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Research Article

Development And Validation Of Stability Indicating Hplc Method For Estimation Of Leuprolide Acetate In Its Parenteral Dosage Form

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Abstract

In this study, A sensitive, accurate and cost effective RP-HPLC method was developed using YMC-Pack ODS-A(150mmX46mm), 3 µ stationary phase for quantitative determination of leuprolide acetate in its 3 month depot formulation. Chromatographic separation was achieved by using Mobile Phase A [Buffer (Triethylamine in milli Q water): Organic mixture (Acetonitrile: n-Propanol)(60:40)] and Mobile phase B (Buffer : Organic mixture)(50 : 50) by Gradient method under UV detection at 220 nm. Retention time was found to be 12 to 15 min. This drug was subjected to stress conditions like Acid, Alkali, Thermal, Photolytic and Humidity Degradation. Linearity was found to be in the range of 80-150 microgram/ml. The accuracy of present method was evaluated at 50%, 100% and 150%. The method was found to be robust. The method enables high throughput and is easy to perform.

Keywords: Leuprolide depot, Leutenising hormone-Releasing hormone, Stability indicating method, RP-HPLC, Forced degradation.

INTRODUCTION[1-3]

Prostate cancer is cancer that occurs in the prostate — a small walnut-shaped gland in men that produces the seminal fluid that nourishes and transports sperm. Leuprolide is a parenterally administered, gonadotropin releasing hormone (GnRH) agonist which causes an inhibition of estrogen and androgen production and is used predominantly to treat advanced prostate cancer. It is a synthetic 9 residue peptide analog of gonadotropin releasing hormone. Leuprolide acetate acts as an agonist at pitutary GnRH receptors. By interrupting the normal pulsatile stimulation of, and the desensitizing, the GnRH receptors, it indirectly down regulates the secretion of gonadotropins leutenizing hormone and folical stimulating hormone, leading to hypogonadism and thus a dramatic reduction in estradiol and testosterone levels in both sexes. Methods such as HPLC, HPTLC, and UV-spectrophotometric method, HPLC-RIA are reported for estimation of Leuprolide acetate alone or in combination with other drugs. A literature search reveals that few analytical methods were reported for Stability indicating RP-HPLC method development and Validation for estimation of leuprolide acetate. Hence a Simple, Rapid, Sensitive, Accurate and Time consuming stability indicating RP-HPLC method was developed for determination of Leuprolide acetae in its Parenteral dosage form.

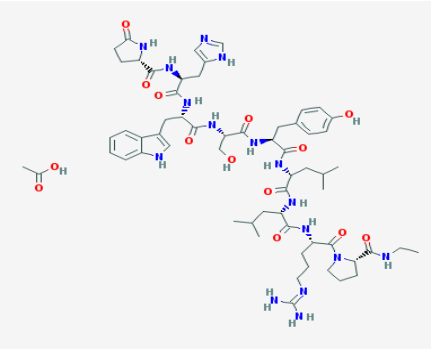

Fig. 1 Leuprolide acetate structure

MATERIALS AND METHODS[7-18]

Material and Reagents

Acetonitrile, Methanol, Dimethyl sulphoxide, Ortho Phosphosphoric Acid, Hydrochloric acid, Sodium hydroxide, Hydrogen peroxide (50%) were procured from Merck and Finar Reagent.

Leuprolide acetate standard Gifted by Zydus Cadila Health Care, Moraiya, Ahmedabad.

Equipment

Chromatographic separation was performed on RP-HPLC system consist of model Shimadzu Prominence-1 LC-2030C 3D series PDA detector with 10µl loop volume. UV spectrophotometer which consists of model Shimadzu UV 1800 Spectrophotometer is also used to measure the wavelength of Leuprolide acetate.

Preparation of Standard Solution

Accurately weigh and transfer of about 20 mg of Leuprolide acetate standard into 200ml volumetric flask. Add about 125 ml of diluent and sonicate to dissolve. Make volume up to the mark with diluents and mix well.

Preparation of diluents:

Prepare a degassed mixture of dimethyl sulphoxide:methanol in the ratio of (50:50)% v/v.

Preparation of Sample solution:

Accurately weigh and transfer sample equivalent to 5 mg of Leuprolide acetate into a 50 ml volumetric flask. Add to it 25 ml of diluents and sonicate to dissolve. Make volume up to the mark with diluents and mix well.

Table 1 Formulation Details

For sterile lyophilized microspheres		
Sr. No.	**List of Ingredients**	**mg/vial**
1	Lyo-Leuprolein for 22.5 mg depot inj.	22.5
2	Resomer R20H	198.6
3	Mannitol IP(injectable grade)	38.9
	Total	260
For diluents(use for reconstitution		
Sr. No.	**List of Ingredients**	**mg/vial**
1	Carbocxymethylcellulose sodium	7.5
2	D-Mannitol	75
3	Polysorbate 80	1.5
4	Water for injection	2.0 ml
5	Glacial acetic acid (To control pH)	q.s.

Chromatographic Conditions

YMC-Pack ODS-A (150mmX46mm),3µ was used as the stationary phase. Elution was Gradient by Mobile Phase A [Buffer (42ml Triethylamine in 1900ml milli Q water):Organic mixture(Acetonitrile: n-Propanol)][(60:40) and Mobile phase B (Buffer : Organic mixture)(50 : 50) It was filtered through 0.45µ (micron) membrane filter and degassed. The mobile phase was pumped at 1.1 ml/min. The eluents were monitored at 220 nm. The injection volumes of sample and standard were 10µl.Total run time was 25 min. Column temperature was 35 ºC. Chromatograms are shown in figure: 2

Validation of developed method

System Suitability

It was demonstrated by making six replicate injections of Standard solution prepared as per the test method. The peak area of Leuprolide acetate was recorded. The theoretical plates and tailing factor were evaluated for the Leuprolide acetate peak. The values of system suitability results obtained are shown in Table 3.

Acceptance criteria

- % RSD of Area of five replicate standard injections should not be more than 2.0.
- Theoretical Plates for the analyte peak should not be less than 2000.
- Tailing factor for the analyte peak should not be more than 2.0.

Linearity

The linearity for Leuprolide acetate is established over the range of 50% -150% of target concentration. These solutions were injected into the HPLC system and area response of the same was recorded. A plot of concentration Vs Y-intercept of the plot were evaluated. The Preparation of linearity solution and observations were shown in Table 2 and 4.

Table 2 Linearity Preparation

Linearity level	Stock soln to be taken in ml	Diluent to volume with diluent	Final conc. In µg/ml
50%	2.5	20.0	50
80%	4.0	20.0	80
100%	5.0	20.0	100
120%	6.0	20.0	120
150%	7.5	20.0	150

Acceptance Criteria

The correlation coefficient should be 0.999.

Method Precision

It was demonstrated by making six replicate injections of Standard solution prepared as per the test method, Injected in Chromatographic system & determined the %Assay of these samples. Evaluate the precision of the method by computing the % RSD.

Precision considered at three levels: Repeatability, Intermediate (Intraday) Precision and Reproducibility (Interday) Precision.

Intraday precision

Solutions containing 80%, 100% & 120% Level of Leuprolide acetate was analysed for three times on same day and % RSD was calculated.

Interday precision

Solutions containing 80%, 100% & 120% Level of Leuprolide acetate was analysed for three times on three different successive days and % RSD was calculated.

Repeatability

Method precision of experiment was performed by preparing the standard solutions of Leuprolide acetate for six times and analysed as per proposed method and % RSD was calculated.

Acceptance criteria

- % RSD of peak area should not be more than 2.0%.
- Data shown in the Table 5,6,7.

Accuracy

The accuracy of the test sample was demonstrated by preparing recovery samples at the level of 50%, 100% & 150% of target conceration. Each solution was injected in triplicate and the % recovery was calculated by measuring the Assay. The observations were shown in Table 8.

Acceptance Criteria

The %Recovery at each level should be 98% to 120% of added concentration.

Robustness

The robustness was studied by analysing the sample of Leuprolide acetate by deliberate variation in method parameters. The change in response of Leuprolide acetate was noted. Robustness of the method was studied by changing flow rate ± 0.2 ml, mobile phase composition and column temperature and pH change. The change in the response of Leuprolide acetate was noted and compare with the original one.

Flow Rate: 0.99 ml and 1.21 ml.

Organic phase composition : 1. +2%v/v, 2 -2 %v/v; **Column Temperature:** 30ºC and 40ºC. **Buffer pH change:** 2.8 and3.2

Acceptance criteria

- Number of theoretical plates for the analyte peak should not be less than 2000.
- Asymmetry value for the analyte peak should not be more than 2.0.
- % RSD for the analyte peak should not be more than 2.0 % .
- Data was shown in Table 9, 10.

Solution Stability

Standard solution was prepared as per test procedure and % Recovery of standard was determined as per proposed RP-HPLC method. Standard solution was stored for 24 hours and 48 hours at 5ºC. The peak area results obtained was compared with the initial value.

Acceptance criteria

The response factor for standard solution should be in between 0.98-1.02. Data were shown in Table 11.

Forced Degradation Study

ICH prescribed stress conditions such as acidic, basic, oxidative, thermal and photolytic stresses were carried out.

Acid degradation condition: 1N HCl 1 ml at 90°c for 2 Hrs.

Sample preparation:

5 mg equivalent weight of leuprolide acetae sample was taken and transfer it in to 50 ml volumetric flask. Add 50ml of diluent (DMSO:Methanol) and sonicate for 10 minute and 1ml of 1N HCl was added and solution was heated for 120 minute in water bath at 90°C for acid hydrolysis. Then the solution was neutralized with 1N NaOH and made volume upto mark with diluent. Then filter the solution through 0.45µm membrane filter and injected into HPLC vial. Chromatograms are shown in figure: 4

Alkali Degradation condition: 1 N NaOH 1 ml at 90°Cfor 2 Hrs.

Sample preparation:

5 mg equivalent weight of leuprolide acetae sample was taken and transfer it in to 50 ml volumetric flask. Add 50ml of diluent (DMSO:Methanol) and sonicate for 10 minute and 1ml of 1N NaOH was added and solution was heated for 120 minute in water bath at 90°C for base hydrolysis. Then the solution was neutralized with 1N HCL and made volume upto mark with diluent. Then filter the solution through 0.45µm membrane filter and injected into HPLC vial. Chromatograms are shown in figure: 5

Peroxide Degradation condition: 30% H_2O_2 5 ml at room temp for 5 Hrs.

Sample preparation:

5 mg equivalent leuprolide acetae sample was weighed accurately and transferred in 50ml volumetric flask. Add 50ml of diluent and sonicate for 10 minute and 5ml of 30% H_2O_2 was added. Then filter the solution through 0.45µm membrane filter and injected into HPLC vial. Chromatograms are shown in figure: 6

Photolaytic Degradation condition: Expose Under UV light.

Sample preparation:

5 mg equivalent leuprolide acetae sample was weighed accurately and transferred in 50 ml volumetric flask. Add 50 ml of diluent and sonicate for 10 mins. sample was put in UV chamber for 48 hours. Then filter the solution through 0.45µm membrane filter and injected into HPLC vial. Chromatograms are shown in figure: 7

Thermal Degradation condition: Expose in Oven at 80°C for 2 Days.

Sample preparation:

Sample was kept at 80°C for 48 hrs. 5 mg equivalent leuprolide acetae sample was weighed accurately and transferred in 50 ml volumetric flask. Add 50ml of diluent and sonicate for 10 mins . filter the solution through 0.45µm membrane filter and injected into HPLC vial. Chromatograms are shown in figure: 8

RESULTS AND DISCUSSION

The detection wavelength was carried out in the UV range of 220 nm. Chromatographic separation was carried out using mobile phase composed by Gradient by Mobile Phase A[Buffer(42ml Triethylamine in 1900ml milli Q water):Organic mixture(Acetonitrile: n-Propanol)][(60:40) and Mobile phase B (Buffer : Organic mixture)(50 : 50) by using YMC-Pack ODS-A(150mmX46mm),3µ as the stationary phase.

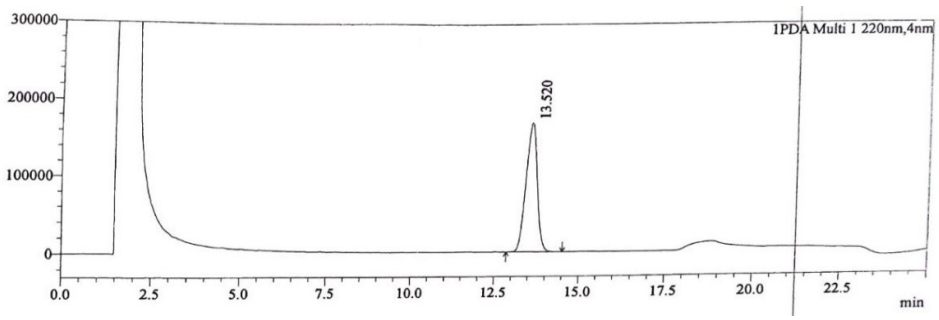

Fig. 2 Chromatogram of leuprolide acetae test sample

Peak	Retention Time	Area	Theoretical Plates	Asymmetry	Name
1	13.52	3916870	7819	1.13	leuprolide acetae

Method validation The described method has been validated which include parameters like System Suitability, specificity, Linearity, Accuracy, Precision, Robustness

System Suitability

System suitability and chromatographic parameters were validated such as Theoretical plates, %RSD of Area and Tailing factor was calculated.

The results are given in table 3.

Linearity

The linearity of this method was evaluated by linear regression analysis and calculated by the least square method and studied by preparing standard solutions of leuprolide acetae at different concentration levels. The calibration curve showed in (Fig. 3) good linearity in the range of 50-150 µg/ml with a correlation coefficient (r^2) of 0.999. The results are given in table 4.

Precision

Intraday precision

Intraday precision was performed by analyzing three different concentrations within linearity range, three times in a day (3*3 determinations).

Interday precision

Interday precision was performed by analyzing three different concentrations within linearity range, on different days.

Repeatability

The repeatability studies were carried out by measuring response for a single concentration for 6 times a day.

Accuracy

Accuracy of the method was confirmed by recovery study of Leuprolide acetate at three levels (50%, 100%, 150%) by standard addition method. The results are given in table 7.

Leuprolide acetate undergoes significant degradation in acid, base, peroxide, thermal and UV. Comparatively, more degradation was found with alkali Degradation. Forced Degradation Summary is given in Table 12.

Table 3 System Suitability Data for leuprolide acetae

Parameters	Observation	Specification
%RSD of Area	0.07%	RSD<2%
Tailing factor(T)	1.14	T ≤ 2
Theoretical plates(N)	8399 ± 25	≥2000

Table 4 Linearity Data of (50-150 µg/ml)

Linearity level	Concentration of Leuprolide acetate in µg/ml	Peak area of analyte
50%	48.4956	1870652
80%	77.5930	2949162
100%	96.9913	3685593
120%	116.3895	4413644
150%	145.4869	5510275

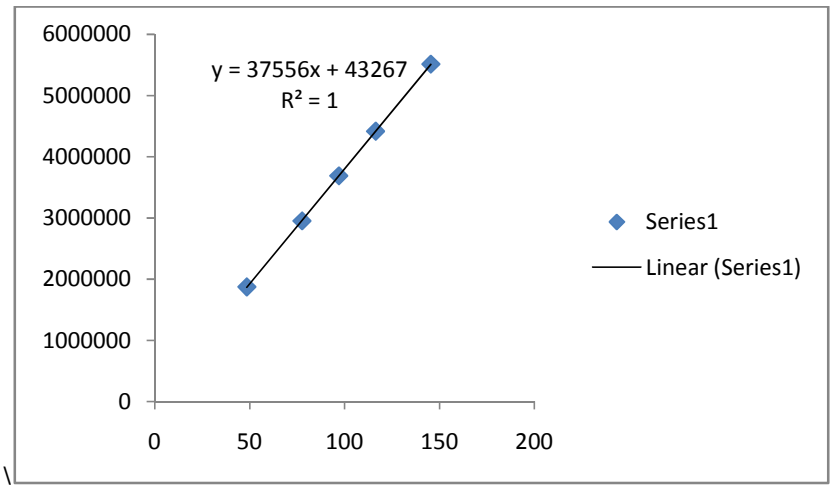

Fig. 3 Calibration curve of Leuprolide acetate

Table 5 Intraday Precision Data for Leuprolide acetate

80% Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	**SD**	**% RSD**
1	80%	2949160	2915421	2932290.5	23857.076	0.813
2	80%	2969587	2924865	2947226	31623.23	1.072
3	80%	2919658	2949588	2934623	21163.71	0.721
100% Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	**SD**	**% RSD**
1	100%	3685593	3639674	3662634	32469.640.886	0.886
2	100%	3639674	3655432	3647553	11142.59	1.095
3	100%	3712688	3658900	3685794	38033.86	1.03
120% Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	**SD**	**% RSD**
1	120%	4498719	4446954	4472837	36603.38	0.818
2	120%	4514299	4472324	4488312	36751.87	0.818
3	120%	4524658	4472394	4498526	36956.23	0.821

Table 6 Inter day Precision Data for Palonosetron Hydrochloride

80% Level						
Set	**Level**	**Day-1**	**Day-2**	**Mean**	**SD**	**% RSD**
1	80%	2949160	2984365	2966762.5	24893.694	0.839
2	80%	2915421	2951324	2933373	25387.25	0.865
3	80%	2934388	2979566	2956977	31945.67	1.083
100% Level						
Set	**Level**	**Day-1**	**Day-2**	**Mean**	**SD**	**% RSD**
1	100%	3672500	3712688	3692594	28417.21	0.769
2	100%	3685593	3629674	3657634	39540.7	1.08
3	100%	3712519	3670432	3691476	29760	0.806
150% Level						
Set	**Level**	**Day-1**	**Day-2**	**Mean**	**SD**	**% RSD**
1	120%	4562682	4493644	4528163	48817.24	1.078
2	120%	4524658	4472652	4498655	36773.8	0.817
3	120%	4505243	4452492	4478868	37300.59	0.832

Table 7 Repeatability Data for Leuprolide acetate

Sr No.	**Leuprolide acetate (0.1mg/ml)**
1	3685593
2	3639674
3	3612532
4	3698974
5	3627694
6	3614275
Mean	3646457
SD	37079.12
% RSD	1.01

Table 8 Accuracy Data for Leuprolide acetate

Set	Level	Amount added	Area	Amount found	%Recovery	Mean recovery	%RSD
1	50%	2.3912	1882901	2.4471	102.3	101.4	0.7
2	50%	2.3912	1859331	2.4165	101.1		
3	50%	2.3912	1855622	2.4117	100.9		
Set	**Level**	**Amount added**	**Area**	**Amount found**	**%Recovery**	**Mean recovery**	**%RSD**
1	100%	4.7823	36540380	4.7490	99.3	99.1	0.2
2	100%	4.7823	3637842	4.7280	98.9		
3	100%	4.7823	3646031	4.7386	99.1		
Set	**Level**	**Amount added**	**Area**	**Amount found**	**%Recovery**	**Mean recovery**	**%RSD**
1	150%	7.1735	5513937	7.1663	99.9	99.7	0.5
2	150%	7.1735	5468942	7.1078	99.1		
3	150%	7.1735	5519082	7.1730	100.0		

Table 9 change in conditions

Parameter	Change	Area	Mean	SD	%RSD
Higher Flow rate	1.21 ml/min	3627581	3630160	2312.129	0.06
	1.21	3630850			
	1.21	3632048			
Lower flow rate	0.99ml/min	4428371	4426189	1971.62	0.044
	0.99 ml/min	4424535			
	0.99 ml/ min	4425662			
Highre column temp	40	3982832	3982596	526.8779	0.013
	40	3982963			
	40	3981992			
Lower column temp	30	4004146	4003682	442.7957	0.011
	30	4003264			
	30	4003636			
Increase organic phase	+2%	3823861	3982596	526.8779	0.013
	+2%	3830572			
	+2%	3825270			
Decrease organic phase	-2%	3845041	3845557	1170.648	0.030
	-2%	384697			
	-2%	3844733			
Parameter	**Change**	**Area**	**Mean**	**SD**	**%RSD**
Increase in pH	2.8	3823937	3820861	5293.472	0.138
	2.8	3823898			
	2.8	3814749			
Decrease in pH	3.2	3833100	3833207	1302.8	0.033
	3.2	3834560			
	3.2	3831961			

Table 10 Robustness data for leeuprolide acetate

Condition	%RSD	Tailing factor	Theoretical plates
Normal	0.06	1.14	8407
Flow rate changed by -10%	0.06	1.16	7267
Flow rate changed by +10%	0.04	1.15	9098
Column temp changed by -5C	0.01	1.14	7715
Column temp changed by -5C	0.01	1.15	7321
Organic phase change by +2%	0.01	1.16	8785
Organic phase change by -2%	0.03	1.15	7773
Buffer pH changed by +0.2 unit	**0.1**	**1.11**	**9049**
Buffer pH changed by -0.2 unit	0.03	1.11	7756

Table 11 Solution Stability data for Standard

Time(hours)	Peak area	% deviation from initial response
Initial	3831273	-
24 hours	3827638	-0.1
48 hours	3835289	0.1

Acid Degradation

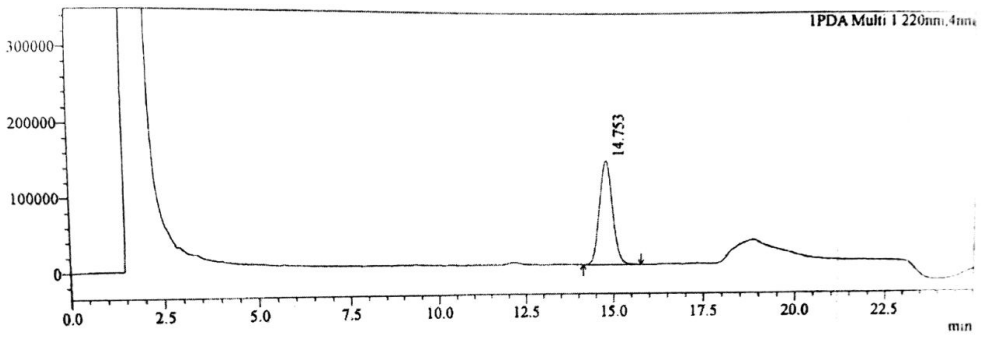

Figure 4 Leuprolide acetate acid degradation

Peak	Area of As such Sample	Area After degradation	As Such As-say	Assay After degra-dation	%degradation
1	3916870	3691567	105.7%	89.27%	4.61%

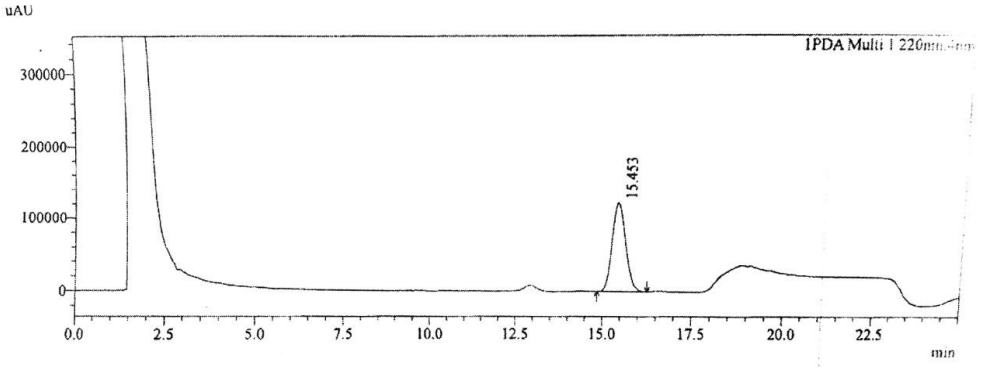

Figure 5 Leuprolide acetate alkali degradation

Peak	Area of As such Sample	Area After degradation	As Such Assay	Assay After degradation	%degradation
1	3916870	3691567	105.7%	89.10%	16.48%

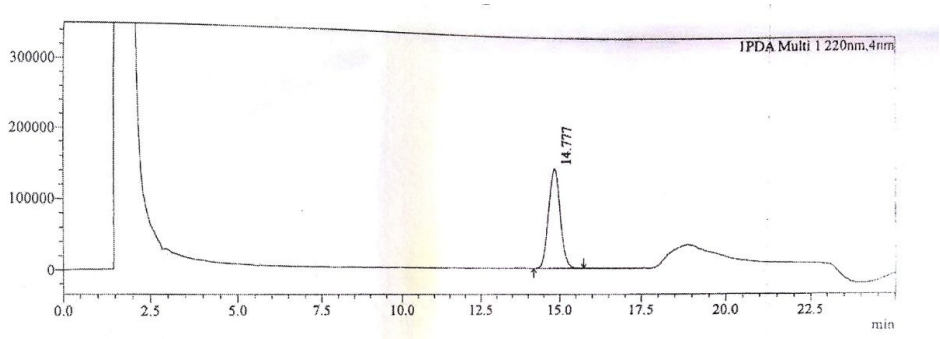

Figure 6 Leuprolide acetate peroxide degradation

Peak	Area of As such Sample	Area After degradation	As Such Assay	Assay After degradation	%degradation
1	3916870	3795888	105.7%	103.60%	1.92%

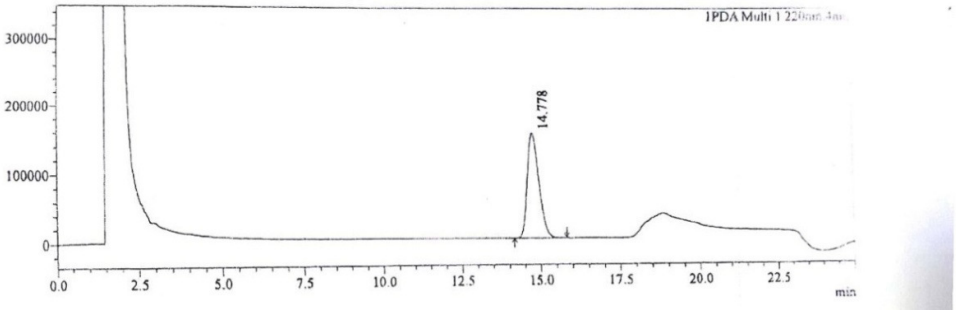

Figure 7 Leuprolide acetate photolytic degradation

Peak	Area of As such Sample	Area After degradation	As Such Assay	Assay After degradation	%degradation
1	3916870	3963838	105.7%	107.30%	2.41%

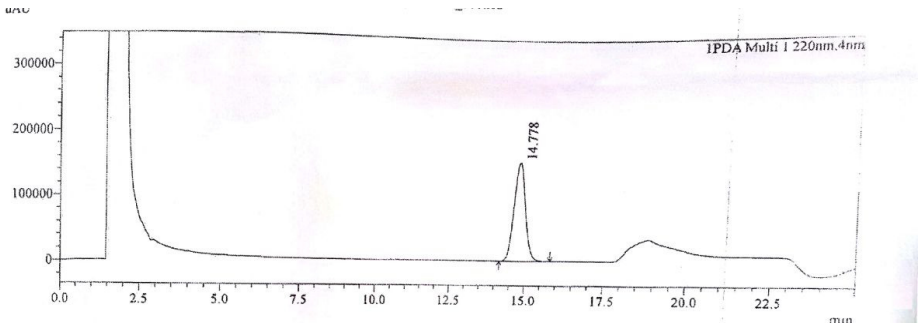

Figure 13 Leuprolide acetate Thermal degradation

Peak	Area of As such Sample	Area After degradation	As Such Assay	Assay After degradation	%degradation
1	3916870	3997183	105.7%	107.90%	3.2%

Table 12 Forced Degradation Summary

Condition	Area	%Assay	% Degradation	Peak Purity
As Such	3916870	105.7	-	-
Acid Degradation	3691567	100.40	4.61	0.999995
Base Degradation	3232282	89.10	16.48	0.999996
Peroxide Degradation	3795888	103.60	1.92	0.999996
Photolytic Degradation	3963838	107.30	2.41	0.999996
Thermal Degradation	3997183	107.90	3.2	0.999995

Hence, a method of the analysis of Leuprolide acetate in Parenteral dosage form shows that the degradation product doesn't interfere with the analytical determination. Hence the proposed analytical method is also useful for the determination of Leuprolide acetate stability in a sample of the pharmaceutical dosage form.

CONCLUSION

Stability indicating RP-HPLC methods have been developed and validated for the determination of Leuprolide acetate in depot formulation. The methods are found to be specific as there as no interference of any co-eluting impurities after stress degradation. The proposed method is found to be Simple, Accurate, Precise, Robust and Time consuming. Hence it can be used successfully for the routine analysis of Leuprolide acetate in Pharmaceutical dosage forms an for analysis of stability samples obtained during accelerated stability study.

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