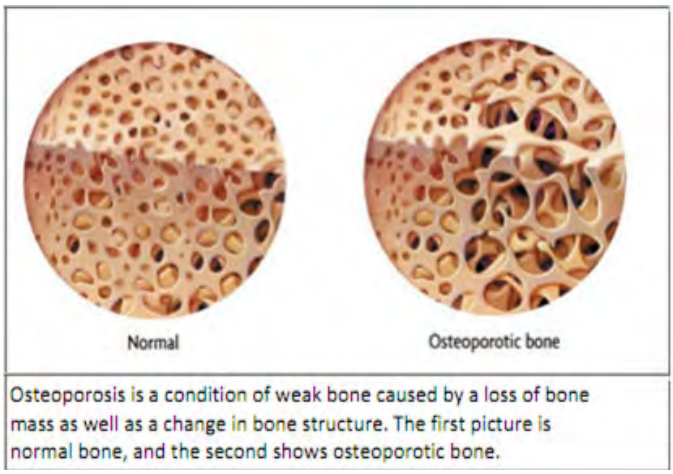
After the menopause, as estrogen levels fall, the rate at which bone is broken down increasingly outstrips new bone production. Bone mass falls even further, the bones thin and osteoporosis has developed.

For this reason osteoporosis mainly affects women after the age of the menopause, although men can develop it too. The risk is increased for a woman if she has an early menopause, has her ovaries removed before the menopause, or has gone for six months or more without periods for as a result of excessive exercising or dieting. For men low levels of testosterone increase the risk.

Strontium ranelate is recommended as an alternative treatment option for the primary prevention of osteoporotic fragility fractures in postmenopausal women:

- who are unable to comply with the special instructions for the administration of alendronate and either risedronate or etidronate, or have a contraindication to or are intolerant of alendronate and either risedronate or etidronate (as defined in section 1.7) and
- Who also have a combination of T-score, age and number of independent clinical risk factors for fracture (see section 1.5) as indicated in the following table?

Figure 1: Comparision of Normal bone & Osteoporosis bone.



Osteoporosis is a condition of weak bone caused by a loss of bone mass as well as a change in bone structure. The first picture is normal bone, and the second shows osteoporotic bone.

T-scores (SD) at (or below) which strontium ranelate is recommended when alendronate and either risedronate or etidronate cannot be taken

Table 1: T-Scores values in S.R treatment.

Age (years)	Number of independent clinical risk factors (section 1.5)		
	o	1	2
65-69	-a	-4.5	-4.0
70-74	-4.5	-4.0	-3.5
75 or older	-4.0	-4.0	-3.0

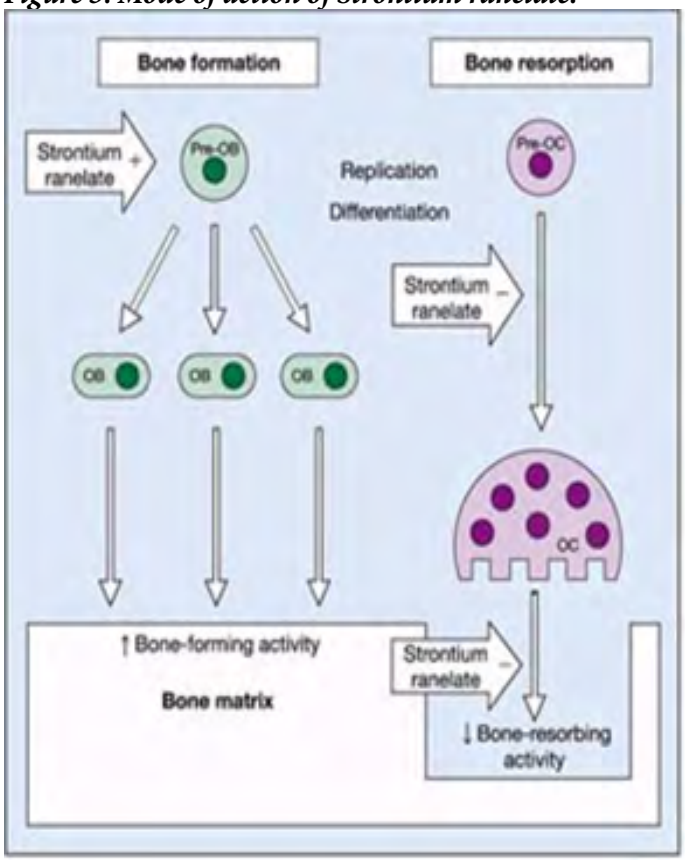
Strontium ranelate is a divalent strontium salt of ranelic acid. It is thought to have a dual effect on bone metabolism, increasing bone formation and decreasing bone resorption. It has a UK marketing authorisation for the treatment of postmenopausal osteoporosis to reduce the risk of vertebral and hip fractures.

The recommended dose is one 2g sachet taken daily as a suspension in water. The absorption of strontium ranelate is reduced by food, milk and products derived from milk. It should therefore be administered between meals, ideally at bedtime and preferably at least 2 hours after eating.

3.1 Dual Mode of Action of Strontium Ranelate:-

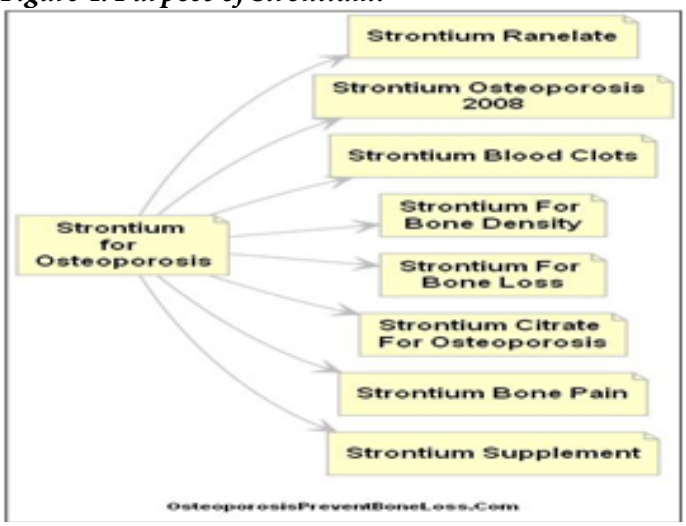
Strontium ranelate has a unique dual mode of action on bone metabolism as it appears to simultaneously increase bone formation and decrease bone resorption. S.R has been shown in vitro to increase bone formation by increasing the replication of pre-osteoblasts (Pre-OBs) into osteoblasts(OBs), leading to an increase in bone matrix synthesis. It has also been shown to decrease bone resorption by reducing the differentiation of pre-osteoclasts(Pre-OCs) into osteoclasts(OCs), and the bone resorbing activity of osteoclasts. The bone created with the use of Strontium ranelate is lamellar (ie, normal) and well mineralized. Bone strength is also increased as a result of improvements in the bone biomechanical properties.

Figure 3: Mode of action of Strontium ranelate.



3.2 Purpose of Strontium in this work:-

Figure 4: Purpose of Strontium.

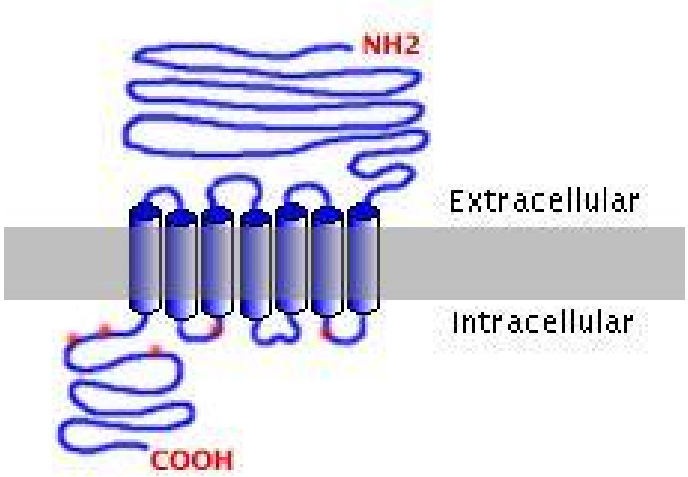


3.3 Significance of the Extra cellular Calcium-Sensing Receptor:-

Maintaining tight control over the concentration of calcium in blood and extracellular fluid is a critical task. It stands to reason that a calcium sensor would evolve as a component of the system responsible for calcium homeostasis. Considering its involvement in modulating so many physiologic processes, calcium itself can be thought of as a type of hormone, and the calcium sensor as its receptor.

The DNA sequence encoding the extracellular calcium sensor was originally isolated from bovine parathyroid gland. Since then, corresponding sequences have been isolated from a broad range of species, enabling serious study of this intriguing membrane protein.

Figure 5: Calcium Sensing Receptor.



The calcium-sensing receptor is a member of the G protein-coupled receptor family. Like other family members, it contains seven hydrophobic helices that anchor it in the plasma membrane. The large (~600 amino acids) extracellular domain is known to be critical to interactions with extracellular calcium. The receptor also has a rather large (~200 amino acids) cytosolic tail. These features are depicted in the figure 5; the red highlights on the intracellular domain correspond to potential protein kinase phosphorylation sites.

5. MATERIALS AND METHODS

5.1 Chemicals used in synthesis:

5.1.1 Active ingredients:-

Di methyl 3 oxoglutatrate, Malononitrile, Sulphur, Methyl bromo acetate, Strontium Hydroxide.

5.1.2 Catalysts:-

Cetrimide, Tri ethyl amine.

5.1.3 Bases:-

Tri ethyl amine, Potassium Carbonate, Lye solution.

5.1.4 Solvents:-

Methanol, Toluene, Acetonitrile, con HCl, Iso propyl alcohol, Distilled Water, De mineralized water.

5.1.5 Miscellaneous:-

Activated Carbon, Nitrogen Gas.

5.2 Glassware used in this work:

Single neck round bottom flask, triple neck round bottom flask, Four necks round bottom flask, Pipette, Stirrer rod, TLC Chamber, Test tubes, Capillary tubes, Condenser, Beaker, Iodine Chamber etc…

5.3 Instruments used in this work:

Stirrer, Condensing Chamber, HPLC, Analytical Balance, Electrical Balance, Agitator, Distillation apparatus, Hot air oven, Melting Range apparatus, Moisture Content Apparatus.

5.3.1 HPLC:

5.3.2 FTIR:

5.3.3 Mass Spectrometry:

5.4 DATABASES:

5.4.1 NCBI: -

The National Center for Biotechnology Information (NCBI) is part of the United States National Library of Medicine (NLM), a branch of the National Institutes of Health.

5.4.2 GenBank

The NCBI has had responsibility for making available the GenBank DNA sequence database since 1992. [a]

5.4.3 DRUG BANK:-

The DrugBank database available at the University of Alberta is a bioinformatics and cheminformatics resource that combines detailed drug (i.e. chemical, pharmacological and pharmaceutical) data with comprehensive drug target (i.e. sequence, structure, and pathway) information. [c]

5.4.4 SWISSPROT:-

UniProt is the Universal Protein resource, a central repository of protein data created by combining the Swiss-Prot, TrEMBL and PIR-PSD databases.

5.4.5 PDB:-

The Protein Data Bank (PDB) is a repository for the 3-D structural data of large biological molecules, such

as proteins and nucleic acids.

5.5 SOFTWARES AND SERVERS:

- Blast
- Homology modeling
- Template selection
- Alignment
- Model building
- Side chain modeling
- Verify 3D
- Ramachandran plot
- Energy minimization
- Force field
- CASTp
- ChemSketch
- Docking
- PK/DB

6. METHOD OF SYNTHESIS

Synthesis Procedure for the Strontium Ranelate

6.1 Step 1: Synthesis of methyl 5-amino-4-cyano-3-(2-methoxy-2-oxoethyl) thiophene-2-carboxylate (S.R-I): (CYCLIZATION)

In a clean and dried R.B flask Methanol (420 ml), Di methyl 3-Oxo Glutarate (1.72 moles) & Malononitrile (1.72 moles) were added at 25-350C under N2 atmosphere and stirred for 10 mins.The reaction mass was cooled to 100C and Cetrimide 0.024 moles was added and again stirred for 10 min at 100 C, slowly added 1 mole of TEA at 10-150C, stirred fro 30 min. Now the temperature was slowly raised for 25-300C, then to 65-700C and refluxed for 2hrs. The reaction mass cooled to 25-350C . 1.3 moles of sulphur was added, stirred for 10 min and the temperature was again raised to 65-700C for 2hrs. The progress of the reaction was checked by TLC, cooled the mass to 45-500C and stirred for 15 mins. Now gradually cooled the reaction mass to 25-350C then upto 100C and maintained for 30 mins. Filtered and washed with 50 ml of chilled DM H2O and dried the product at 60-650C. (Limit: Moisture content NMT 1%).

6.1.1 Purification of SR-I by Methanol:-

The above dried material was treated with Methanol (170ml) at 25-35oC, stirred for 15mins. Slowly raised the temperature up to reflux & maintained for 1hr. Cooled the mass to 25-35oC, then to 0-5oC and maintained for 1hr, filtered and washed with chilled Methanol (28.5 ml) and dried the product at 60-65oC. (Limit: Moisture content NMT 0.5%).

6.1.2 Removal of Moisture content of S.R-I by Toluene:

The above dried product was treated with Toluene (270ml) at 25-35oC, stirred for 15mins slowly raised the temperature upto reflux, maintained for 30mins at reflux temperature 110-112oC, Cooled the mass to 25-35oC then to 10-15oC, maintained for 1hr at 10-15oC. The material was filtered and washed with Toluene (50ml) and dried at 60-65oC (Limit: Moisture content NMT 0.5%).

6.2 Step 2: Synthesis of methyl 5-[bis (2-methoxy-2-oxoethyl) amino]-4-cyano-3-(2-methoxy-2-oxoethyl) thiophene-2-carboxylate (SR-II): (ESTERIFICATION)

In a clean and dry R.B flask K2CO3 (0.46 moles), Methyl bromo Acetate (2.13 moles) were added at 30-350C under the N2 atmosphere and stirred for 15 mins. Acetonitrile (326 ml) and Cetrimide (0.028 moles) were added one by one separately and stirred for 15 mins at 30-350C. Now slowly added S.R-I (0.79 moles) at 30-320C and stirred for 2 hours. Slowly raised the temperature upto reflux at 80-820C and stirred for 4 hours. The progress of the reaction was checked by the TLC [Limit: S.R-I NMT 0.2%]. Cooled the mass to 25-350C and removed the N2 atmosphere and filtered the reaction mixture. Collected the filtrate and kept aside and then washed the salts with Acetonitrile (25 ml) and collected the filtrate. Mixed the above two filtrates and distilled out the solvent under the vacuum at 80-850C for 1-2 hrs. The crude mass was cooled to 40-450C and added Methanol (200 ml) stirred for 15 mins at that temperature only. Gradually cooled the mass to 40-450C and then to 300C. Finally the temperature was maintained at 5-100C for 1 hour. Filtered the product (SR-II), washed with chilled Methanol (25 ml) and dried at 50-550C. (Limit: Moisture content NMT 0.5%).

1st Methanol slurry:

The above dried crude material of S.R-II was taken and Methanol (520ml) was added at 25-35oC, slowly raised the temperature upto reflux i.e.60-65oC till the material completely dissolves. Activated carbon (5.2gm) was added, refluxed at 60-65oC for 30mins, filtered the mass through hyflow bed, and washed the bed with Methanol (65ml). Methanol (423 ml) was distilled out from the above clear filtrate at 60-65oC. Cooled the mass to25-35oC, and observed the product formation. Again the mass was cooled to 5oC, maintained at 5-10oC for 1hr, filtered the material and washed with methanol (13ml). The product was dried at 50-55oC (Limit: Moisture content NMT 0.5%).

2nd Methanol slurry:

The above dried crude material of S.R-II was taken and Methanol (520ml) was added at 25-35oC,

slowly raised the temperature upto reflux i.e.60-65^{0}C till the material completely dissolves. Activated carbon (5.2gm) was added, refluxed at 60-65^{0}C for 30mins, filtered the mass through hyflow bed, and washed the bed with Methanol (65ml). Methanol (423 ml) was distilled out from the above clear filtrate at 60-65^{0}C. Cooled the mass to25-35^{0}C, and observed the product formation. Again the mass was cooled to 5^{0}C, maintained at 5-10^{0}C for 1hr, filtered the material and washed with methanol (13ml). The product was dried at 50-55^{0}C (Limit: Moisture content NMT 0.5%).

Toluene slurry:

The above dried crude material of S.R-II was taken and particle free Toluene (350 ml) was added & stirred for 10mins at 25-35^{0}C, slowly raised the temperature at 70-75^{0}C till the material completely dissolves. Now the mass was cooled to 25-35^{0}C, again the mass was cooled to 0^{0}C, maintained 1hr at 0-5^{0}C. Filtered the product and washed with chilled particle free Toluene (35ml) and the product was dried at 50-55^{0}C (Limit: Moisture content NMT 0.5%).

6.3 Step 3: Synthesis Procedure for the Strontium Ranelate (S.R)

(HYDROLYSIS & SALT FORMATION)

In a clean and dry R.B flask S.R-II (0.25 moles) and Methanol (100 ml) were added and stirred for 15 mins. Slowly added LYE solution (NaOH 10 gm +H2O 150 ml) and stirred for 1 hour at 35-370C. PH was adjusted to 1.5 – 2.0 by using Con-HCl. Now added activated carbon and stirred for 1 hour at 35±20C then filter the activated carbon under hyflow bed and washed with 10 ml H2O and collected the clear filtrate kept aside. ----------→ (A)

Strontium Hydroxide solution:

In a clean and dry R.B flask Sr (OH)2 (2.25 moles) and DM H2O (1:10 ratio) heat to reflux the reaction mixture at 95-1000C for ½ hour and filtered the reaction mixture to remove the undissolved matter and washed with DM H2O and collected the clean (B)

Take a clean and dry R.B flask added (B) solution, heated at 60-65^{0}C, added the (A) solution and slowly raised the temperature at 80-85^{0}C, stirred for 2 hrs. Now the reaction mixture was cooled to 70^{0}C and filtered the reaction mixture, washed with hot DM H_2O and suck dry.

1st Water slurry:

Added the above solid material in a clean R.B flask and added particle free DM H_2O (1: 3 ratio) heat to reflux at 80-85^{0}C and stirred for 2hrs. The reaction mixture was cooled to 70-75^{0}C and filtered the solution. Finally washed the product with hot DM H_2O.

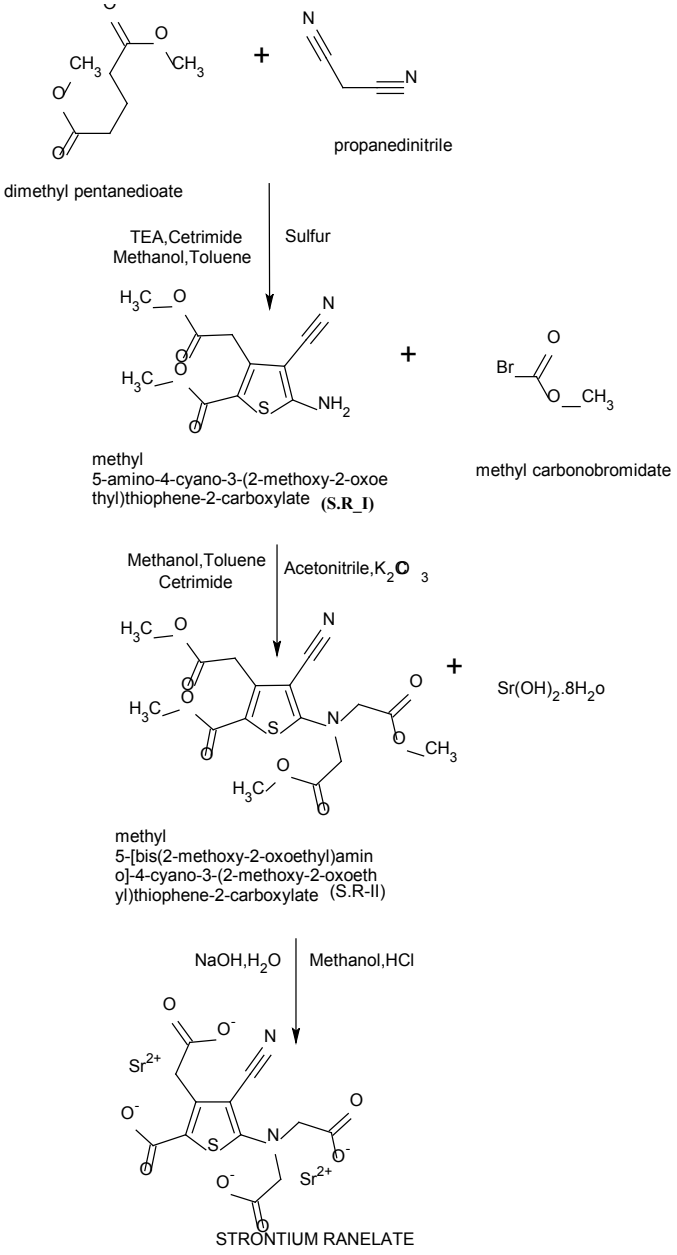
2nd Water slurry:

Added the above solid material in a clean and dry R.B flask and added particle free DM H2O (1: 3 ratio) heat to reflux at 80-85oC and stirred for 2hrs. The reaction mixture was cooled at 70-75oC and filtered the solution. Finally washed the product with hot DM H2O and suck dry.

IPA slurry:

Added the above solid material in a clean and dry R.B flask and added 20% IPA solution (80% IPA + 20% H2O) in 1: 3 ratios, now raised the temperature upto reflux, stirred for 2hrs at 80-85oC. Reaction mixture was cooled to room temperature and filters the solution, washed the solid with 20% IPA and suck dry.

6.4 REACTION SCHEME:



6.5 TEST PROCEDURE FOR THE DESCRIPTION

6.5.1 SOLUBILITY:

Take 1gm of sample in a 100ml glass beaker and added 30ml of 1N HCl and observed the solubility of the sample.

Take 0.1gm of sample in a 200ml glass beaker and added 100ml of H2O and observed the solubility of the sample.

6.5.2 IDENTIFICATION TEST:

The specification for the finished product at release and shelf life includes tests for characterization, average mass, uniformity of mass, PH, Identification(Organic part and strontium), assay(HPLC), degradation products assay of Strontium and dissolution. All tests included in the specification have been satisfactorily described and validated.

6.5.2.1 LOSS ON DRYING:

Dry the glass stopper bottle for 30minutes at 150oC, remove the bottle from the oven, and cool it in a desiccator. Weigh the bottle with stopper (W1). Transfer 1gm of sample, stopper it and weigh it accurately (W2).

Place the bottle with sample in a hot air oven at 150oC for 3 hours, remove the bottle from the oven keep it in a desiccator till becomes cool, weigh the bottle (W3), and check the loss on drying. Check Loss on drying twice for confirmation of the result. Calculate the percentage of loss on drying by given formula.

% Loss on drying = L x 100/W

Where:

L = Loss of weight in gm (W2-W3)

W= Weight of sample taken in gm (W2-W1)

6.5.3 Assay of Strontium Ranelate by HPLC:

Chromatographic conditions:

Column	: Inertsil ODS 3V 250mm X 4.6mm X 5µm or equivalent.
Column Temperature	: 25oC
Detection	: UV at 236nm.
Flow rate	: 0.8ml/min.
Injection Volume	: 10µl
Run time	: 50min.
Diluent	: Water

6.5.3.1 Preparation of mobile phase:

Preparation of the buffer solution:

Accurately about 6.81 gm of potassium di hydrogen phosphate was taken in 1000ml of water. Adjusted the PH to 3.0 ± 0.05 with phosphoric acid.

6.5.3.2 Preparation of mobile phase A:

Buffer and Methanol were taken in the ratio of 80: 20

6.5.3.3 Preparation of mobile phase B:

Buffer and Methanol were taken in the ratio of 25: 75

6.5.8.4 Gradient program:

Table 2: Gradient Program

Time (min)	% Mobile phase A	% Mobile phase B
0	90	10
15	25	75
25	10	90
30	10	90
35	25	75
38	25	75
45	90	10
50	90	10

6.5.3.5 Preparation of test sample solution:

Accurately about 10mg of test sample was weighed and taken in 20ml volumetric flask containing 10ml water, dissolved and diluted to the volume with same solvent.

6.5.3.6 Preparation of standard solution:

Accurately about 10mg of standard Strontium ranelate was weighed and taken in a 20 ml volumetric flask containing 10ml water, dissolved and diluted to the volume with same solvent.

6.5.3.7 Procedure:

Injected 10µl of water as blank for one time.
Injected 10µl of standard solution for six times.
Injected 10µl of test sample solution two times.

6.5.3.8 Acceptance criteria:

The RSD (%) of the area responses for six replicate injections should not be more than 2.0.

Result:

Calculate any impurity (known or unknown) by area normalization method.

$$\frac{\text{Avg area of sample X Wt of standard X (100- std LOD) X Wt assay}}{\text{Avg area of standard X Wt of sample X (100- sample LOD)}}$$

9. RESULTS AND DISCUSSION

Figure 8: S.R-I HPLC

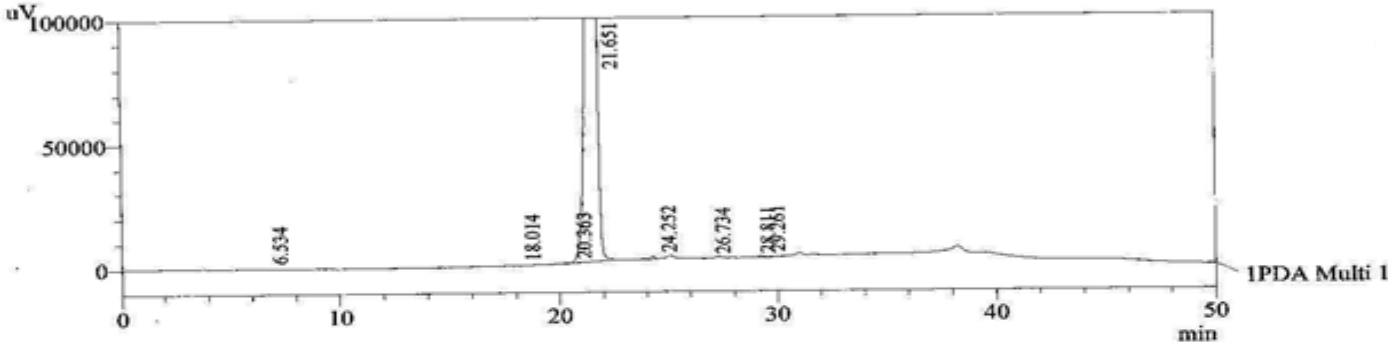
RA CHEM PHARMA LTD
HPLC REPORT

Sample Information

Sample Name	: SR-I
Sample ID	:20-29-08-10 Toluene purification
Tray#	:1
Vail#	:2
Injection Volume	: 10 uL
Data Filename	: 221210_SR-I_20-29-08-10 Toluene purification.lcd
Method Filename	: SR.Gr.m.lcm
Date Acquired	: 12/22/2010 12:54:07 PM

HPLC Chromatogram

D:\2010\DATA\SR\221210_SR-I_20-29-08-10 Toluene purification.lcd



1 PDA Multi 1 / 236nm 4nm

PeakTable

PDA Ch1 236nm 4nm

Peak#	Name	Ret. Time	Area	Height	Area %
1	RT6.534	6.534	1728	160	0.009
2	RT18.014	18.014	1675	172	0.009
3	RT20.363	20.363	1575	166	0.008
4	RT21.651	21.651	18698993	1265791	99.847
5	RT24.252	24.252	14805	1081	0.079
6	RT26.734	26.734	304	32	0.002
7	RT28.811	28.811	747	57	0.004
8	RT29.261	29.261	7908	520	0.042
Total			18727735	1267980	100.000

Figure 9 : S.R-II HPLC report.

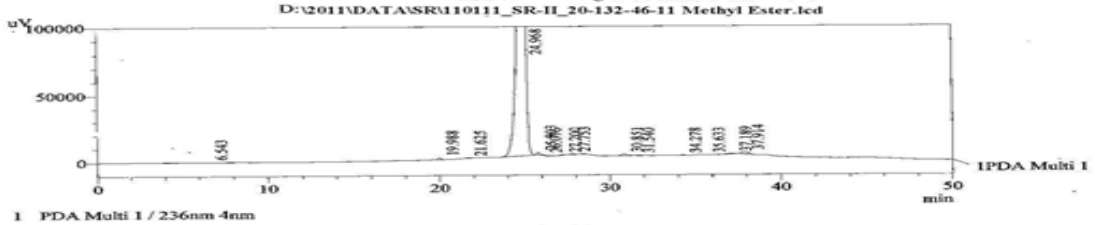
RA CHEM PHARMA LTD
HPLC REPORT

Sample Information

Sample Name	: SR-II
Sample ID	:20-132-46-11 Methyl Ester.
Tray#	: 1
Vail#	: 3
Injection Volume	: 10 uL
Data Filename	: 110111_SR-II_20-132-46-11 Methyl Ester.lcd
Method Filename	: SR.Gr.m.lcm
Date Acquired	:01/11/2011 1:45:38 PM

HPLC Chromatogram

D:\2011\DATA\SR\110111_SR-II_20-132-46-11 Methyl Ester.lcd



1 PDA Multi 1 / 236nm 4nm

PeakTable

PDA Ch1 236nm 4nm

Peak#	Name	Ret. Time	Area	Area %	ative Retention T
1	RT6.543	6.543	1528	0.009	0.26
2	RT19.988	19.988	12778	0.078	0.80
3	RT21.625	21.625	3232	0.020	0.87
4	RT24.968	24.968	16281082	99.123	1.00
5	RT25.803	25.803	19955	0.121	1.03
6	RT26.076	26.076	2440	0.015	1.04
7	RT27.200	27.200	6078	0.037	1.09
8	RT27.753	27.753	8111	0.049	1.11
9	RT30.851	30.851	17620	0.107	1.24
10	RT31.540	31.540	4767	0.029	1.26
11	RT34.278	34.278	4384	0.027	1.37
12	RT35.633	35.633	4196	0.026	1.43
13	RT37.189	37.189	13627	0.083	1.49
14	RT37.914	37.914	45331	0.276	1.52
Total			16425129	100.000	

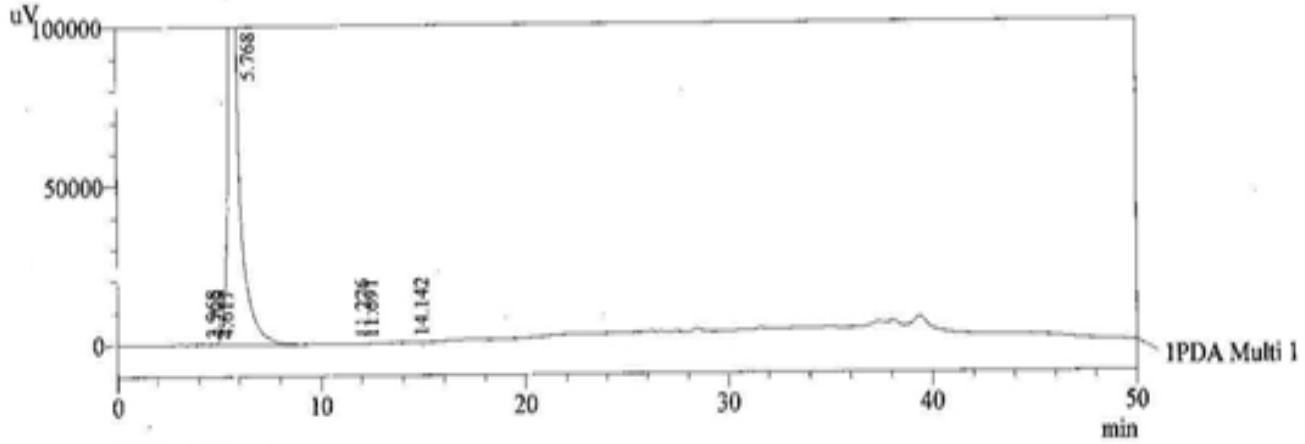
Figure 10: S.R HPLC report

RA CHEM PHARMA LTD
HPLC REPORT

Sample Information

Sample Name	: SR-III
Sample ID	:20-120-43-11 IPA Slurry
Tray#	: 1
Vail#	: 2
Injection Volume	: 10 uL
Data Filename	: 080311_SR-III_20-120-43-11 IPA Slurry.lcd
Method Filename	: SR.Gr.m.lcm
Date Acquired	: 03/08/2011 12:04:05 PM

HPLC Chromatogram
D:\2011\DATA\SR\080311_SR-III_20-120-43-11 IPA Slurry.lcd



1 PDA Multi 1 / 236nm 4nm

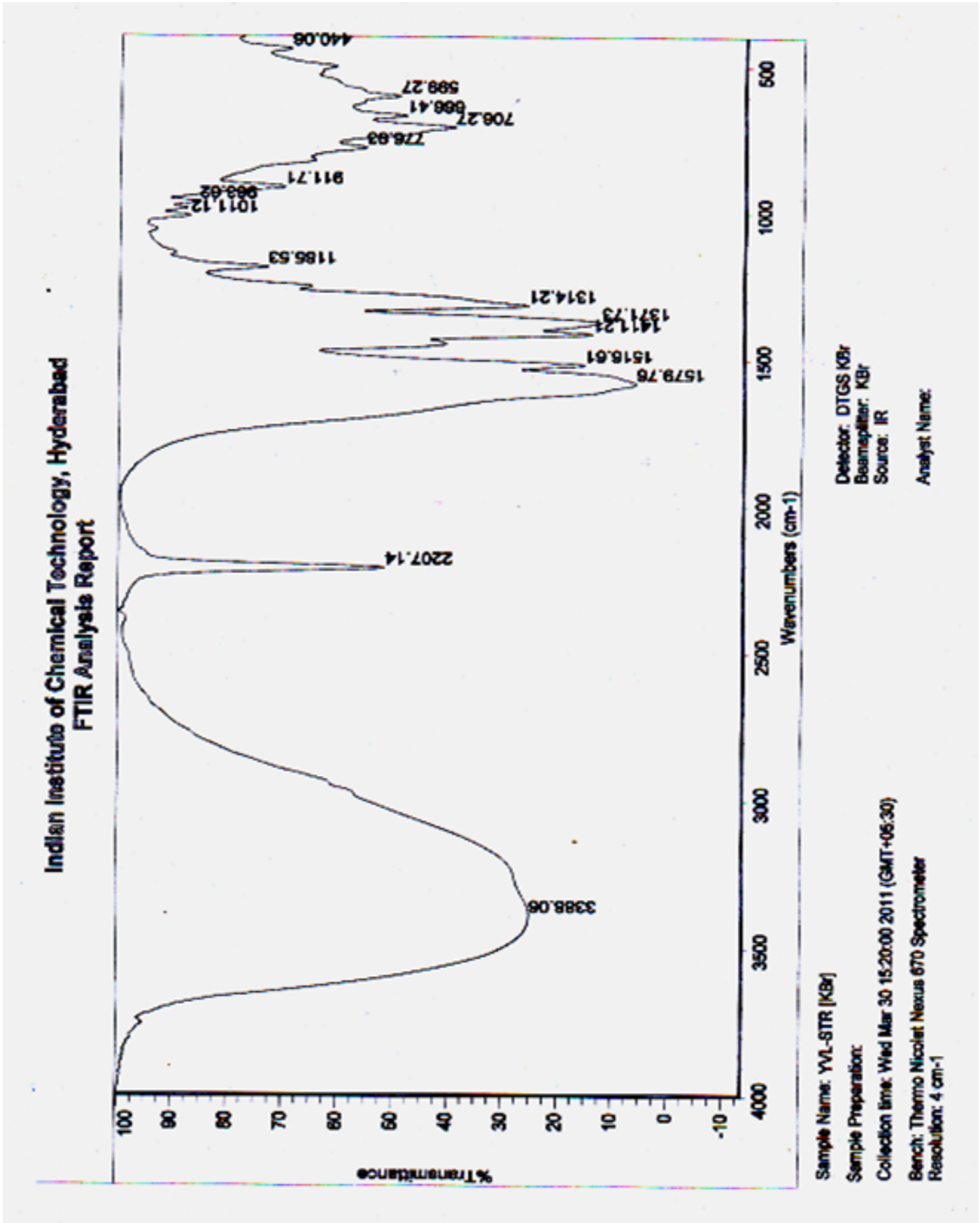
PeakTable

PDA Ch1 236nm 4nm

Peak#	Name	Ret. Time	Area	Area %	ative Retention T
1	RT3.968	3.968	2102	0.022	0.69
2	RT4.209	4.209	2523	0.026	0.73
3	RT4.617	4.617	2507	0.026	0.80
4	RT5.768	5.768	9723414	99.885	1.00
5	RT11.276	11.276	2368	0.024	1.95
6	RT11.691	11.691	838	0.009	2.03
7	RT14.142	14.142	846	0.009	2.45
Total			9734598	100.000	

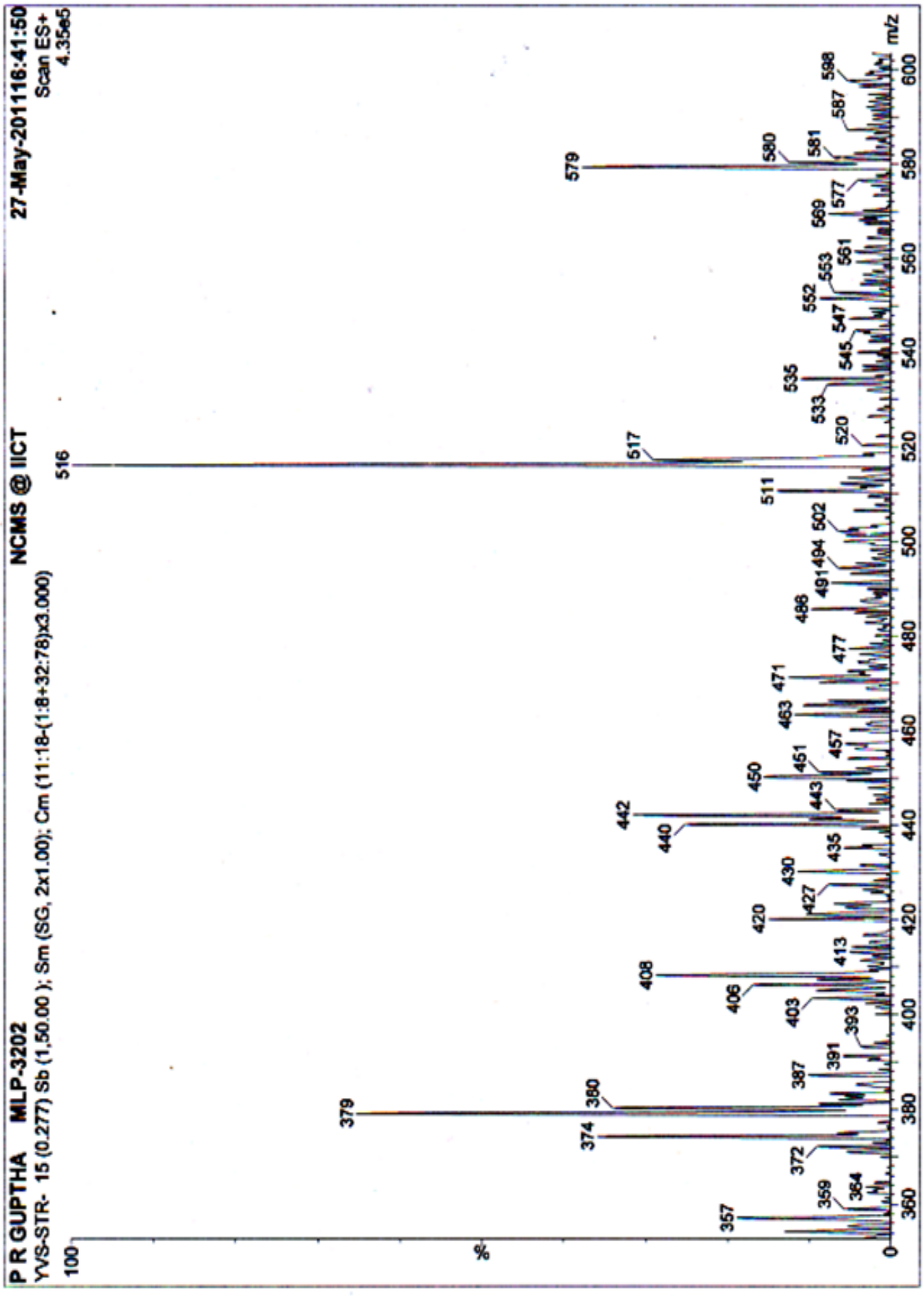
FTIR

Figure 11: FTIR report for the Strontium Ranelate.



MASS SPECTROMETRY:-

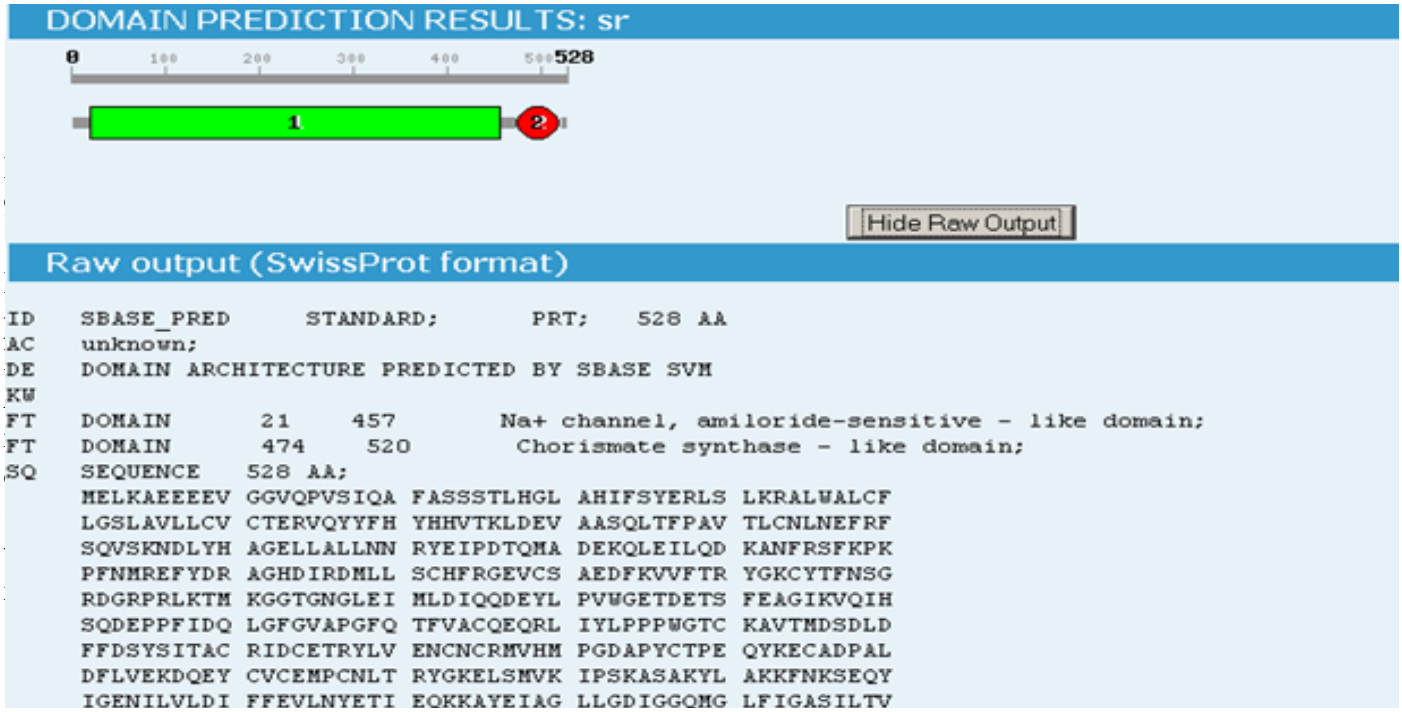
Figure 12: Mass Report for Strontium Ranelate.

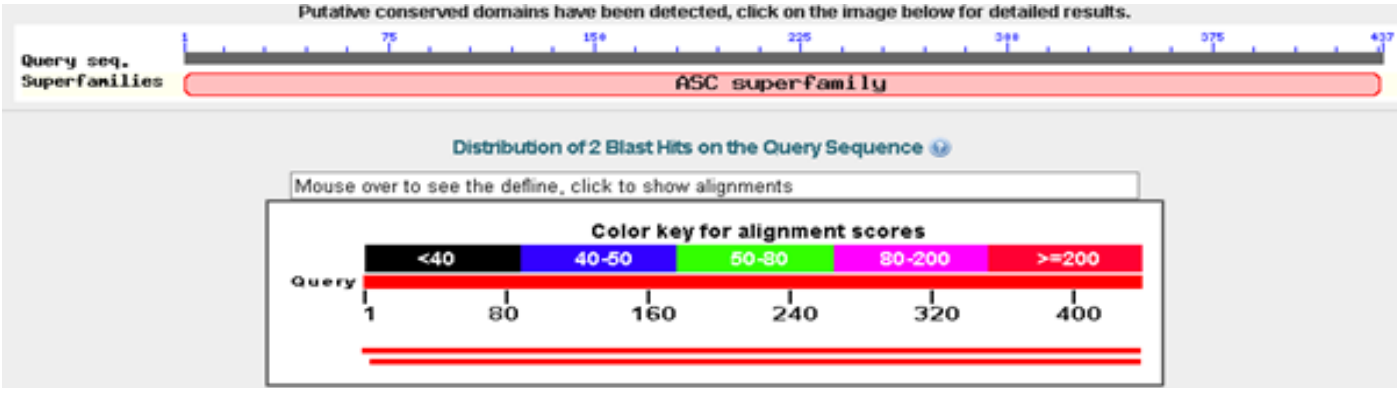


9.4 Homology Modeling of ASCC2N Protein (P78348)

A high level of sequence identity should guarantee more accurate alignment between the target sequence and template structure. In the results of BLAST search against PDB, only one-reference protein 3HGC chain A has a high level of sequence identity and the identity of the reference protein with the ASCC2N protein are 90%. Structurally conserved regions (SCRs) for the model and the template were determined by superimposition of the two structures and multiple sequence alignment.

Figure 13: identification of domain region by using SBASE server





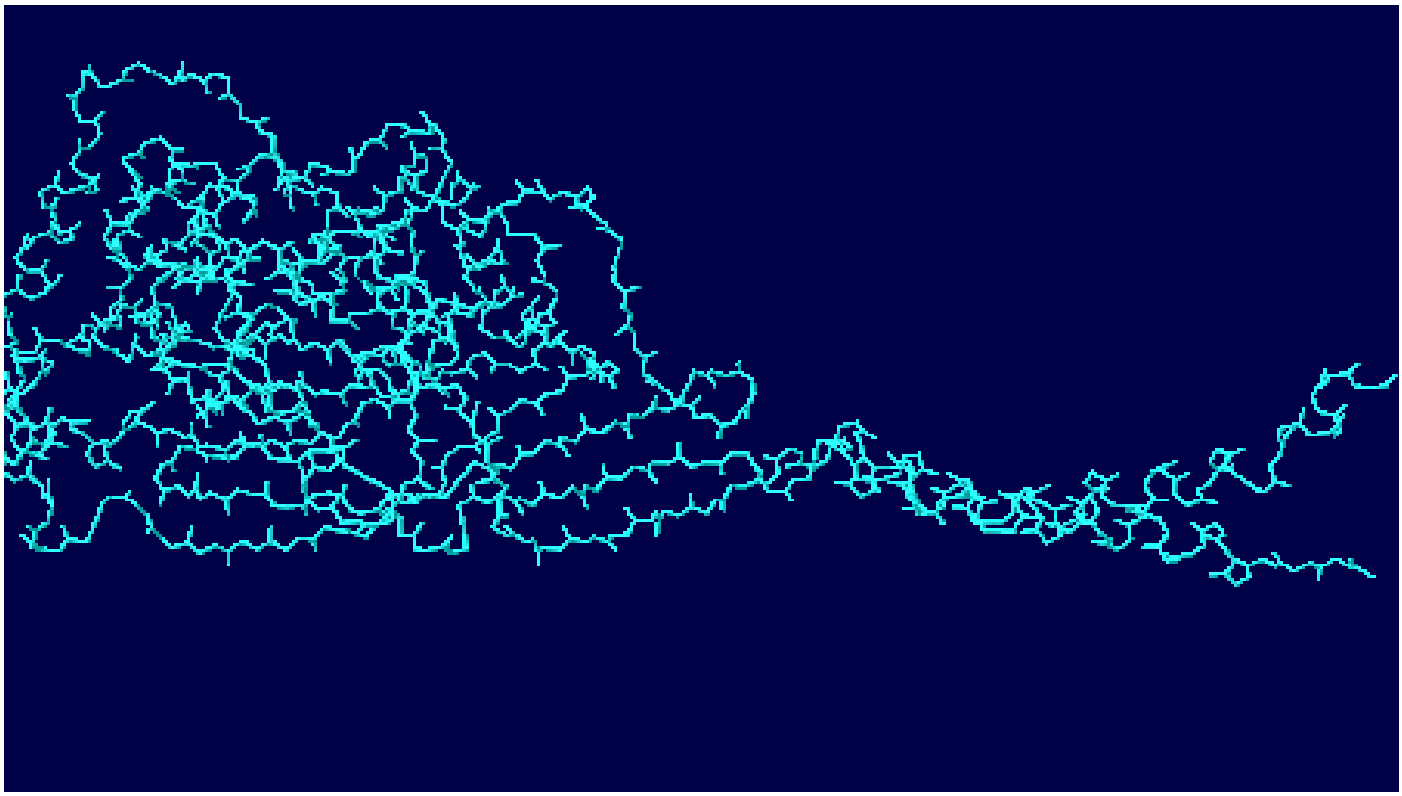
Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value
3HGC_A	Chain A, Crystal Structure Of A Functional Acid Sensing Ion	832	832	100%	0.0
2QTS_A	Chain A, Structure Of An Acid-Sensing Ion Channel 1 At 1.9	824	824	99%	0.0

Figure 14: Blast result with a similar template having 90% identity with ASCC2N.

```
>pdb|3HGC|A Chain A, Crystal Structure Of A Functional Acid Sensing Ion Channel
In The Desensitized State
 pdb|3IJ4|A Chain A, Cesium Sites In The Crystal Structure Of A Functional
Acid Sensing Ion Channel In The Desensitized State
Length=465

 Score =  832 bits (2148),  Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 394/437 (90%), Positives = 416/437 (95%), Gaps = 2/437 (0%)

Query  1    FASSSTLHGLAHIFSYERLSLKRALWALCFLGSLAVLLCVCTERVQYYFHYHHVTKLDEV  60
            FASSSTLHG++HIFSYERLSLKR +WALCF+GSLA+L  VCT R+QYYF Y HVTKLDEV
Sbjct  21   FASSSTLHGISHIFSYERLSLKRVVWALCFMGSLALLALVCTNRIQYYFLYPHVTKLDEV  80

Query  61   AASQLTFPAVTLCNLNEFRFSQVSKNDLYHAGELLALLNNRYEIPDTQMADEKQLEILQD  120
            AA++LTFPAVT CNLNEFRFS+V+KNDLYHAGELLALLNNRYEIPDTQ ADEKQLEILQD
Sbjct  81   AATRLTFPAVTFCNLNEFRFSRVTKNDLYHAGELLALLNNRYEIPDTQTADEKQLEILQD  140

Query  121  KANFRSFKPKPFNMREFYDRAGHDIRDMLLSCHFRGEVCSAEDFKVVFTRYGKCYTFNSG  180
            KANFR+FKPKPFNM EFYDRAGHDIR+MLLSC FRGE CS EDFKVVFTRYGKCYTFN+G
Sbjct  141  KANFRNFKPKPFNMLEFYDRAGHDIREMLLSCFFRGEQCSPEDFKVVFTRYGKCYTFNAG  200

Query  181  RDGRPRLKTMKGGTGNGLEIMLDIQQDEYLPVWGETDETSFEAGIKVQIHSQDEPPFIDQ  240
            +DG+PRL TMKGGTGNGLEIMLDIQQDEYLPVWGETDETSFEAGIKVQIHSQDEPP IDQ
Sbjct  201  QDGKPRLITMKGGTGNGLEIMLDIQQDEYLPVWGETDETSFEAGIKVQIHSQDEPPLIDQ  260

Query  241  LGFGVAPGFQTFVACQEQRLIYLPPPWGTCKAVTMDSDLDFFDSYSITACRIDCETRYLV  300
            LGFGVAPGFQTFV+CQEQRLIYLPPPWG CKA T DS  +F+D+YSITACRIDCETRYLV
Sbjct  261  LGFGVAPGFQTFVSCQEQRLIYLPPPWGDCKATTGDS--EFYDTYSITACRIDCETRYLV  318

Query  301  ENCNCRMVHMPGDAPYCTPEQYKECADPALDFLVEKDQEYCVCEMPCNLTRYGKELSMVK  360
            ENCNCRMVHMPGDAPYCTPEQYKECADPALDFLVEKD EYCVCEMPCN+TRYGKELSMVK
Sbjct  319  ENCNCRMVHMPGDAPYCTPEQYKECADPALDFLVEKDNEYCVCEMPCNVTRYGKELSMVK  378

Query  361  IPSKASAKYLAKKFNKSEQYIGENILVLDIFFEVLNYETIEQKKAYEIAGLLGDIGGQMG  420
            IPSKASAKYLAKK+NKSEQYIGENILVLDIFFE LNYETIEQKKAYE+AGLLGDIGGQMG
Sbjct  379  IPSKASAKYLAKKYNKSEQYIGENILVLDIFFEALNYETIEQKKAYEVAGLLGDIGGQMG  438

Query  421  LFIGASILTVLELFDYA  437
            LFIGASILTVLELFDYA
Sbjct  439  LFIGASILTVLELFDYA  455
```

Figure 15. The stable structure of the 3HGC chain A protein obtained is shown.



In the following study, we have chosen 3HGC chain A as a reference structure for modeling ASCC2N domain. Coordinates from the reference protein (3HGC chain A) to the SCRs, structurally variable regions (SVRs), N-termini and C-termini were assigned to the target sequence based on the satisfaction of spatial restraints.

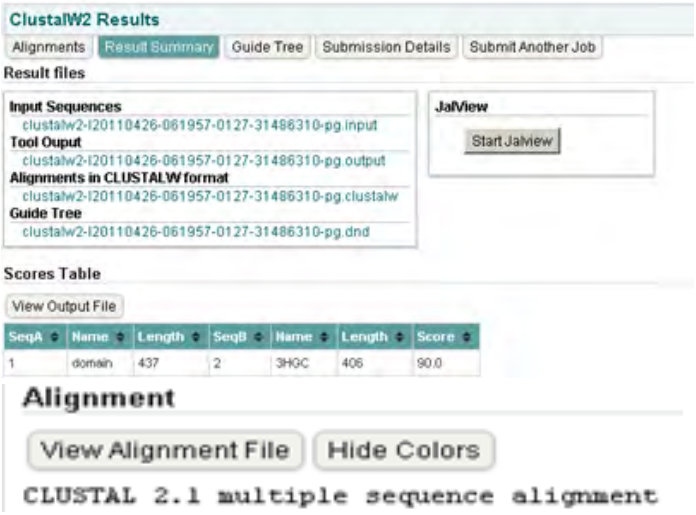
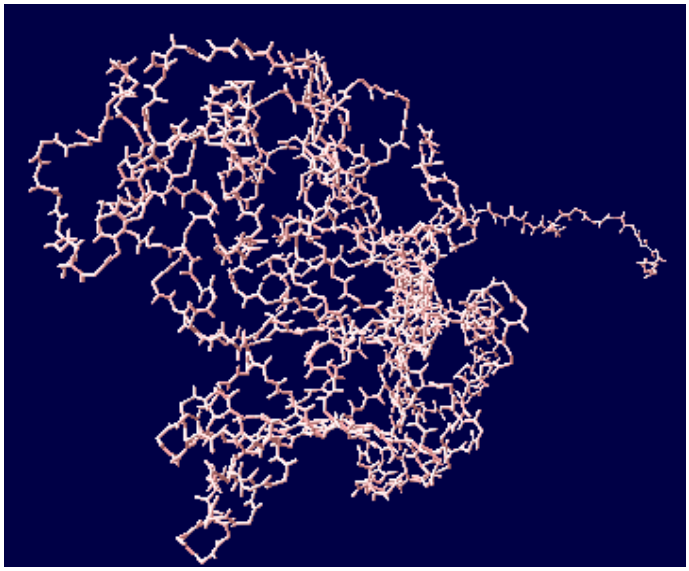


Figure 17: 3D structure of ASCC2N generated by Modeller9V8



By the help of SPDBV it is evident that Amiloride sensing Cation channel 2, neuronal has 11 helices and 15 sheets and it is shown in the Figure 6.

```
domain          FASSSTLHGLAHIFSYERLSLKRALWALCFLGSLAVLLCVCTERVQYYFHYHHVTKLDEV 60
3HGC            ------------------------VWALCFMGSLALLALVCTNRIQYYFLYPHVTKLDEV 36
                                         :*****:****:*  ***:*:**** * ********

domain          AASQLTFPAVTLCNLNEFRFSQVSKNDLYHAGELLALLNNRYEIPDTQMADEKQLEILQD 120
3HGC            AATRLTFPAVTFCNLNEFRFSRVTKNDLYHAGELLALLNNRYEIPDTQTADEKQLEILQD 96
                **::********:*********:*:************************ ***********

domain          KANFRSFKPKPFNMREFYDRAGHDIRDMLLSCHFRGEVCSAEDFKVVFTRYGKCYTFNSG 180
3HGC            KANFRNFKPKPFNMLEFYDRAGHDIREMLLSCFFRGEQCSPEDFKVVFTRYGKCYTFNAG 156
                *****.******** ***********:*****.**** **.*****************:*

domain          RDGRPRLKTMKGGTGNGLEIMLDIQQDEYLPVWGETDETSFEAGIKVQIHSQDEPPFIDQ 240
3HGC            QDGKPRLITMKGGTGNGLEIMLDIQQDEYLPVWGETDETSFEAGIKVQIHSQDEPPLIDQ 216
                :**:*** **************************************************:***

domain          LGFGVAPGFQTFVACQEQRLIYLPPPWGTCKAVTMDSDLDFFDSYSITACRIDCETRYLV 300
3HGC            LGFGVAPGFQTFVSCQEQRLIYLPPPWGDCKATTGDS--EFYDTYSITACRIDCETRYLV 274
                ************:************ ***.* **  :*:*:**************** 

domain          ENCNCRMVHMPGDAPYCTPEQYKECADPALDFLVEKDQEYCVCEMPCNLTRYGKELSMVK 360
3HGC            ENCNCRMVHMPGDAPYCTPEQYKECADPALDFLVEKDNEYCVCEMPCNVTRYGKELSMVK 334
                ************************************:**********:**********

domain          IPSKASAKYLAKKFNKSEQYIGENILVLDIFFEVLNYETIEQKKAYEIAGLLGDIGGQMG 420
3HGC            IPSKASAKYLAKKYNKSEQYIGENILVLDIFFEALNYETIEQKKAYEVAGLLGDIGGQMG 394
                ************:******************.************:**********

domain          LFIGASILTVLELFDYA 437
3HGC            LFIGASILTVLE----- 406
```

Figure 16: alignment of ASCC2N with template 3HGCchain A

In the modeler we will get a 20 PDB out of which we select a least energy .The energy unit will be in kilo joule .All side chains of the model protein were set by rotamers. The final stable structure of the Amiloride sensing Cation channel 2, neuronal protein obtained is shown in Figure 5.

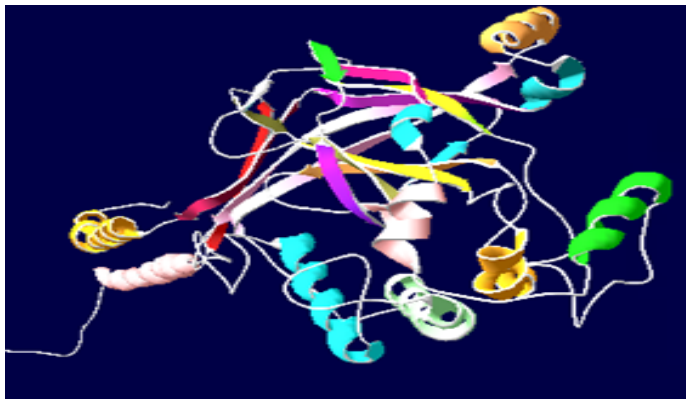


Figure 18: 3D structure of ASCC2N Protein with helices and sheets

The structure having the least energy with low RMSD (Root Mean Square Deviation) which was obtained by the NAMD is in water molecule (TIP3).

Figure 19: ASCC2N with water molecule (TIP3)

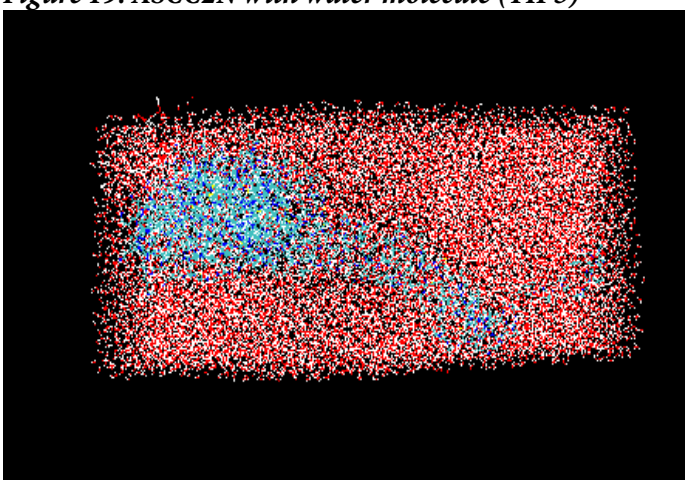
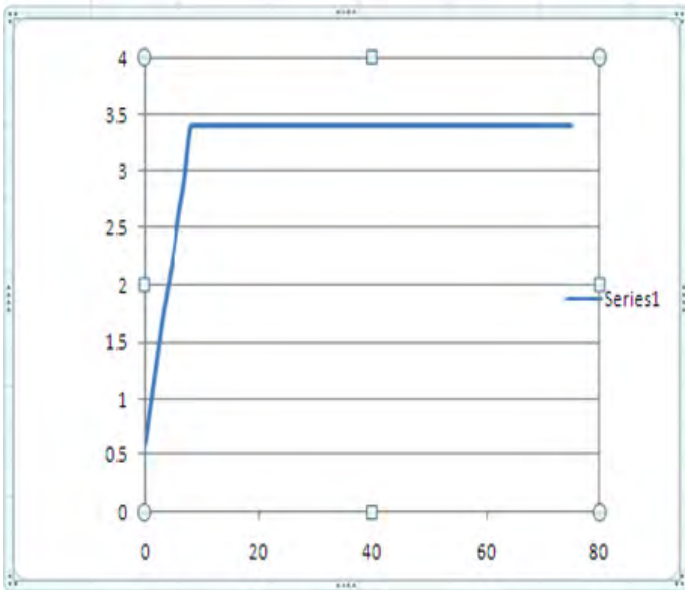
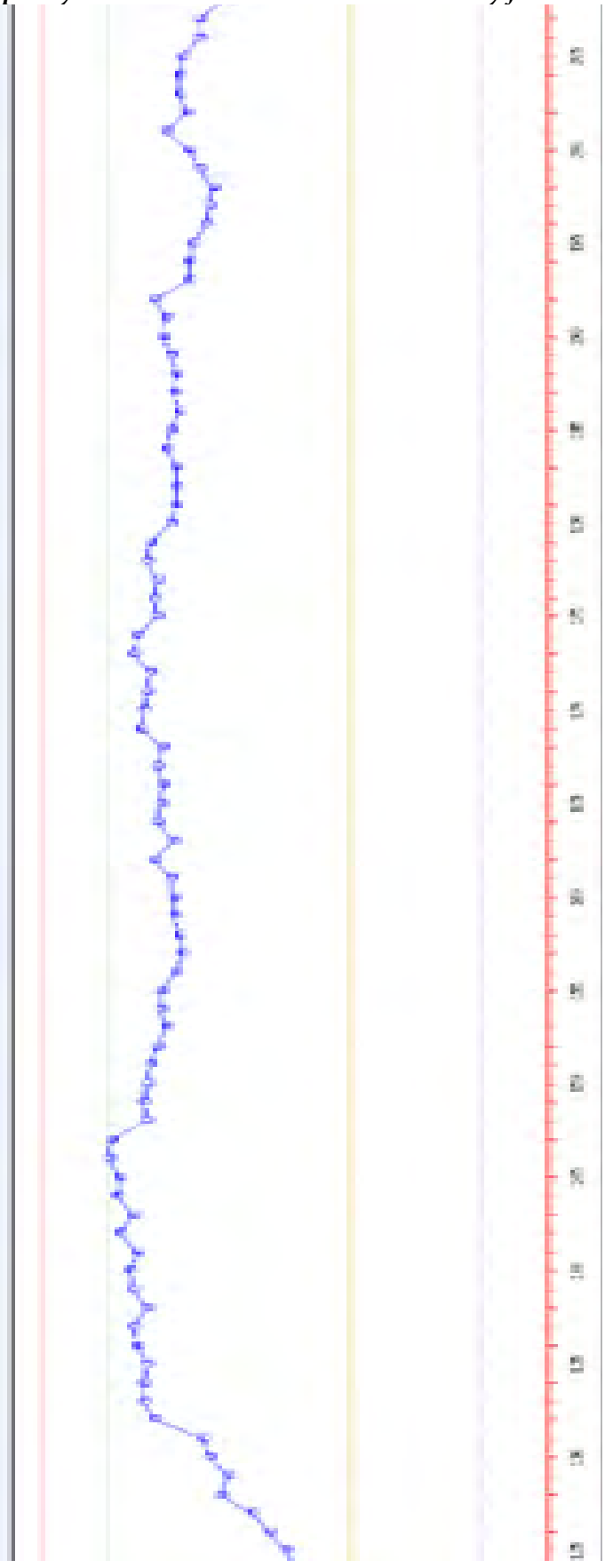


Figure 20: RMSD values



The final structure was further checked by verify 3D graph and the results have been shown in Figure 8. The overall scores indicates acceptable protein environment.

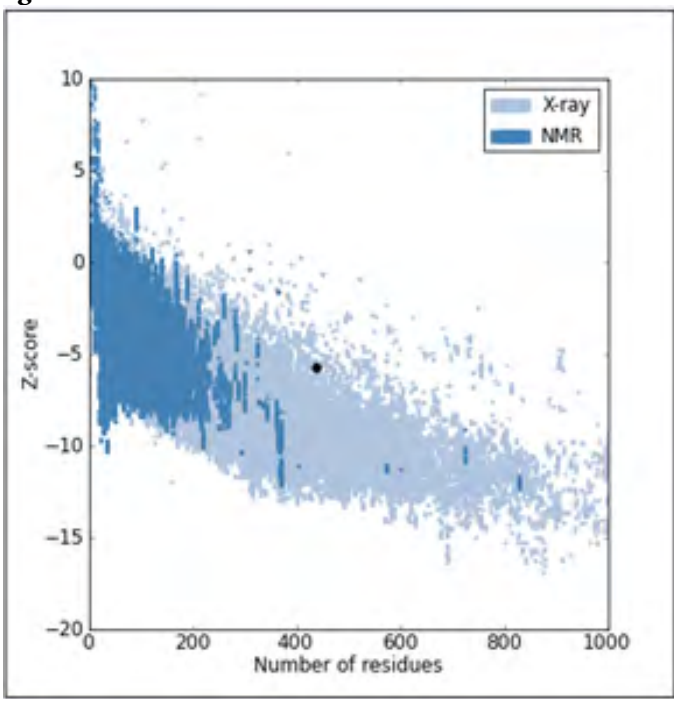
Figure 21: The 3D profiles verified results of Amiloride sensing Cation channel 2, neuronal model; overall quality score indicates residues are reasonably folded.



Validation of Protein

After the refinement process, validation of the model was carried out using Ramachandran plot calculations computed with the PROCHECK program. The π and ψ distributions of the Ramachandran plots of non-glycine, non-proline residues are summarized in Table 1. Altogether 70.6% of the residues of Amiloride sensing Cation channel 2, neuronal were in favored and allowed regions. The overall PROCHECK G-factor of ASCC2N and verify3D environment profile were good.

Figure 22: Prosa results Z score value -5.68



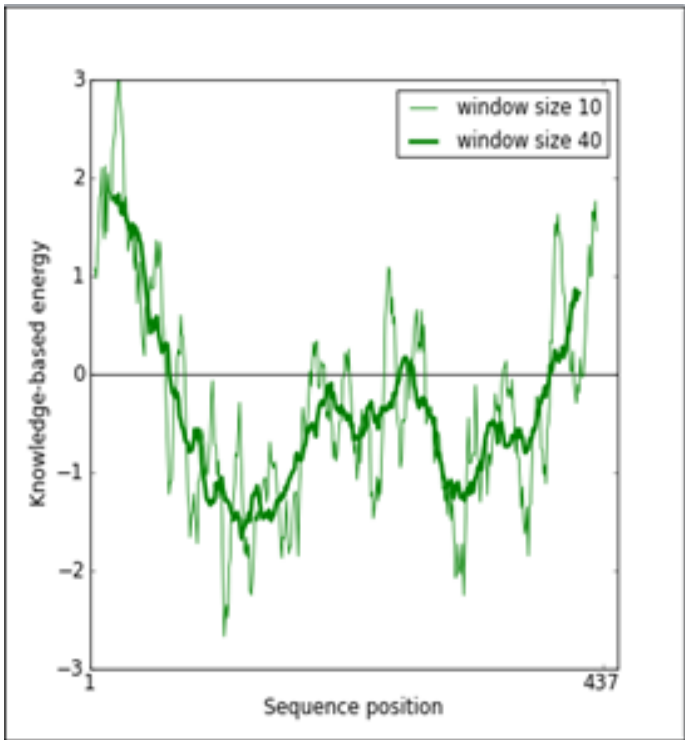
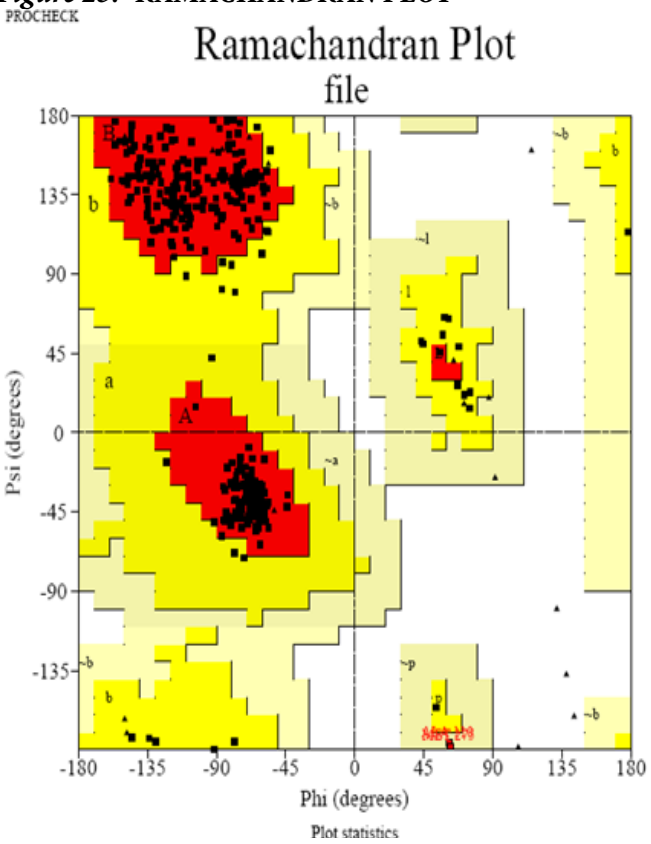


Figure 23:- RAMACHANDRAN PLOT



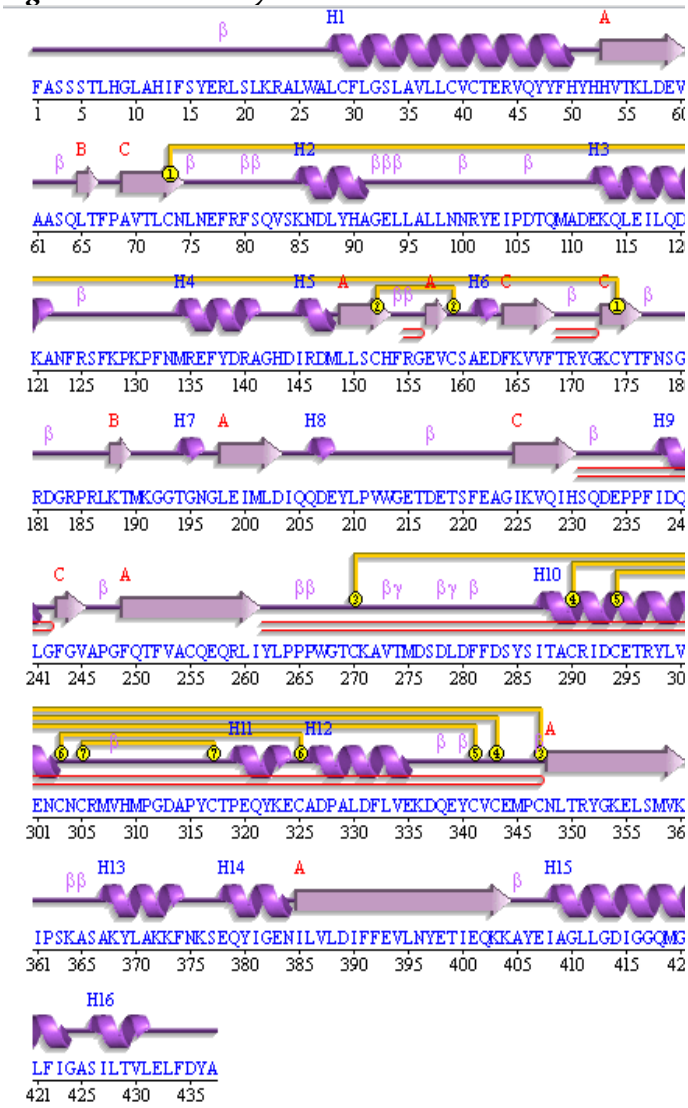
Plot statistics

Residues in most favoured regions [A,B,L]	357	91.5%
Residues in additional allowed regions [a,b,l,p]	31	7.9%
Residues in generously allowed regions [~a,~b,~l,~p]	2	0.5%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	390	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	27	
Number of proline residues	18	
Total number of residues	437	

Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.

SECONDARY STRUCTURE:-

Figure 24:- Secondary Structure



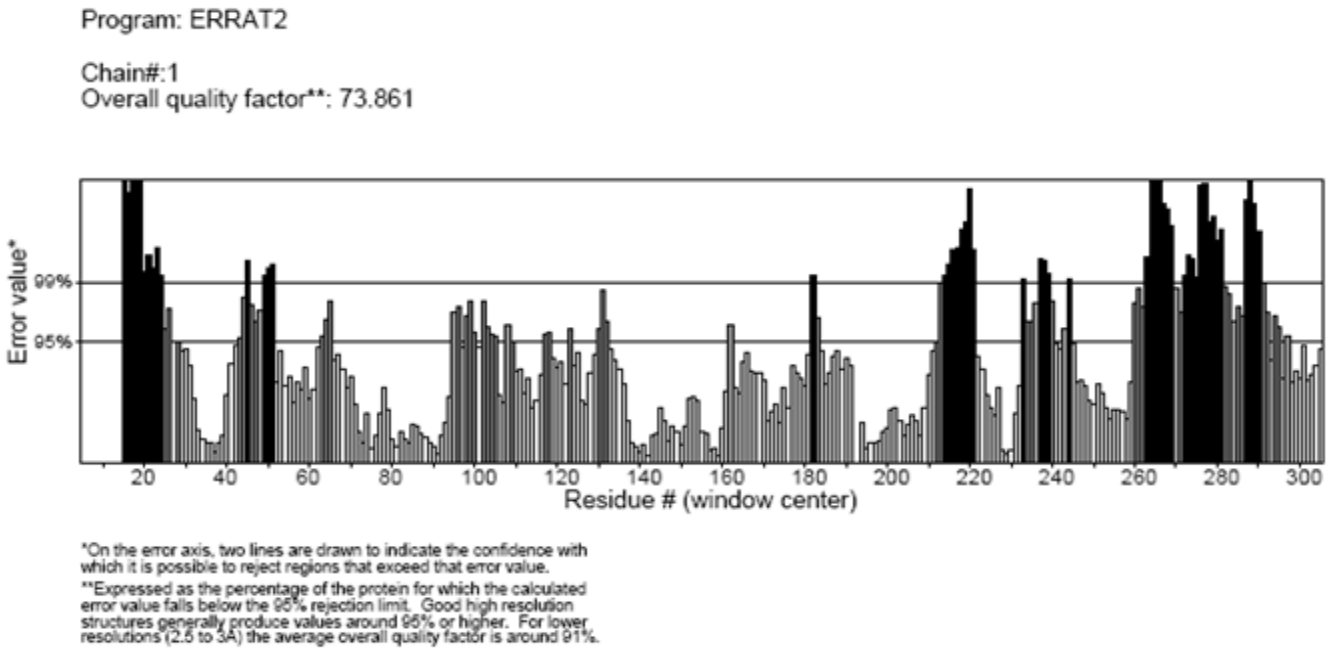
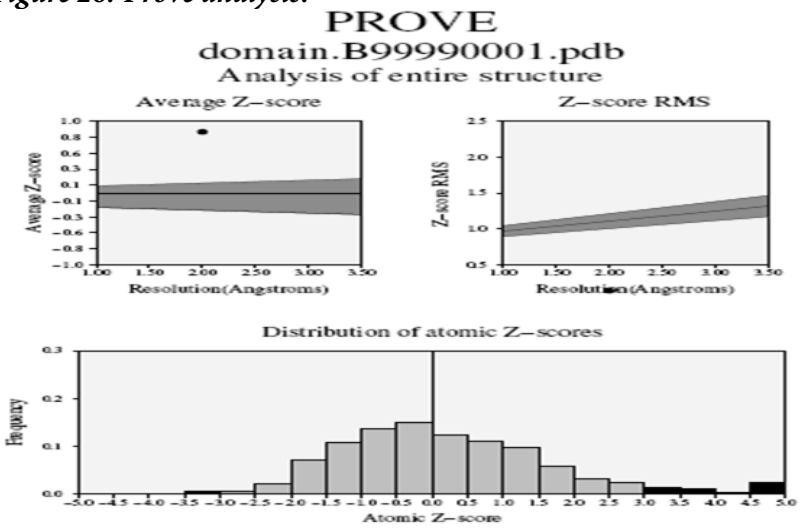


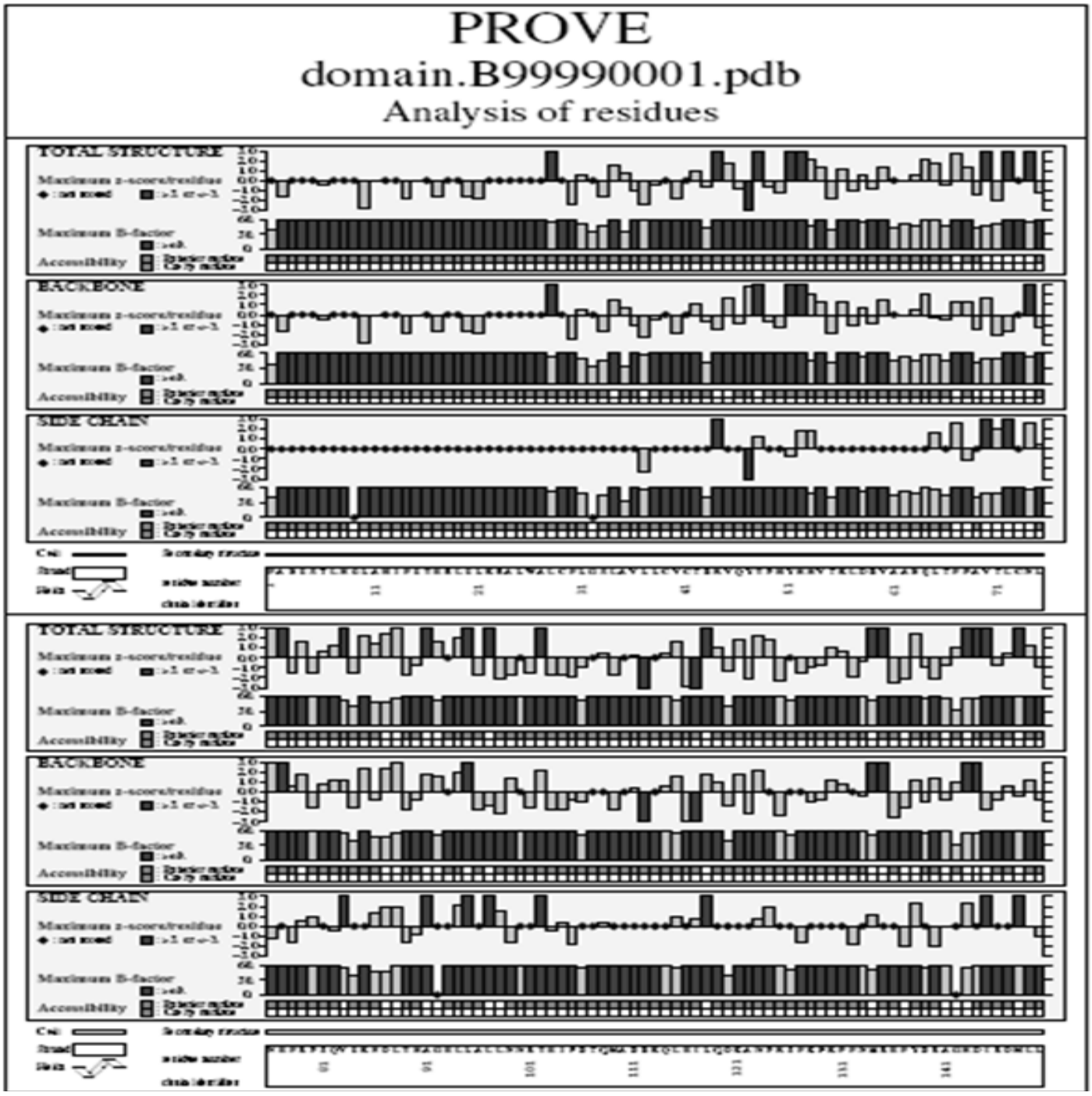
Figure 25:- Errat prediction.

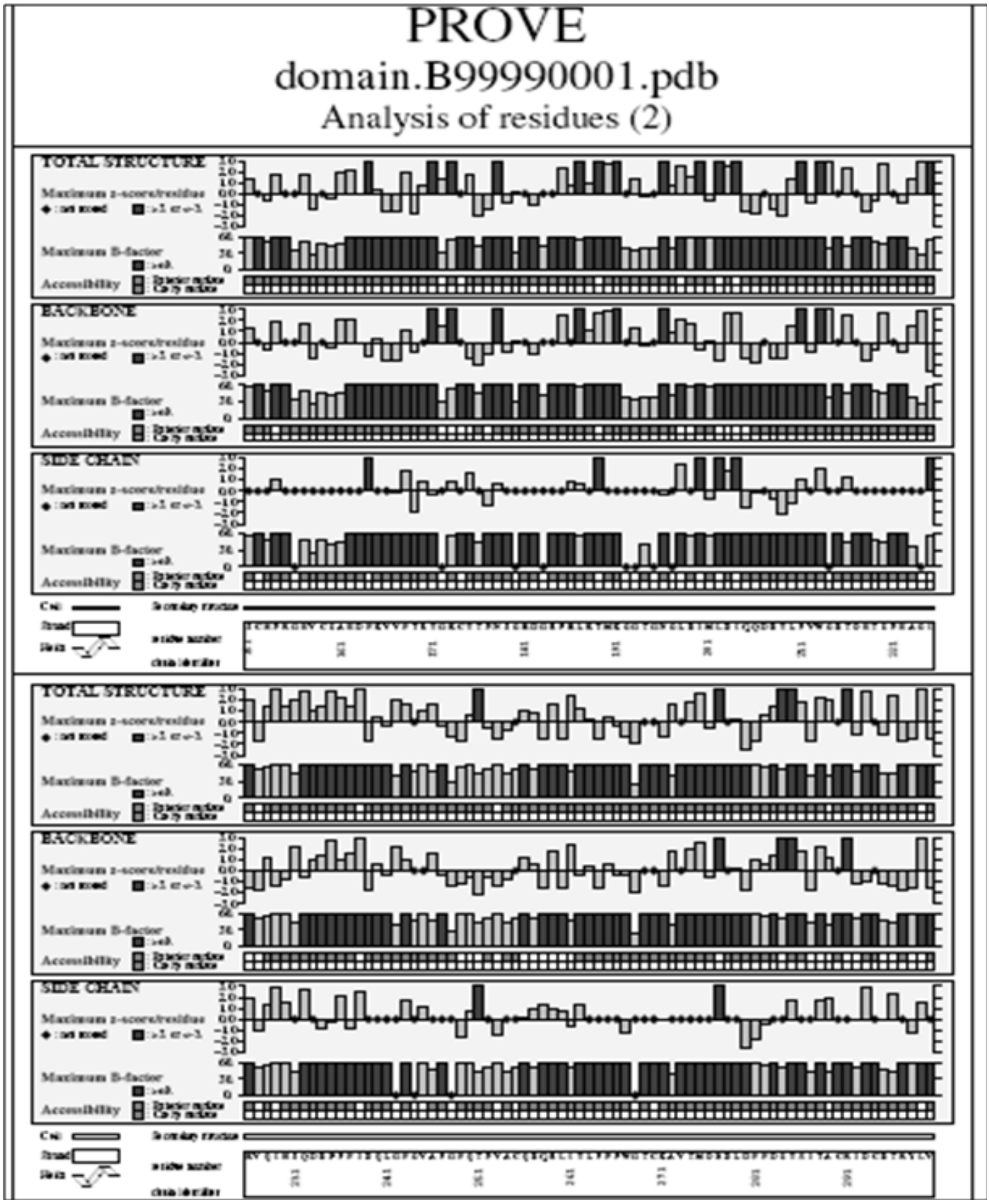
Figure 26:-Prove analysis.

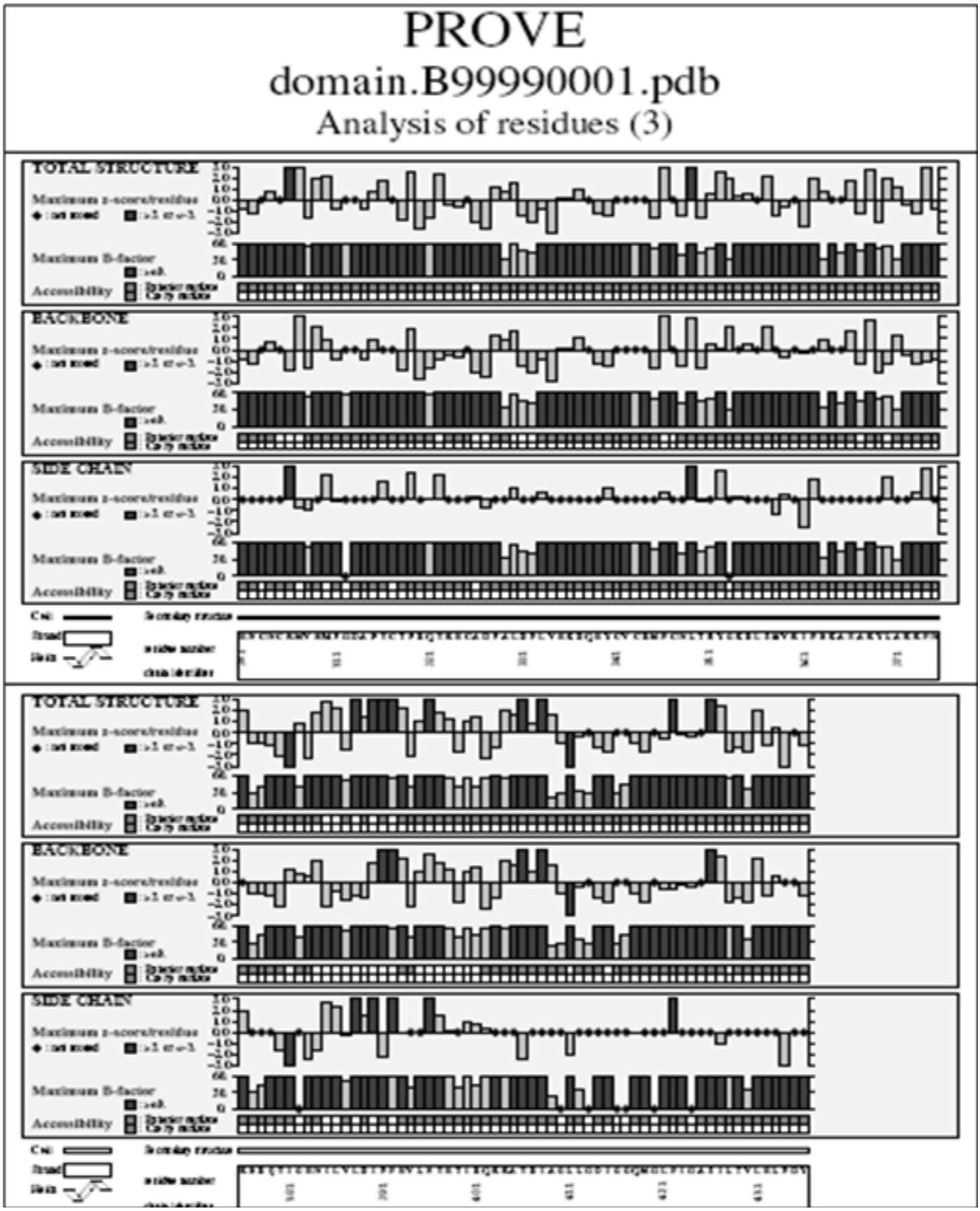


Z–score mean	0.854
Z–score stddev	26.802
Z–score RMS	26.806
# scored atoms	1393
# outliers	57
% outliers	4.100

Figure 27:- Analysis of All Residues.




PROVE
domain.B99990001.pdb
Analysis of residues (2)
TOTAL STRUCTURE
BACKBONE
SIDE CHAIN
Maximum z-score/residue
Maximum B-factor
Accessibility
TOTAL STRUCTURE
BACKBONE
SIDE CHAIN
Maximum z-score/residue
Maximum B-factor
Accessibility



9.5 Superimposition of 3HGC chain A with ASCC2N

The structural superimposition of C trace of template and Amiloride sensing Cation channel 2, neuronal is shown in Figure. The weighted root mean square deviation of C α trace between the template and final refined models 0.48Ao. This final refined model was used for the identification of active site and for docking of the substrate with the protein ASCC2N.

Figure 28: Super imposition of ASCC2N and 3HGC chain A

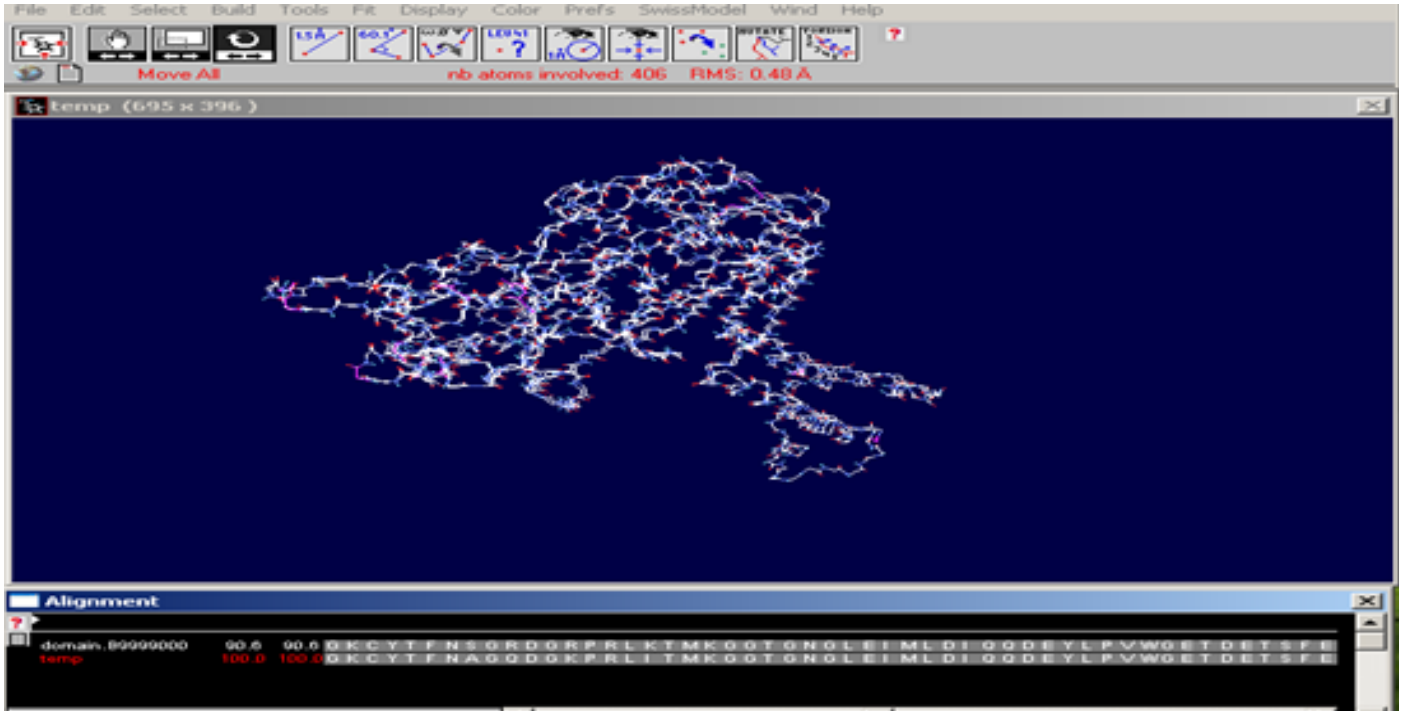
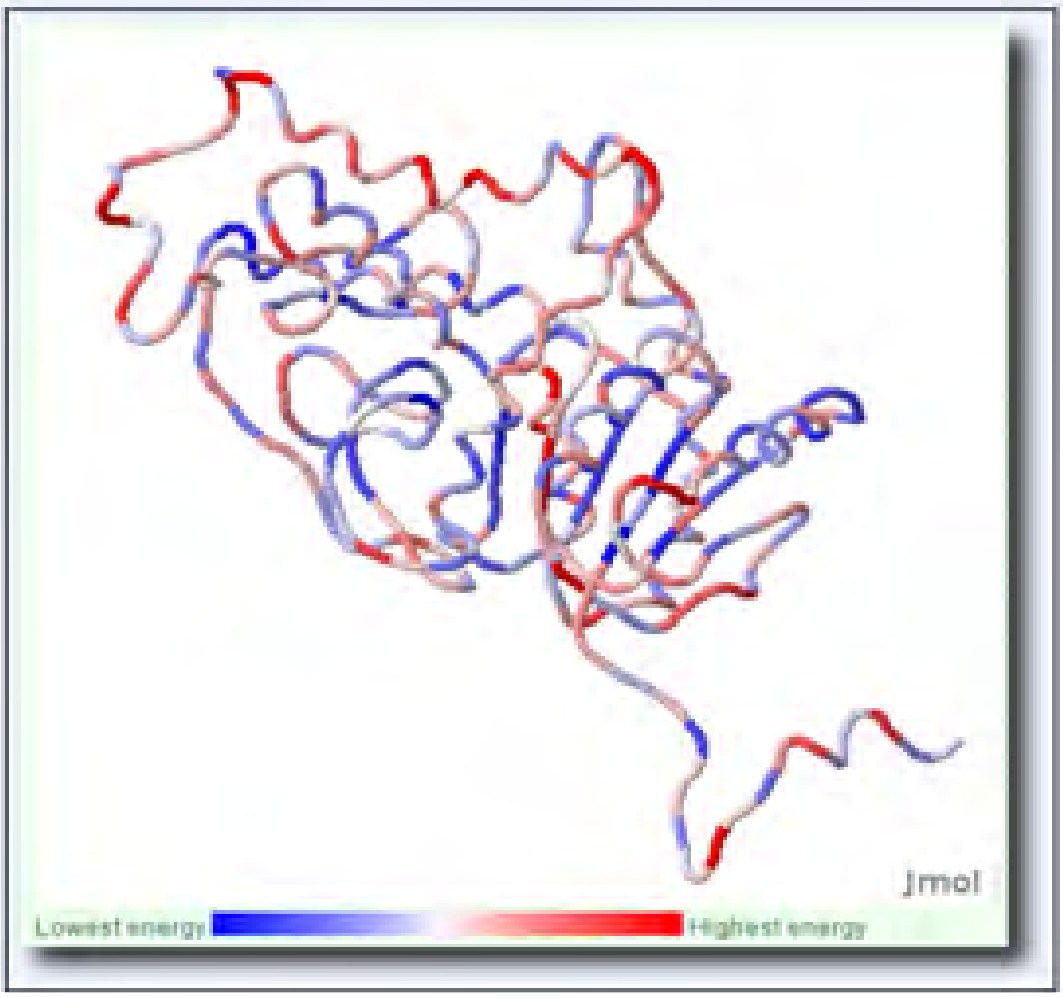


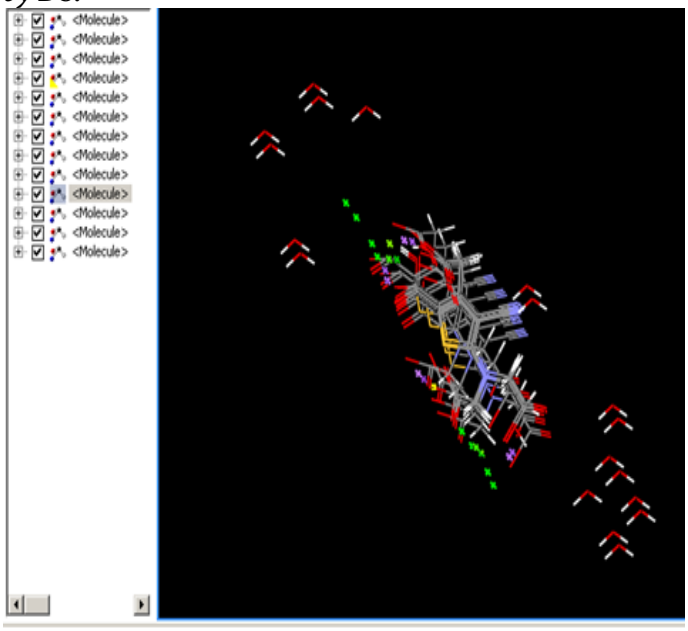
Figure 29: Interactive molecular viewer.



9.6 Superimposition of S.R derivatives:

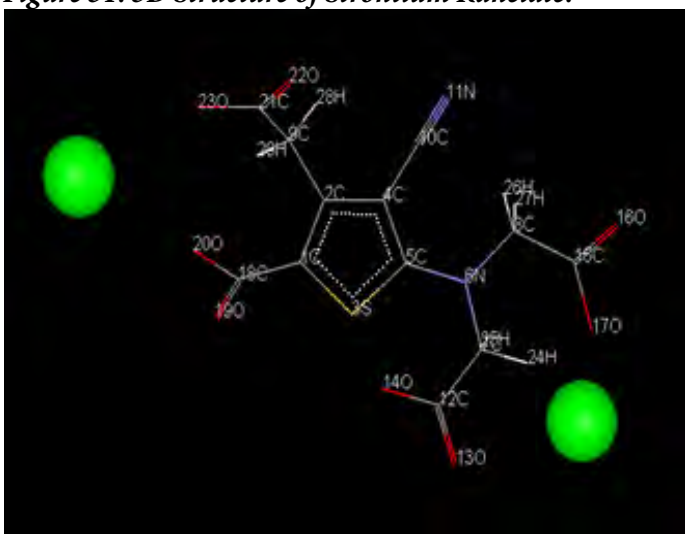
The Strontium Ranelate derivatives were superimposed by Discovery Studio.

Figure 30: Super imposition of Ranelic acid derivatives by DS.



3D Structure of Strontium Ranelate:-

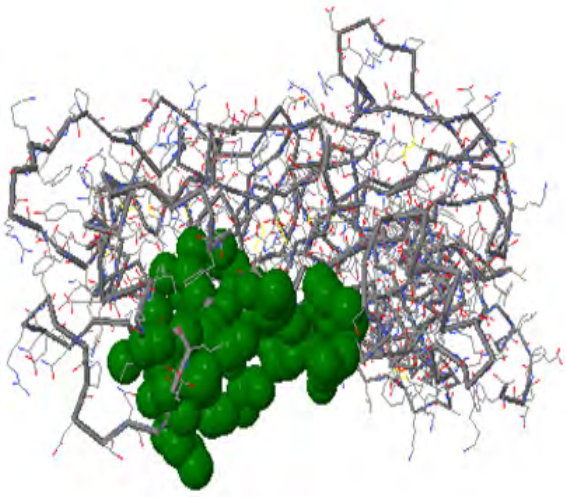
Figure 31: 3D Structure of Strontium Ranelate.



9.7 Active site Identification of Amiloride sensing Cation channel 2, neuronal protein

After the final model was built, the possible binding sites of Amiloride sensing Cation channel 2, neuronal was searched based on the structural comparison of template and the model build and also with CASTp server and was shown in Figure 17. Since, Amiloride sensing Cation channel 2, neuronal and the 3HGC chain A are well conserved in both sequence and structure; their biological function should be identical. Infact from the structure-structure comparison of template, final refined model of Amiloride sensing Cation channel 2, neuronal protein using SPDBV program and was shown in Figure6. It was found that secondary structures are highly conserved and the residues Leu75, Asn76, Glu77, Phe78, Gln81, Leu95, Met109, Ala110, Asp111, Lys113, Glu114, Leu115, Leu118, Gln206, Asp207, Tyr209, Leu210, Pro211, Val212, Trp213, Glu218, Thr219, Ser220, Phe221, Glu222, Ala223, Gln379, Tyr380, Glu383, Asn384.

Figure 32: active site of ASCC2N



9.8 The Ligand (agonist) molecules used for Docking studies by GOLD:

Docking was carried out using GOLD (Genetic Optimization of Ligand Docking) software which is based on genetic algorithm (GA). This method allows as partial flexibility of protein and full flexibility of ligand. The compounds are docked to the active site of the CASR. The interaction of these compounds with the active site residues are thoroughly studied using molecular mechanics calculations. The parameters used for GA were population size (100), selection pressure (1.1), number of operations (10,000), number of island (1) and niche size (2). Operator parameters for crossover, mutation and migration were set to 100, 100 and 10 respectively. Default cutoff values of 3.0 A° (Dh-X) for hydrogen bonds and 6.0 A° for vanderwaals were employed. During docking, the default algorithm speed was selected and the ligand binding site in the alpha glucosidase was defined within a 10 A° radius with the centroid as CE atom of PHE220. The number of poses for each inhibitor was set 100, and early termination was allowed if the top three bound conformations of a ligand were within 1.5A° RMSD. After docking, the individual binding poses of each ligand were observed and their interactions with the protein were studied. The best and most energetically favorable conformation of each ligand was

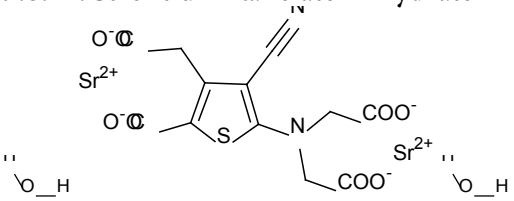
selected.

9.8.1-13 compounds of Ranelic acid derivatives were design in chemsketch and docked with ASCC2N

By modifying ligand molecules, 13 agonists are designed and they are listed below:

The best docked one is Strontium Ranelate Di hydrate.

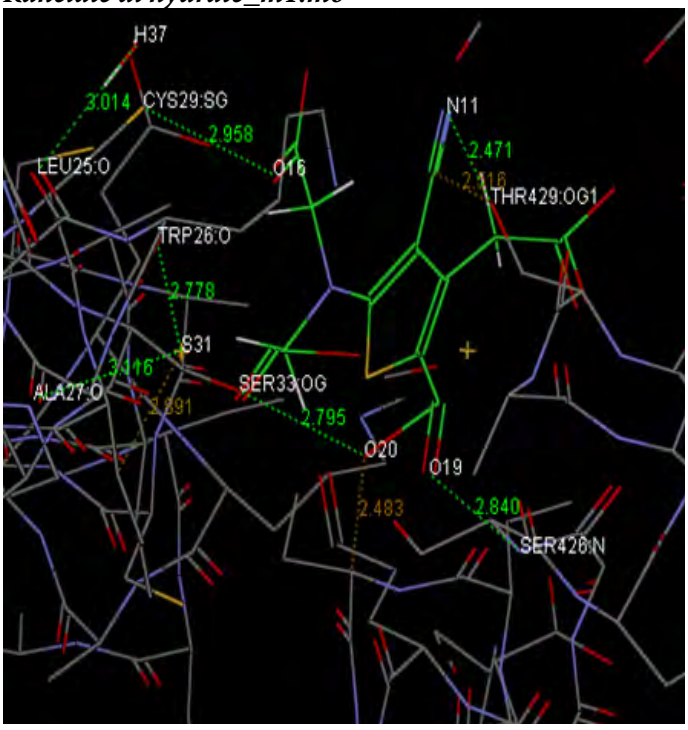
9.8.11. Strontium Ranelate Di hydrate*



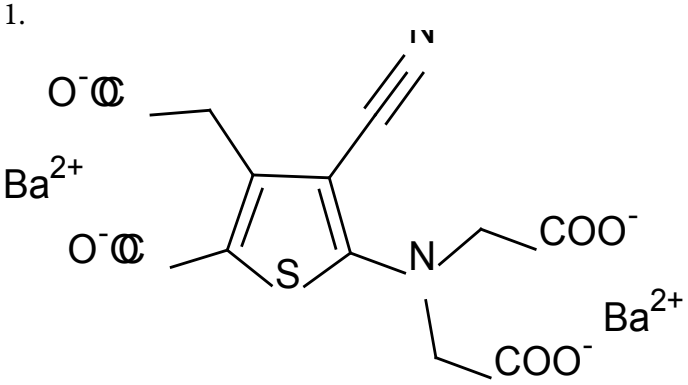
distrontium5-[bis(2-oxido-2-oxoethyl)amino]-4-cyano-3-(2-oxido-2-oxoethyl)thiophene-2-carboxylate di hydrate

Docking results of Strontium Ranelate di hyadrate

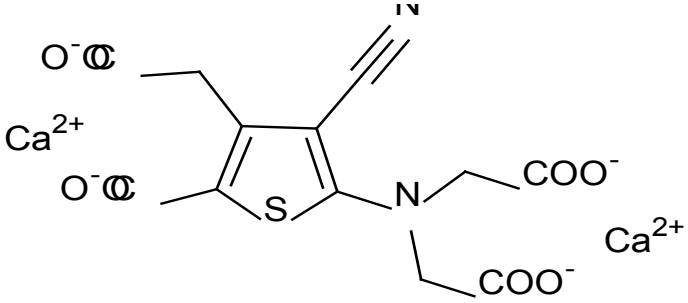
Figure 33: Fitness list for ligand file of gold_Strontium Ranelate di hydrate_m1.mo



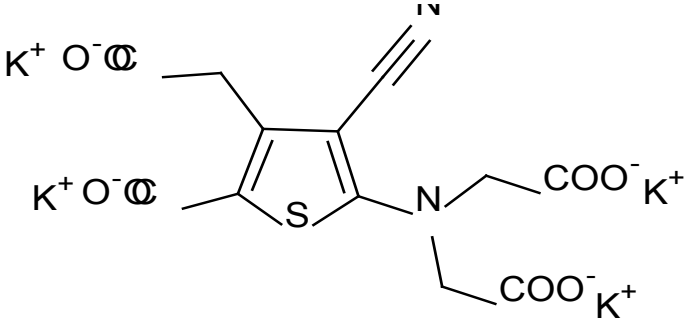
1.



2.



3.



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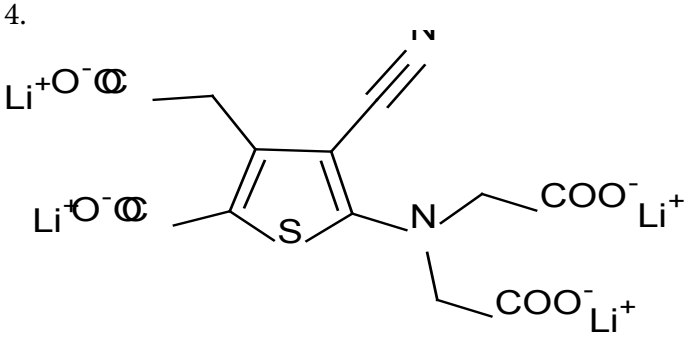

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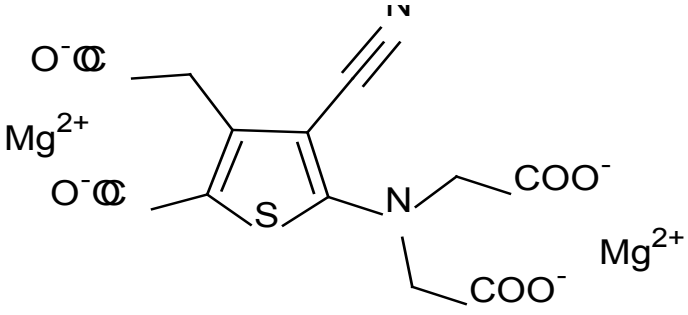
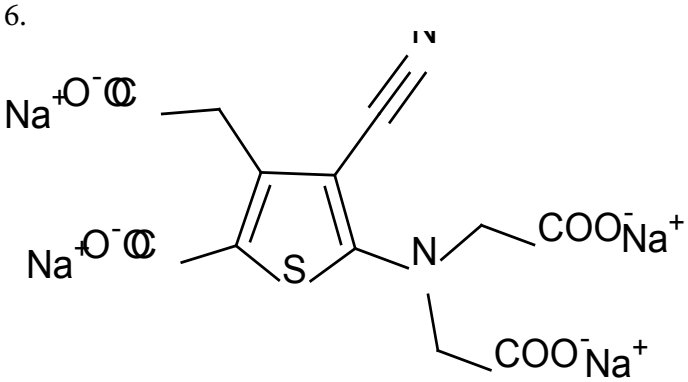
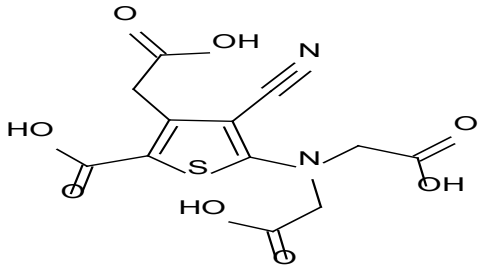


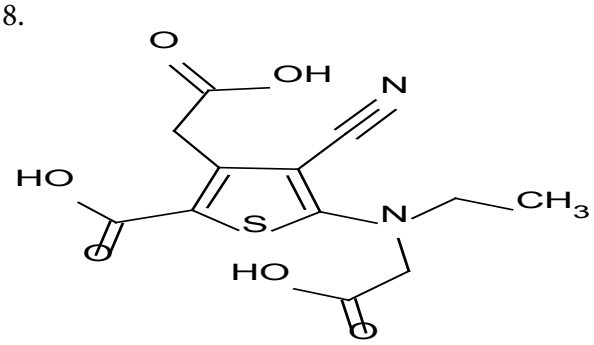
6.



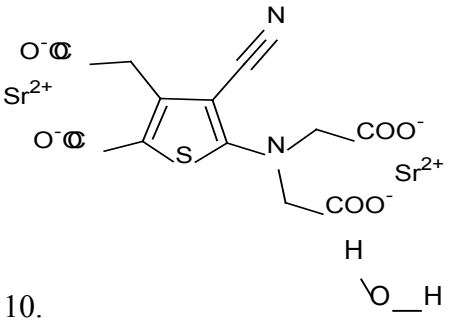
7.


O
OH
N
HO
O
S
N
O
OH
HO

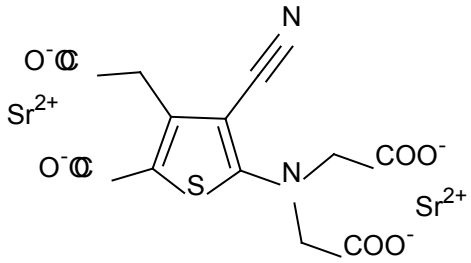
8.


O
OH
N
HO
CH3
S
N
HO

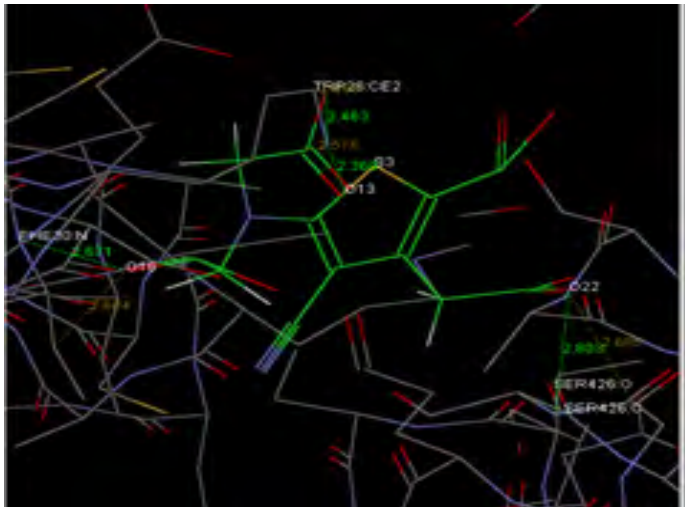
9.


N
O-C
Sr2+
O-C
S
N
COO-
Sr2+
COO-
H
O—H

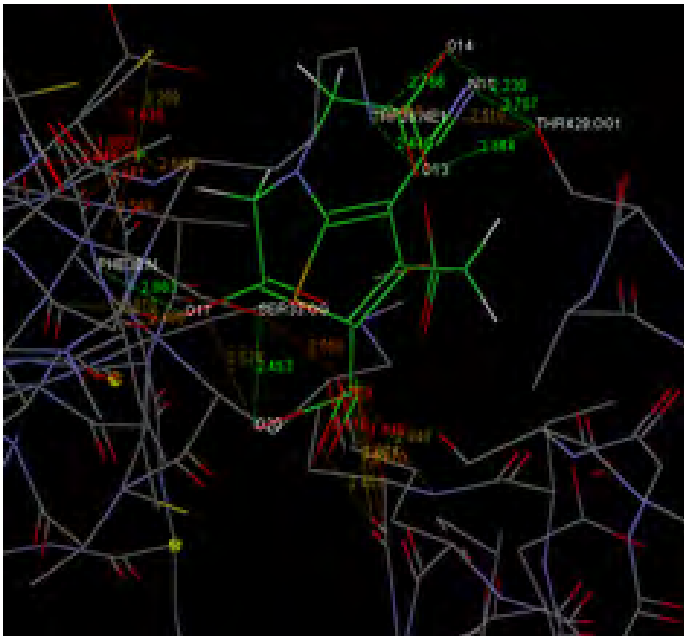
10.


N
O-C
Sr2+
O-C
S
N
COO-
Sr2+
COO-

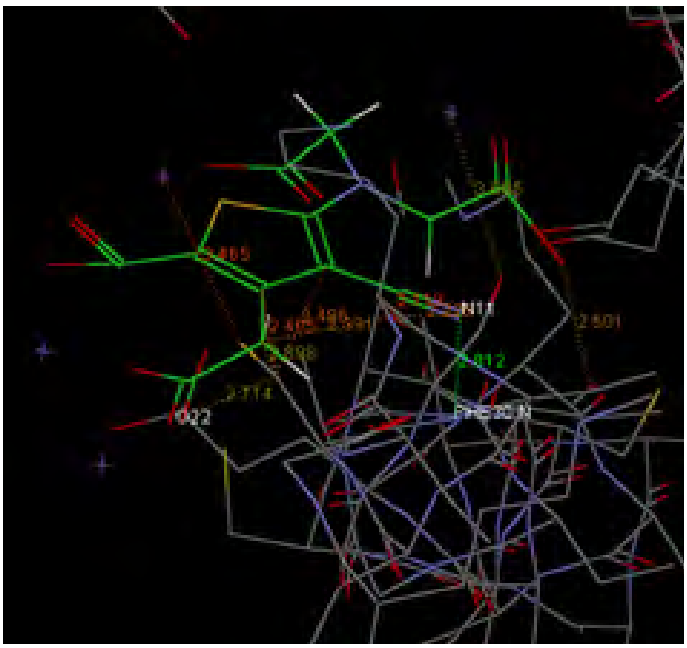
1.


TRP28:CE2
O13
O22
SER426:O

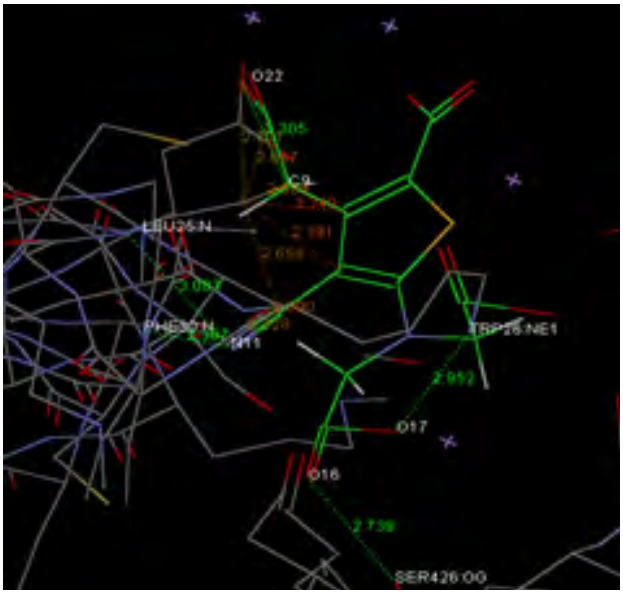
2.


O14
THR429:O01
O13
PHE30:N
O17
O22

3.


N11
O22
PHE30:N

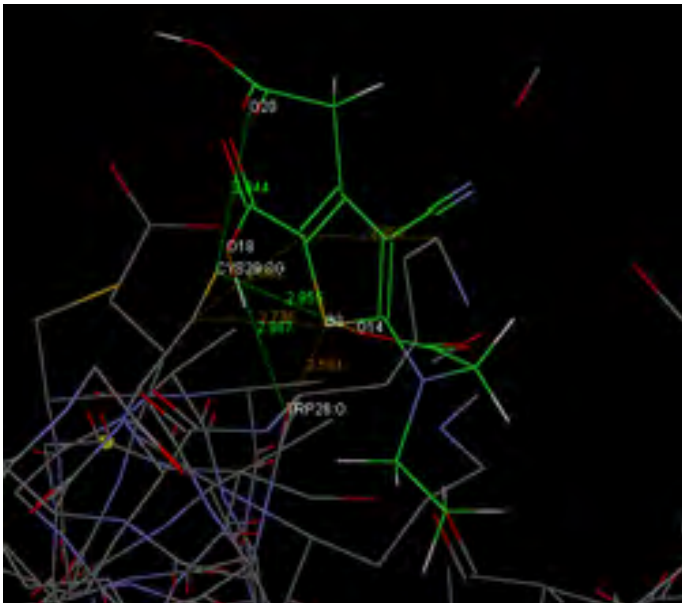
4.


O22
C9
LEU25:N
PHE30:N
N11
TRP28:NE1
O17
O16
SER426:OG

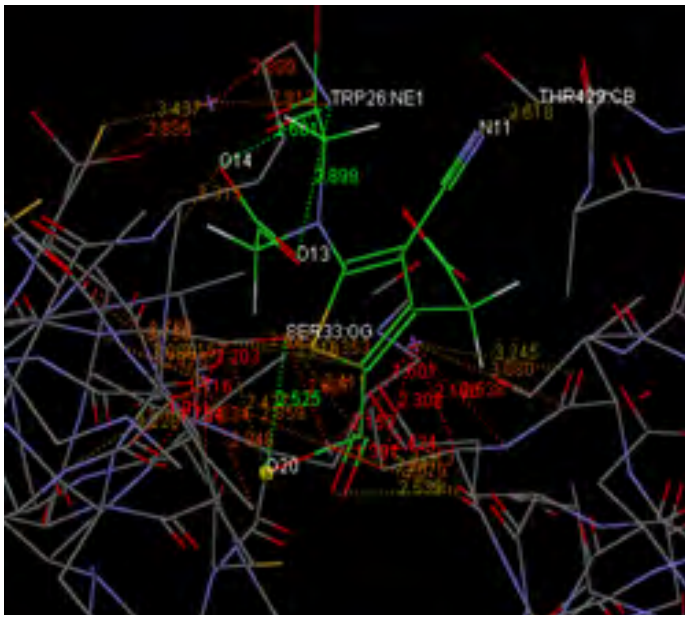
5.


O14
TRP26:NE1
O13
O17
PHE30:N
VAL430:N
VAL430:CA
N11
SER426:OG
SER426:C

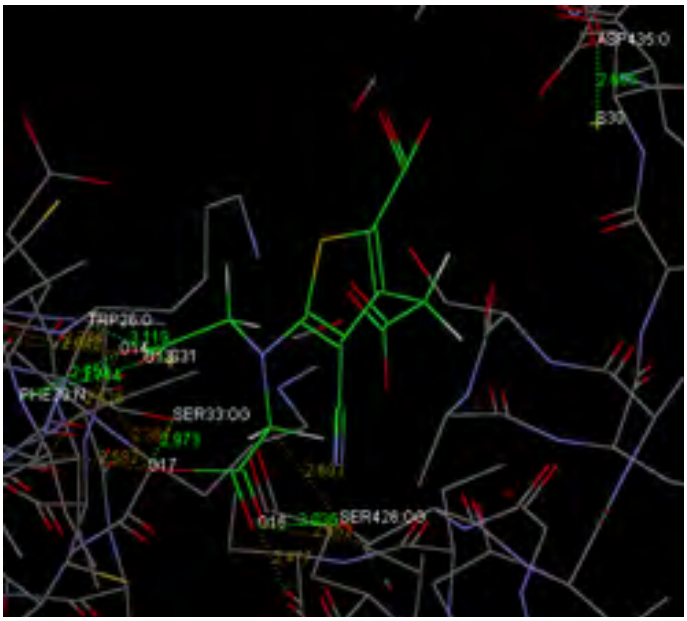
8.


O20
O18
CYS29:O
O14
TRP26:O

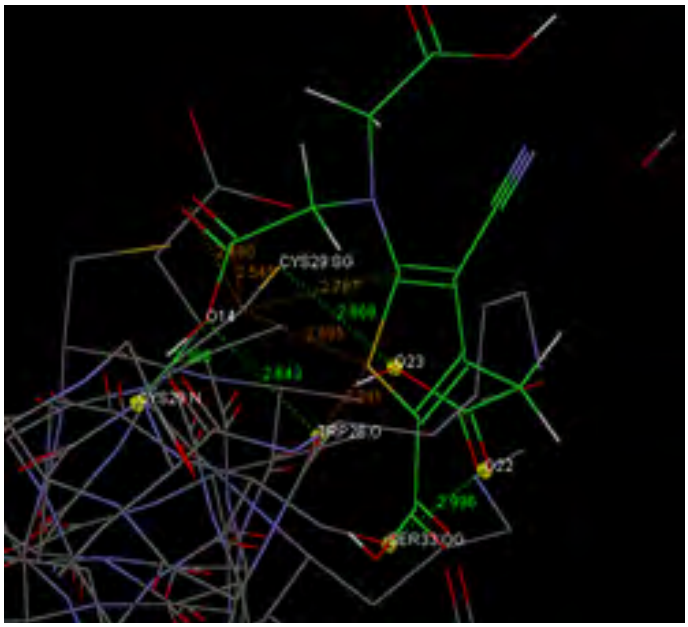
6.


TRP26:NE1
THR429:CB
N11
O14
O13
SER33:OG
O20

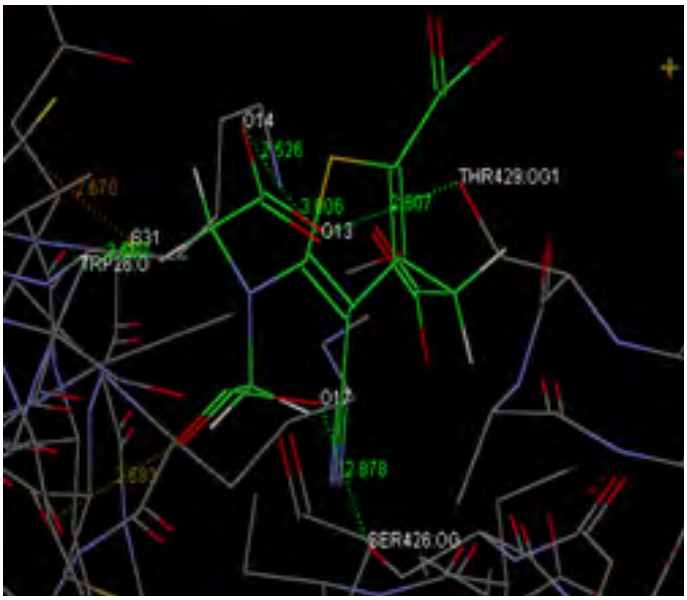
9.


ASP426:O
B30
TRP26:O
SER33:OG
O17
SER426:OG

7.


CYS29:O
O14
O23
TRP26:O
O22
SER33:OG

10.


O14
THR429:OG1
O13
TRP26:O
O12
SER426:OG

Gold Scoring Results:-

Table 11: Gold scoring results

S.No	FITNESS	S(hb ext)	S(vdw ext)	S(hb int)	S(int)	File Name	Ligand name
1	28.12	0.00	30.27	0.00	-13.50	Sai\gold_ soln_Ba R	Ba R
2	26.37	0.00	28.71	0.00	-13.11	Sai\gold_ soln_Ca R	Ca R
3	25.21	0.00	27.71	0.00	-12.89	Sai\gold_ soln_K R	K R
4	26.33	0.00	26.79	0.00	-10.51	Sai\gold_ soln_Li R	Li R
5	26.13	0.00	28.83	0.00	-13.51	Sai\gold_ soln_Mg R	Mg R
6	24.92	0.00	26.53	0.00	-11.56	Sai\gold_ soln_Na R	Na R
7	31.46	4.30	30.00	0.00	-14.09	Sai\gold_ soln_Ran	Ran
8	29.95	1.26	28.72	0.00	-10.80	Sai\gold_ soln_Rane	Rane
9	32.20	0.00	32.30	0.00	-12.22	Sai\gold_ soln_SR	S R
10	28.98	0.00	31.51	0.00	-14.35	Sai\gold_ soln_SR1	S R1

The high active molecule having docked at the allosteric site (active site) of ASCNN2 protein with the dock score of 39.25k.cal/mol accordingly might show agonist activity against Osteoporosis diseases.

Docking of agonist with the active site of Amiloride sensing Cation channel 2, neuronal

Docking of the agonist with ASCC2N was performed using GOLD 3.0.1, which is based on Genetic algorithm. This program generates an ensemble of different rigid body orientations (poses) for each compound conformer within the binding pocket and then passes each molecule against a negative image of the binding site. Poses clashing with this 'bump map' are eliminated. Poses surviving the bump test are then scored and ranked with a Gaussian shape function. We defined the binding pocket using the ligand-free protein structure and a box enclosing the binding site. This box was defined by extending the size of a co-crystallized ligand by 4 Å. This dimension was considered here appropriate to allow, for instance, compounds larger than the co-crystallized ones to fit into the binding site. One unique pose for each of the best-scored compounds was saved for the subsequent steps. The compounds used for docking was converted in 3D with SILVER. To this set, the substrate corresponding to the modeled protein was added.

10. CONCLUSION

Strontium Ranelate is used for the treatment of osteoporosis in post menopausal women. Unlike existing therapies, it has a dual mode of action; it increases the bone formation and decreases the bone resorption. It to be effective in reducing the incidence of vertebral and non vertebral fractures in post menopausal women. It can be consider as a second line treatment of postmenopausal osteoporosis. In this work, we are synthesized the strontium ranelate molecule with high purity and yield. The wet cake was dried to yield 143 grams of the Strontium salt. The % yield of the SR is 94.48% and the purity of the SR is 99.885%. The synthesis compound is conformed by FTIR and MASS.

Amiloride sensing Cation channel 2, neuronal (ASCC2N) is a member of recently identified family of transcription factors that activate gene transcription in response to a number of different cytokines in Homo sapiens. In this work, we are constructed a 3D model of ASCC2Ndomain, using the MODELLER software and obtained a refined model after energy minimization. The final refined model was further assessed by ERRAT and PROCHECK program, and the results show that this model is reliable. The stable structure of ASCC2N is further used for docking with modified ligand molecules. Docking results indicate that conserved amino-acid residues ASCC2N, main

play an important role in maintaining a functional conformation and are directly involved in donor substrate binding. The interaction between the domain and the agonist proposed in this study are useful for understanding the potential mechanism of domain and the agonist binding. As is well known, hydrogen bonds play important role for the structure and function of biological molecules. In this study it was found that, Ile 153, Leu 179, Val 182, Met 183, Gln 186, Phe 187, Leu 195, Gln 199, Met 202, Leu 203, Phe 223, Try 236, Trp 238, Leu 239, Glu 240, Ile 242, Leu 243, Ile 246 are important for strong hydrogen bonding interaction with the inhibitors. To the best of our knowledge MET1, MET3, ARG4, THR5 are conserved in this domain and may be important for structural integrity or maintaining the hydrophobicity of the inhibitor-binding pocket. The molecule Strontium Ranelate Di hydrate showed best docking results with target protein.

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