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RESEARCH ARTICLE

IDENTIFICATION, ISOLATION AND CHARACTERIZATION OF PROCESS AND DEGRADATION IMPURITIES OF FUDOSTEINE AND ITS STABILITY INDICATING HPLC METHOD VALIDATION.

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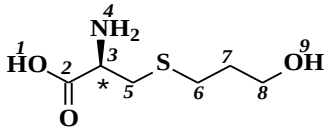
Abstract

Fudosteine [(-)-(*R*)-2-amino-3-(3-hydroxypropylthio) propionic acid] is a synthetic derivative of L-cysteine, a novel expectorant. During the synthesis of Fudosteine, four process impurities were detected along with two degraded impurities in HPLC-UV analysis at a level ranging from 0.05 to 0.1%. Stress studies were conducted to assess the nature of the impurity using LCMS analysis. These detected impurities were isolated by preparative LC and a thorough study was undertaken to characterize these impurities. Based on the spectral data obtained by ^{1}H-NMR, ^{13}C-NMR, DEPT and 2D-NMR, Mass, elemental composition and IR, the structures were characterized as , Impuirty-I (R)-2-amino-3-mercaptopropionic acid, Impurity-II (2R,2´R)-3,3´-disulfanediylbis(2-aminopropanoic acid), Impurity-III(R)-2(3-hydroxypropyl) amino-3-3(3-hydroxypropylthio)propionic acid, Impurity-IV (R)-2-amino-3-[3-(3-hydroxypropoxy) propylthio] propionic acid, Impurity-V (R)-3-(3-hydroxypropylsulfinyl)-2-aminopropionic acid, Impurity-VI (2R,2´R)-3-3´-(propane-1,3-diyldisulfanediyl)bis(2-aminopropionic acid. The developed stability indicating RP-LC method was validated with respect to Specificity, linearity, precision, accuracy, range, robustness, ruggedness, LOD, LOQ and response factor (RF) respectively.

Introduction:-

Fudosteine, 2-(R)-Amino-3-(3-hydroxypropylsulfonyl)propionic acid (Fig.1) was approved in Japan in the year 2001. Fudosteine is a low molecular weight cysteine derivative expectorant. It has a therapeutic effect against mucus hyper secretion caused by an increase in the number of globlet cells in chronic respiratory disease such as bronchial asthma, pulmonary emphysema, pulmonary tuberculosis, a typical micro bacterial disease and diffuse panbronchiolitis [1-3].



** asymmetric centre*

Fig.1:-

Fudosteine has less retention in ODS HPLC column due to its more polarity and lack of UV absorption and Fluorescent functional groups. Methods to achieve better sensitivity with fluorescent detection on HPLC [4] and

liquid chromatography-electro spray ionization mass spectrometry [5,6]. All these methods have time consuming derivatization procedure with long run time. Few methods have been published to measure fudosteine in human plasma with 96-well protein precipitation [7] and FMOC derivatization of fudosteine detection with LC/MS/MS[8]. Some more methods have been published for the separation of fudosteine and its enantiomer on Achiral C18 column with chiral ligand exchange selectors as mobile phase additives (cupric sulphate & L-phenyl alanine), enantiospecific analogue of fudoseine by RP-HPLC with chiral pre-column derivatization[9,10].

The aim of the present study was to establish inherent stability of Fudosteine through stress studies under a variety of ICH recommended test conditions [11-12]. It is mandatory requirement for regulatory authorities that the impurity profile study has to be carried out for every product and the identification, isolation, characterization of any impurities at a level as low as 0.05% followed by validation of the analytical method as per the ICH guidelines [13-15]. During the analysis of lab batches from process development of fudosteine synthesis (Fig.2), the imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 were found (Fig.3) and the levels of the detected impurities are ranged from 0.05 to 0.1%. A comprehensive study was undertaken to isolate and characterize these impurities by the spectroscopic techniques. The current study of fudosteine and its six impurities are observed in Japanese interview forum (both process and degradation impurities) but the impurities isolation and characterization and the current HPLC method validation was not reported till date to my best of Knowledge.

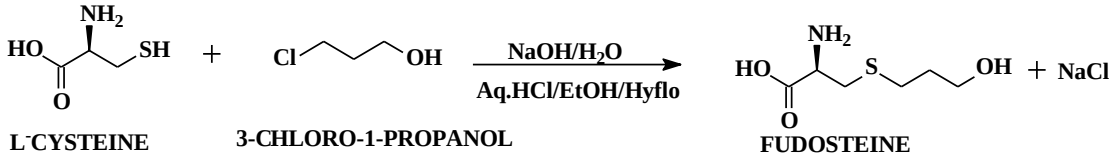


Fig.2:- Synthetic scheme of Fudosteine.

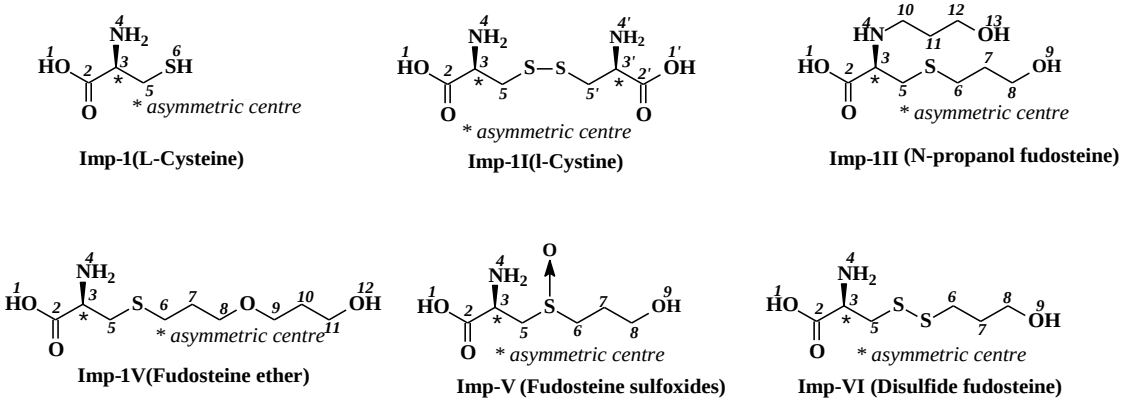


Fig.3:- Impurities structure and its numbering for structural elucidation.

Material and Methods:-

Chemicals, Reagents and Samples:-

The investigated samples of Fudosteine and crude samples were synthesized in APL Research Centre (a unit of Aurobindo Pharma Ltd., Hyderabad.). Perchloric acid, Acetonitrile, Hydrogen peroxide, Hydrochloric acid, Ammonium acetate, sodium hydroxide (AR grade) and Potassium bromide (IR spectroscopy grade) were procured from Merck (India) Limited and highly pure milli-Q water was used with the help of Millipore purification system.

High Performance liquid Chromatography (Analytical):-

A Waters Alliance 2695 separation module equipped with 2996 Photodiode array detector with Empower pro data handling system [Waters Corporation, MILFORD, MA 01757, USA] was used. The analysis was carried out on Atlantis T3, 250mm long, 4.6mm i.d., 5µ particle diameter column. Mobile phase-A was 5ml of perchloric acid in 1000ml of water. Mobile phase-B was acetonitrile. Mobile phase- A was used as diluent. UV detection was carried out at 210nm and flow rate was kept at 1.0ml/min and data acquired for 55min. Pump mode was gradient and the programme was as follows:

Time (min) / A (v/v): B (v\v); T0.01/100:0, T25/95:5, T40/80:20, T50/75:25, T55/75:25, T57/100:0, T65/100:0.

Preparative Liquid Chromatography:-
A Shimadzu LC-8A preparative liquid chromatography equipped with SPD-10A VP, UV-Vis detector [Shimadzu corporation, Analytical instruments Division, Kyoto, Japan] was used. Hypersil HS C18 500mm long, 30mm i.d., make: Thermo Scientific preparative column packed with 10µ particle size was employed for isolation of Impurity-III, IV & VI. The mobile phase-A consists of 0.1% of trifluoroacetic acid solution (method-1) and 0.1% formic acid solution (method-2) and mobile phase-B was Acetonitrile for both the methods. Flow rate was set 20 & 30ml/min respectively for both the methods and the UV detection was carried out at 210nm.

The gradient programme was as follows,
Method 1: Time(min)/A(v/v): B(v/v); T0.01/100:0, T20/95:5, T40/90:10, T50/30:70 (impurity-III).
Method II: Time(min)/A(v/v): B(v/v); T0.01/100:0, T15/90:10, T30/80:20, T45/70:30, T60/30:70 (impurity-IV & VI).

MS/LC-MS:-
MS/LC-MS analysis was carried out using a Perkin Elmer triple quadruple mass spectrometer (API 2000, PE SCIEX) coupled with a Shimadzu HPLC equipped with SPD10 AT VP UV-Vis detector and LC 10 AT VP pump(Foster city, CA). Analyst software was used for data acquisition and data processing. The turbo ion spray voltage was maintained at 5.5kv and temperature was set at 375ºC. The auxiliary gas and curtain gas used was high pure Nitrogen. Zero air was used as nebulizer gas. LC-MS spectra were acquired from m/z 100-1000 in 0.1 amu steps with 2.0s dwell time. The analysis was carried out by using Atlantis DS, 250mm long, 4.6mm i.d., 5µm particle diameter column. Mobile phase-A consists of 0.1% formic acid in H_2O and mobile phase-B consists of 0.1% formic acid in methanol. UV detection was carried out 210nm, temperature maintained at 45ºC and the flow rate was kept as 0,7ml/min. Data acquisition was 60min. The gradient programme was as follows,

Time (min)/A(v/v): B(v/v); T0.01/100:0, T10/100:0, T20/80:20, T30/60:40, T50/40:60, T52/0:100, T60/0:100.

NMR Spectroscopy:-
The ^{1}H-NMR, ^{13}C-NMR (proton decoupled) and DEPT spectra were recorded on Bruker 300 MHz [Bruker AG industries, Fellenden, Switzerland] & Varian 500MHz [Varian Deutschland GmbH, Darmstadt, Germany, now taken over by Agilent Technologies) NMR spectrometer using Deuterium oxide (D_2O) as solvent.

FT-IR Spectroscopy:-
IR spectra ware recorded as KBr pellet on Perkin Elmer Instrument model-Spectrum one.

Analytical method validation:-
The method was validated using samples of Active Pharmaceutical Ingredient by determination of the following parameters: Specificity, Linearity, Precision, Accuracy, LOD, LOQ, Robustness and system suitability test following the ICH guidelines [12].

Specificity:-
A stability indicating method is defined as an analytical method that accurately quantifies the active ingredients without any interference from the process related impurities, degradation products or any other potential impurities. The method capability was evaluated by subjecting the API sample solution to accelerated degradation by acidic, basic, peroxide, thermal, photolytic and humidity condition and checked the interference in the quantitation of Fudosteine. The acidic degradation was conducted by keeping the sample solution in 5M HCl at 85ºC for about 120 minutes after which solution was cooled and neutralized with base. The sample in 5M NaOH solution at 85ºC for about 120 min was used for base hydrolysis evaluation. Peroxide degradation was performed by preparing the sample solution in 1% H_2O_2 at room temperature for about 30min.The sample was heated to 105ºC for about 120 hrs. used for evaluating the thermal degradation. Photolytic conditions of white fluorescence light, 1.2 million lux hours and UV light, 200watt-hour/m2 was used to study the photolytic degradation. The sample was subjected to expose at 90% relative humidity at 25ºC for about 120 hours and screened the data. The method capability was established by determining the peak purity of fudosteine in the degraded samples using the PDA detector. Additionally the sample solutions were subjected to LC/MS study for identifying the impurities based on the m/z

values and its degradation pathway and further to prepare the impurities synthetically or isolation by preparative depending on the feasibility.

LOD & LOQ:-
The limit of detection (LOD) and the limit of quantitation (LOQ) of Fudosteine and its related substances were determined using the values of slope, standard deviation and responses of individual analytes that have been obtained from the linearity study carried out from 1% to 150% of specification level. The predicted concentrations of LOD and LOQ for the known related substances and fudosteine were verified for precision by preparing the solutions containing fudosteine and its related substances at about these predicted concentrations. Injecting each solution six times, using the test method conditions. The RSD for LOQ is 10.0% and 33.0% for LOD. The determined LOD, LOQ for all the impurities are tabulated in table.4.

Linearity:-
Linearity was determined by preparing the series of solutions using fudosteine reference standard and its related substances, concentration levels from 1% to 150% of specification level. From this data after establishing LOQ level for all the related substances, the linearity has been deduced from LOQ level to 150% of specification level. The correlation coefficient is more than 0.990 for Fudosteine and its related substances.

Precision:-
The precision of the method was determined by the following three parameters.

System precision:-
Standard solution was injected into HPLC system for six times and calculated the RSD for the peak areas and they are below 5%.

Method Precision:-
Six sample solutions were prepared using single fudosteine batch sample spiked with all the related substances at specification level and injected to check the precision.

Intermediate precision (Ruggedness):-
Sample solutions were prepared in duplicate by spiking the related substances at specification level for six days by two analysts using different columns and different HPLC systems to check the ruggedness of the method.

Accuracy:-
Sample solutions were prepared in triplicate with fudosteine and its related substances at levels LOQ 50%, 100% and 150% of specification level as per the methodology. The recovery found between 85% to 115% for LOQ level and up to 0.2% specification level and 90% to 110% for those with specification level more than 0.2% specification.

Range:-
The range of the analytical method was obtained from the linearity, precision and accuracy data which is from LOQ to 150% of specification.

Stability of the standard and sample solution:-
Standard and sample solutions were prepared as per the test procedure and analyzed initial and different time intervals and check the % difference in the areas obtained at initial and different time intervals are within the limits and are stable for at-lest 48hrs at room temperature.

Robustness:-
The robustness of the analytical procedure refers to its ability to remain unaffected by small deliberate variations in method parameters and provides an indication of its reliability for routine analysis. The altered conditions include change in flow rate by 10%, detection wave length by 5nm, gradient programme of buffer and acetonitrile by 2% and column oven temperature by 5ºC from the methodology values. The system suitability test was carried out to evaluate the resolution, reproducibility of the system for the analysis to be performed. The parameters measured were peak area, retention time, resolution, tailing factor (peak symmetry). Response factor has been calculated for all the related substances and are tabulated in table.4.

Table4:- (Comparative study of RRT, RF, LOD & LOQ for fudosteine and its impurities)

Name	RRT	Response Factor	LOD (% w/w)	LOQ (% w/w)
L-Cystine	0.32	0.79	0.005	0.010
Fudosteine sulfoxides	0.37	0.77	0.005	0.010
L-Cysteine	0.41	1.90	0.010	0.020
Fudosteine	1.00	1.00	0.006	0.013
N-Propanol Fudosteine	1.54	1.55	0.010	0.020
Fudosteine ether	3.07	1.49	0.010	0.020
Disulfide	2.07	0.70	0.005	0.010

Results and discussion:-

Identification and Isolation:-

Fudosteine was analyzed by HPLC as per the analytical conditions mentioned sec.2.2. The chromatogram displayed seven peaks at relative retention times compared to fudosteine at 0.32, 0.37, 0.41, 1.54, 2.07 and 3.07. The LC-MS analysis showed six peaks having m/z values 240, 195, 121, 237, 237 and 211. Out of six impurities, three impurities were isolated by preparative HPLC as per the conditions mentioned in sec.2.3. Two impurities were prepared synthetically, and one impurity was outsourced as a raw material. All these impurities were co-injected with fudosteine sample to confirm the retention time. HPLC chromatogram of fudosteine and the spiked chromatogram with all these impurities were shown in fig.4a & 4b.

Figure 4a:- (A Typical representative HPLC chromatogram of Fudosteine drug substance)

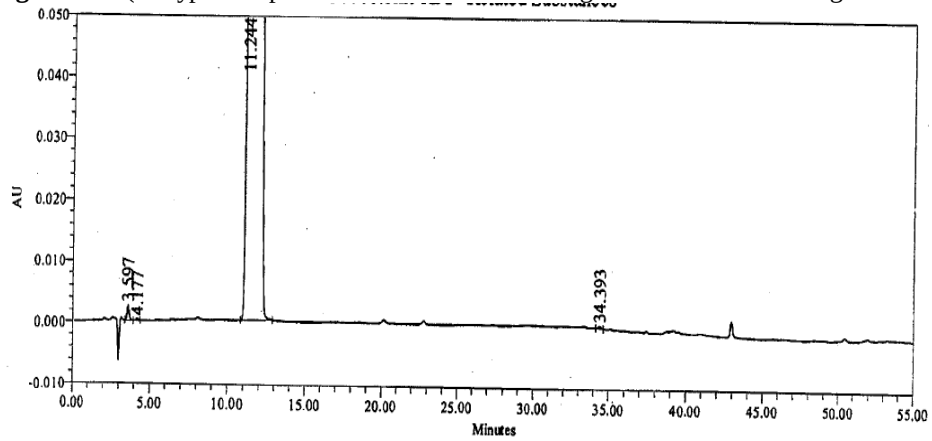
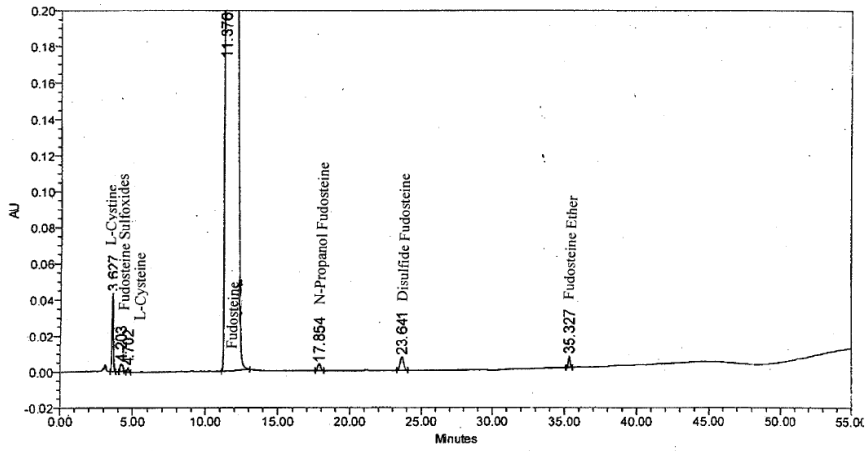


Figure 4b:- (A Typical representative HPLC chromatogram of Fudosteine spiked with all the six impurities)



Characterization and Origin of impurities:-

Impurity-I:-

The ESI mass spectrum of impurity-I displayed the molecular ion m/z 120 in the negative ion mode. Therefore the molecular weight of this impurity was considered as 121 which was less by 58 amu than fudosteine. This impurity

RRT and its molecular ion peak completely resembles with that of the key raw material (L-Cysteine). Which in turn indicated the origin of this impurity was oxidative degradation of fudosteine. The elemental composition, theoretical values: C 29.74% H 5.82% N 11.56% S 26.46%, found: C 29.70% H 5.79% N 11.52% S 26.40% supports the structure. The complete structural interpretation of this impurity by ^{1}H-NMR &^{13}C-NMR was tabulated in table-1 & 2. Based on the above structural data this impurity is characterized as (R)-2-amino-3-mercaptopropionic acid (L-Cysteine). Comparative IR characterization has been discussed in table.3.

Table 1:-Comparative ^{1}H-NMR assignments for fudosteine and its impurities.

Position[a]	Fudosteine	Impurity I	Position[a]	Impurity II	Impurity III	Impurity IV	Impurity V	Impurity VI
	***δ* (*ppm*), multiplicity**	***δ* (*ppm*), multiplicity**		***δ* (*ppm*), multiplicity**	***δ* (*ppm*), multiplicity**	***δ* (*ppm*), multiplicity**	***δ* (*ppm*), multiplicity**	***δ* (*ppm*), multiplicity**
1	-	-	1,1′	-	-	-	-	-
2	-	-	2,2′	-	-	-	-	-
3	3.88 (m, IH)	3.97 (t, 1H)	3,3′	4.06 (dd, 2H)	3.87 (t, 1H)	3.89 (m, 1H)	4.24 (m,1H)	4.10 (m, 1H)
4	-	-	4,4′	-	-	-	-	-
5	2.98 & 3.09 (ABq, 2H)	2.99 & 3.09 (ABq, 2H)	5,5′	2.86 & 3.03 (ABq, 4H)	3.10 & 3.18 (ABq, 2H)	2.99 & 3.11 (ABq, 2H)	3.00-3.51 (m, 2H)	3.10 & 3.34 (ABq, 2H)
6	2.63 (t, 2H)	-		-	2.64 (t, 2H)	2.64 (m, 2H)	3.00-3.51 (m, 2H)	2.87 (m, 2H)
7	1.81 (m, 2H)	-		-	1.80 & 1.93 (ABq, 2H)	1.77-1.87 (m, 2H)	1.99 (m, 2H)	1.97 (m, 2H)
8	3.64 (t, 2H)	-		-	3.64 & 3.71 (ABq, 2H)	3.57-3.66 (m, 2H)	3.72 (t, 2H)	3.71 (t, 2H)
9	-	-		-	-	3.57-3.66 (m, 2H)	-	-
10	-	-		-	3.10 & 3.18 (ABq, 2H)	1.77-1.87 (m, 2H)	-	-
11	-	-		-	1.80 & 1.93 (ABq, 2H)	3.57-3.66 (m, 2H)	-	-
12	-	-		-	3.64 & 3.71 (ABq, 2H)	-	-	-

[a]Refer to Figure 3 for numbering. t, triplet; ; dd, doublet of doublet; m, multiplet; ABq, AB quartet.

Table 2:- Comparative ^{13}C (Proton decoupled) and DEPT NMR assignments for fudosteine and its impurities:

Position[a]	Fudosteine	Impurity I	Position[a]	Impurity II	Impurity III	Impurity IV	Impurity V	Impurity VI	DEPT
	(*ppm*), multiplicity	***δ* (*ppm*), multiplicity**		***δ* (*ppm*), multiplicity**	***δ* (*ppm*), multiplicity**	***δ* (*ppm*), multiplicity**	***δ* (*ppm*), multiplicity**	***δ* (*ppm*), multiplicity**	
1	-	-	1,1′	-	-	-	-	-	-
2	173.3	172.5	2,2′	172.5	171.1	173.2	171.2, 171.3	172.6	C
3	53.9	55.9	3,3′	53.2	60.3	53.8	50.1, 50.9	53.3	CH
4	-	-	4,4′	-	-	-	-	-	-
5	32.4	24.8	5,5′	36.7	31.2	32.3	49.0, 50.8	37.7	CH_2
6	28.2	-		-	28.0	28.6	48.2, 50.4	33.7	CH_2
7	31.3	-		-	30.7	31.5	24.5, 24.6	30.6	CH_2
8	60.5	-		-	59.6	69.2	59.8, 59.9	59.9	CH_2
9	-	-		-	-	67.6	-	-	CH_2
10	-	-		-	45.4	28.6	-	-	CH_2
11	-	-		-	28.4	59.0	-	-	CH_2
12	-	-		-	60.3	-	-	-	CH_2
13	-	-	-	-	-	-	-	-	-

Impurity-II:-

The ESI mass spectrum of impurity-II displayed the molecular ion at m/z 241 in the positive ion mode. Therefore the molecular weight of this impurity was considered as 240 which was 61 amu more than that of fudosteine. This even number molecular weight indicated the presence of even nitrogen atoms. The ^{1}H-NMR spectra was very close comparison with that of the key raw material L-Cysteine, but with double integral values (symmetrical protons), presumed that could be the dimer of L-Cysteine. Based on the above data and the elemental composition, theoretical: C 29.99% H 5.03% N 11.66% O 26.63% S 26.68% and found: C 29.89% H 5.10% N 11.66% S 26.70%, the origin of this impurity was due to the oxidation of L-Cysteine results the L-Cystine (Imp-II) and has been characterized as (2R,2´R)-3,3´-disulfanediylbis(2-aminopropanoic acid). This impurity can be controlled if we can able to prepare the fudosteine in nitrogen atmosphere to minimize the oxidation. The complete structural interpretation with numbering (Figure-3) of this impurity by ^{1}H-NMR &^{13}C-NMR was tabulated in table-2 & 3.

Impurity-III:-

The ESI mass spectrum of impurity-III displayed the molecular ion at m/z 238 in the positive ion mode. Therefore the molecular weight of this impurity was considered as 237 which was 58 amu more than that of fudosteine. Based on the elemental composition, theoretical: C 45.55% H 8.07% N 5.90% O 26.97% S 13.51% and found: C 45.55% H 8.0% N 5.91% O 26.87% S 13.41%. The major fragment ions at m/z-163 and 147 support the one more additional propanol moiety in the structure. The ^{1}H-NMR spectra shows additional signals at δ 1.93 ppm (multiplet with 2 proton integral value), δ 3.18 ppm (multiplet with 2 proton integral value and is assigned to N-CH_2) and at δ 3.71 ppm (multiplet with 2 proton integral value and is assigned to O-CH_2) and in ^{13}C-NMR spectra shows additional carbon signals at δ45.4, 28.4 and 60.3ppm corresponds to three methylene protons of position at 10, 11 & 12. Based on the above data, the origin of this impurity was due to the N-alkylation of fudosteine results the N-propanol fudosteine (Imp-III) and has been characterized as (R)-2(3-hydroxypropyl)amino-3-3(3-hydroxypropylthio)propionic acid. This impurity is having higher solubility in aqueous ethanol, remains in the mother liquor and can be controlled. The complete structural interpretation with numbering (Figure-3) of this impurity by ^{1}H-NMR &^{13}C-NMR was tabulated in table-1 & 2.

Impurity-IV:-

The ESI mass spectrum of impurity-III displayed the molecular ion at m/z 238 in the positive ion mode. Therefore the molecular weight of this impurity was considered as 237 which was 58 amu more than that of fudosteine. The additional 58 amu will be the attributable to additional propanol moiety. Based on the elemental composition, theoretical: C 45.55% H 8.07% N 5.90% O 26.97% S 13.51% and found: C 45.55% H 8.07% N 5.85% O 26.90% S 13.49%. The impurity-III and IV were showing same molecular mass of m/z-237 but with different retention time in HPLC. The impurity IV is more non-polar than the impurity-III. This indicates that the propanol moiety will beattached at hydroxyl group, the total molecule will be non-polar than the impurity-III. The ^{1}H-NMR spectra shows additional signals at δ 1.87 ppm (multiplet with 2 proton integral value) and at δ 3.57 – 3.66 ppm (multiplet with 4 proton integral value and is assigned to two O-CH_2) and in ^{13}C-NMR spectra shows additional carbon signals at δ 67.6, 28.6 and 59.0, corresponds to carbon signals of position at 9, 10 & 11. Based on the above data, the origin of this impurity was due to the condensation of L-Cysteine with 3-(3-chloropropoxy)propanol impurity present in the 3-chloro propanol raw material results the fudosteine ether impurity (Imp-IV) and has been characterized as (R)-2-amino-3-[3-(3-hydroxypropoxy)propylthio]propionic acid. This impurity can be controlled by limiting the 3-(3-chloropropoxy)propanol impurity in the 3-chloropropanol raw material .The complete structural interpretation with numbering (Figure-3) of this impurity by ^{1}H-NMR &^{13}C-NMR was tabulated in table-1 & 2.

Impurity-V:-

The ESI mass spectrum of impurity-III displayed the molecular ion at m/z 194 in the negative ion mode. Therefore the molecular weight of this impurity was considered as 195 which was 16 amu more than that of fudosteine. The additional 16 amu will be the attributable to additional oxygen moiety. Based on the elemental composition, theoretical: C 36.91% H 6.71% N 7.17% O 32.78% S 16.42% and found: C 36.89% H 6.66% N 7.12% O 32.68% S 16.40%. The impurity-V is more polar than fudosteine as per the HPLC retention time. The ^{1}H-NMR spectra of this impurity showed the number of protons is similar with that of fudosteine. But the proton chemical shift values are different. The down field shift of protons in this impurity at position 3,5,6,7 & 8 at δ4.24ppm, δ 3.00-3.51ppm, δ 1.99 & δ 3.72 from the δ 3.88ppm, (2.98 & 3.09ppm), δ 2.63ppm and δ3.64ppm of fudosteine molecule indicates that the complete moiety near to the ‘s’ atom was shifted down field due to the formation of sulfoxide. In ^{13}C-NMR spectra also significant down field shift observed for the Sulphur adjacent two methylene carbon signals of position at 5 & 6 at 49.0, 50.8, 48.2 and 50.4 (both α & β) in comparison with the fudosteine ^{13}C-NMR signals. 2D-NMR (^{1}H-^{1}H-COSY, HSQC) data was able to differentiate the pairs of protons and their carbon signals. Based on the above data, the origin of this impurity was due to the oxidative

degradation of fudosteine results the fudosteine sulfoxide impurity (Imp-V) and has been characterized as (R)-3-(3-hydroxypropylsulfinyl)-2-aminopropionic acid. This impurity can be controlled by synthesizing the fudosteine in nitrogen atmosphere .The complete structural interpretation with numbering (Figure-3) of this impurity by ^{1}H-NMR &^{13}C-NMR was tabulated in table-1 & 2.

Impurity-VI

The ESI mass spectrum of impurity-III displayed the molecular ion at m/z 210 in the negative ion mode. Therefore the molecular weight of this impurity was considered as 211 which was 32 amu more than that of fudosteine. The additional 32 amu will be attributable to additional Sulphur atom. Based on the elemental composition, theoretical: C 34.11% H 6.20% N 6.63% O 22.72% S 30.35% and found: C 34.11% H 6.10% N 6.62% S 30.35. The impurity-VI is non-polar than fudosteine as per the HPLC retention time. The ^{1}H-NMR spectra of this impurity showed the number of protons is similar with that of fudosteine. But the proton chemical shift values are different. The down field shift of protons at position 3,5,6,7 & 8 at δ4.10ppm, (δ3.10&3.34ppm), δ2.87, δ1.97 & δ 3.71 from the δ 3.88ppm, (2.98 & 3.09ppm), δ 2.63ppm, δ1,81ppm and δ3.64ppm of fudosteine molecule. In ^{13}C-NMR spectra also the carbon signals shows significant down field shift of δ37.7 & δ33.7 for signal position 5 & 6.Based on the above data impurity has been characterized as (2R,2´R)-3-3´-(propane-1,3-diyldisulfanediyl)bis(2-aminopropionic acid. The complete structural interpretation with numbering (Figure-3) of this impurity by ^{1}H-NMR &^{13}C-NMR was tabulated in table-1 & 2.

Table 3:- FT – IR spectral data for Fudosteine and impurities.

S.No.	Compound	IR (KBr) absorption bands, *(Cm^{-1})*
1	Fudosteine	3322 (br & s) NH &OH stretch, 2934 (s) aliphatic CHstretch, 1594 (s) C=O stretch, 1418, 1396 (s) aliphatic CH deformations.
2	Impurity-I	3176 (br & s) NH &OH stretch, 2963 (s) aliphatic CHstretch, 2551 (s) SH stretch, 1586 (s) C=O stretch, 1423, 1393 (s) aliphatic CH deformations.
3	Impurity-II	3023 (br & s) NH &OH stretch, 2916 (s) aliphatic CHstretch, 1583 (s) C=O stretch, 1407, 1381 (s) aliphatic CH deformations.
4	Impurity-III	3335 (br & s) NH &OH stretch, 2937 (s) aliphatic CHstretch, 1574 (s) C=O stretch, 1424, 1376 (s) aliphatic CH deformations.
5	Impurity-IV	3331 (br & s) NH &OH stretch, 2942, 2927 (s) aliphatic CHstretch, 1597 (s) C=O stretch, 1418, 1356 (s) aliphatic CH deformations.
6	Impurity-V	3389 (br & s) NH &OH stretch, 2947 (s) aliphatic CHstretch, 1642 (s) C=O stretch, 1389, 1354 (s) aliphatic CH deformations.
7	Impurity-VI	3332 (br & s) NH &OH stretch, 2924 (s) aliphatic CHstretch, 1596 (s) C=O stretch, 1419, 1390 (s) aliphatic CH deformations.

w - weak, s - strong, m- medium.

Method validation:-

Forced degradation was performed to provide the indications of the stability indicating properties of an analytical method. Particularly when there was no information about the potential degradation impurities. There was no significant decrease of Fudosteine area in acid, base, thermal, photolytic, humidity conditions. Significant degradation observed in the peroxide degradation results the formation of impurity-III. The validated method is specific, sensitive, linear, precise, accurate and robust for the determination of related substances in fudosteine drug substance.

Conclusions:-

During Fudosteine synthesis, found six impurities by HPLC and identified by LC/MS, isolated by preparative HPLC, characterized all the impurities by spectroscopic techniques (NMR, MS, IR, elemental composition and proposed the structures. Impurity-II & IMPURITY-V are considered as process/degradant and remaining four are process related impurities. Validated the simple & precise, LC/MS compatible, time saving, stability indicating HPLC method for the quantitative analysis of fudosteine and its impurities to assure therapeutic efficacy.

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