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# Reverse Phase High Performance Liquid Chromatographic Method for Determination of Thymalfasin in Formulated Bulk Solution and Finished Dosage Form

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A simple, fast and sensitive reverse phase high performance liquid chromatographic method has been developed for the determination of thymalfasin by gradient elution in its dosage form. The separation of drug and all degradation products under various stress conditions was achieved using mixture of sodium phosphate buffer and acetonitrile at 1.0 mL/min flow rate on RP $C_{18}$ column. The detection was carried out at 210 nm. The method was statistically validated for its linearity, accuracy and precision. The intra- and inter-day variation was found to be less than 1% showing high precision of the assay method. The calibration curve was linear in the concentration range 50 to 150% of 400 ppm. Due to its specificity, high precision and accuracy, the proposed method may be used for determining Thymalfasin in formulated bulk solution or its finished dosage form.

**Key Words: High Performance Liquid Chromatography, Thymalfasin.**

## INTRODUCTION

Thymalfasin[1] is a thymus hormone; it is a component of thymosin fraction 5 (a crude thymus gland extract), which has been synthesized. Thymalfasin[2] is used alone or in combination with interferon as an immunomodulator for the treatment of chronic hepatitis B and C. It is administered by giving subcutaneous injection in a dose of 1.6 mg twice-weekly up to 12 months. It is also used to enhance the effectiveness of influenza vaccines in immunocompromised or elderly patients and of influenza and hepatitis B vaccines in chronic haemodialysis patients.

Thymalfasin[3] is a cell free, lymphocytopoietic hormone biologically active thymic factor, comprising a family of heat-stable polypeptides, that play an important part in the development of T-cells (thymus-dependent lymphocytes). The formulation is available in the market (*e.g.*, Zadaxin) in its injection form. The HPLC method for the determination of thymalfasin α-1 is not reported. In the present work a new, simple, rapid and accurate HPLC method for the determination of thymalfasin α-1 by gradient elution in its formulated form is studied.

## EXPERIMENTAL

The LC system (Agilent HP), consisted of Binary Pump solvent delivery system, a Peltier cooling autosampler, column oven and a photodiode array detector. The output signal was monitored and processed using a Chemstation software on a pentium computer (Hewlett-Packard).

Following is the gradient composition for 20 min run of the system.

| Time (min) | Solvent A (%) | Solvent B (%) | Flow rate (mL/min) | Column pressure (psi) |
|---|---|---|---|---|
| 00.0 | 90 | 10 | 1.0 | 1500 |
| 08.0 | 75 | 25 | 1.0 | 1500 |
| 10.0 | 90 | 10 | 1.0 | 1500 |
| 20.0 | 90 | 10 | 1.0 | 1500 |

Thymalfasin α-1 was supplied by Kopran Research Laboratory Limited. HPLC grade Acetonitrile (JT Baker), AR grade sodium dihydrogen phosphate, ortho-phosphoric acid and triethylamine were procured from E. Merck.

**Standard stock preparation:** Carefully prepared 4 mg/mL solution of standard using diluent in 5 mL standard volumetric flask. Shake well and store the sample in subdued atmosphere (away from the direct light) below 4°C.

In this experiment, severe stress stability was used in order to induce formation of degradation products and the study was conducted whether in all stress conditions the method is reliable. The result of this study shows that thymalfasin α-1 is stable at dry heat and acidic conditions. The strong degradation was observed during exposure to light, oxidation and basic treatment. The purity of the peaks of thymalfasin α-1 and other interfering potential compounds such as its degradation products and excipients were checked using a PDA detector and peak purity method. The following stress conditions were selected for degradation of thymalfasin α-1.

| Stress | Medium/conditions | Duration (min) |
|---|---|---|
| Acidic | 1 M hydrochloric acid solution | 60 |
| Basic | I M sodium hydroxide solution | 15 |
| Oxidation | 1% hydrogen peroxide | 30 |
| Photostability | 765 W/m$^3$ | 16 |
| Dry heat | 80°C | 36 |

## Method Linearity and Range

This experiment is carried out to demonstrate the response obtained with thymalfasi n α-1 is linear over specified range of concentration, *i.e.* any minor increase or decrease in the concentration of thymalfasin α-1 in the sample will be reflected by proportional increase or decrease in the detector response or in the areas under corresponding main peak. The system was studied for its ability to precisely detect the range of concentration of thymalfasin α-1. The concentration range for thymalfasin α-1 is selected as given below:

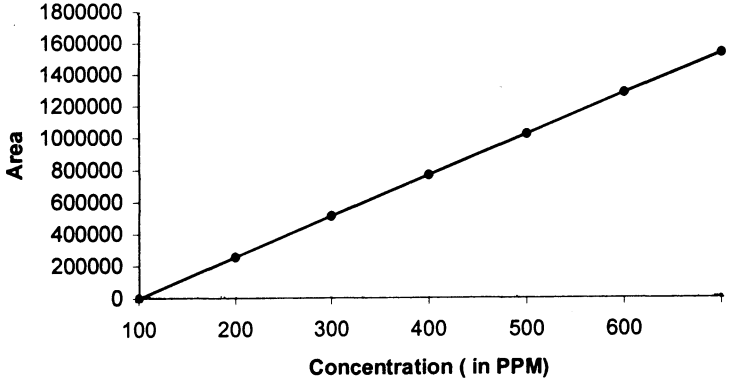
## Preparation of Linearity Solutions

Five linearity levels for thymalfasin α-1 were prepared by diluting standard stock solution as given below in the table:

| Dilutions | Linearity level (ppm) | Volume taken of dilutions | Volume made up to (mL) |
|---|---|---|---|
| One | 200 | 2.5 mL of dilutions three | 5 |
| Two | 300 | 2.5 mL of dilutions five | 5 |
| Three | 400 | 1.0 mL of std stock solution | 5 |
| Four | 500 | 2.5 mL of std stock solution | 5 |
| Five | 600 | 3.0 mL of std stock solution | 5 |

**Central value :** 400 ppm
**Range selected:** 200 ppm, 300 ppm, 400 ppm, 500 ppm and 600 ppm

Each 10 µL sample preparation was injected into the liquid chromatograph and the subsequent areas were recorded. Tables 1 and 2 show the regression analysis and graph 1 shows linearity graph of thymalfasin α-1. The plot ratio of area of thymalfasin α-1 *vs.* respective concentration (in ppm) is found to be linear in the range of 200 ppm to 600 ppm with coefficient of correlation 1.0. The intercept value was found to be not significantly deficient than zero.




Graph 1: Linearity graph of Thymalfasin α-1.

TABLE-1
LINEARITY DETERMINATION

| Observation No. | Concentration level (in ppm) | Area obtained due to thymalfasin α-1 |
|---|---|---|
| 1 | 200 | 512663 |
| 2 | 300 | 768994 |
| 3 | 400 | 1025325 |
| 4 | 500 | 1285655 |
| 5 | 600 | 1537988 |

TABLE-2
LINEARITY REGRESSION ANALYSIS

| No. | Parameter | Thymalfasin α-1 |
|---|---|---|
| 1. | Equation of the least square line | Y = 2567.31X–799.4 |
| 2. | Slope of the line | 2567.31 |
| 3. | Y-intercept at the line | –799.4 |
| 4. | Correlation coefficient | 0.99999 |

**Method Accuracy and Recovery:** The experiment was done to demonstrate to what extent the experimental value is closed to true value (in terms of amount). This is performed by measuring the accuracy of the method reported as percentage recovery by adding varying amounts of thymalfasin α-1 solution to known weight of thymalfasin α-1 stock solution.

**Preparation of standard solution:**

| Concentration of sample solution (ppm) | Concentration level (ppm) | Volume of std. stock solution added (mL) | Volume made up to (mL) |
|---|---|---|---|
| 400 | 20 | 1.0 | 20 |
| 400 | 30 | 1.5 | 20 |
| 400 | 40 | 1.0 | 10 |

The above dilutions were injected into the chromatograph and the responses obtained with test solution were compared with those of reference solution to calculate the amount of thymalfasin α-1 recovered and were expressed in terms of % recovery. The values of recovery experiment were presented in tabular form indicating added amount, amount recovered and percentage recovery (Table-3). The table is prepared containing mean percentage recovery, standard deviation of percentage recovery and relative standard deviation of percentage recovery.

TABLE-3
ACCURACY/RECOVERY OF THYMALFASIN α-1

| Concentration level (in ppm) added externally | Area observed due to thymalfasin α-1 std solution | Area obtained due to 400 ppm of thymalfasin α-1 | Area observed due to added thymalfasin α-1 | Accuracy (%) |
|---|---|---|---|---|
| 20 | 51209 | 1024325 | 10753639 – 1024325 = 51038 | 99.67 |
| 30 | 76814 | 1024325 | 1100969 – 1024325 = 76644 | 99.78 |
| 40 | 102416 | 1024325 | 1126571 – 1024325 = 102246 | 99.83 |
| | | Mean | | 99.76 |
| | | Standard Deviation | | 0.081 |
| | | Relative Standard Deviation | | 0.082 |

## Method Precision

Three injections of three different concentrations were injected into the chromatograph on the same day and the values of relative standard deviation were calculated to determine the intra-day precision. The same study was repeated after 1 week to determine inter-day precision.

## Procedure

1. Separately 3 different solutions of 200, 400 and 600 ppm of thymalfasin α-1 were prepared.
2. These were then diluted to the mark with the diluent.
3. Then 10 μL of each solution was injected once.

## Evaluation

Individual results are listed in tabular form. Table-4 is prepared to demonstrate mean and relative standard deviation, which shows the method and gives reproducible results during inter-day and intra-day precision.

TABLE-4
PRECISION

| Inter-day 1 precision | 200 ppm | 400 ppm | 600 ppm |
|---|---|---|---|
| Area observed due to thymalfasin α-1 std solution | 512122 | 1023569 | 1523698 |
| | 512275 | 1024442 | 1536887 |
| | 512325 | 1024562 | 1535691 |
| Mean (n = 3) | 512240.7 | 1024191 | 1532092 |
| Standard deviation | 105.77 | 542.00 | 7293.97 |
| RSD | 0.02 | 0.05 | 0.48 |

| Inter-day 7 precision | 200 ppm | 400 ppm | 600 ppm |
|---|---|---|---|
| Area observed due to thymalfasin α-1 std solution | 512715 | 1025563 | 1523785 |
| | 512359 | 1024542 | 1526953 |
| | 512553 | 1023212 | 1525562 |
| Mean (n = 3) | 512542.3 | 1024439 | 1525433 |
| Standard deviation | 178.24 | 1178.88 | 1587.91 |
| RSD | 0.03 | 0.12 | 0.10 |
| Intra-day precision* | 200 ppm | 400 ppm | 600 ppm |
| Mean (n = 6) | 512391.5 | 1024315 | 1528763 |
| Standard deviation | 210.91 | 831.78 | 5965.80 |
| RSD | 0.04 | 0.08 | 0.39 |

*For the calculation of intra-day precision the area of all three replicate injections are taken from both inter-day precision procedure.

## RESULTS AND DISCUSSION

The regression value was found to be 1.0 for thymalfasin α-1 which shows the response is linear from 200 to 600 ppm. Selectivity experiment showed that there is no interference or overlapping of peaks either due to its degradation products or excipients or main peak of thymalfasin α-1. High percentage of recovery shows that the method is free from interference of thymalfasin α-1. The RSD values for intra-day precision study are less than 0.5% and for the inter-day study are 1.0%, which confirm that the method is sufficiently precise. The proposed method is simple, accurate, precise, specific and selective. Thus, the proposed method can be used for the routine assay quantification or determining the uniformity of contents in quality control of thymalfasin α-1 in its finished dosage form.

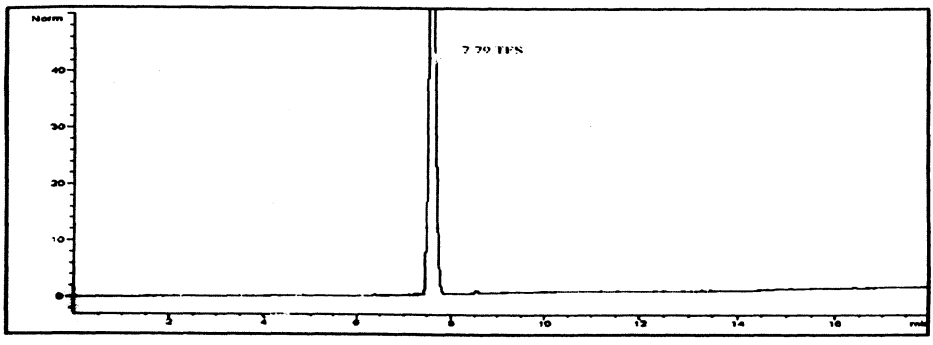



Fig. 1. Thymalfasin α-1 standard solution

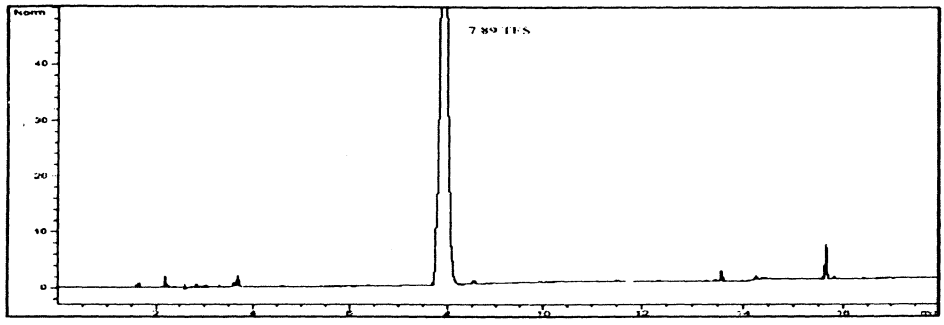


Fig. 2. Thymalfasin α-1 treated with 1M HCl for 60 min

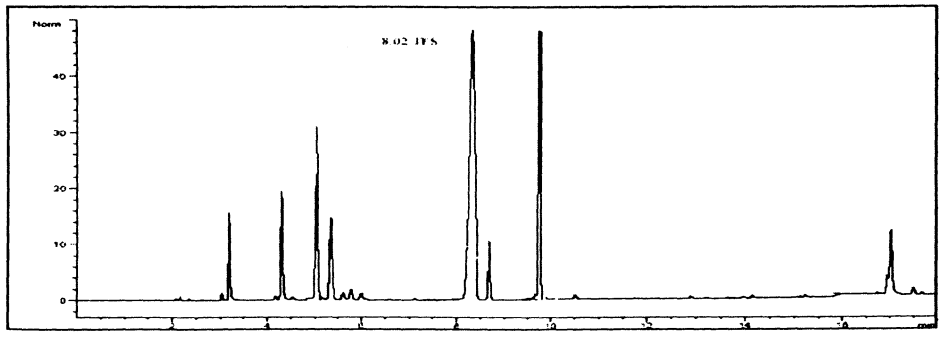


Fig. 3. Thymalfasin α-1 treated with 1M NaOH for 15 min

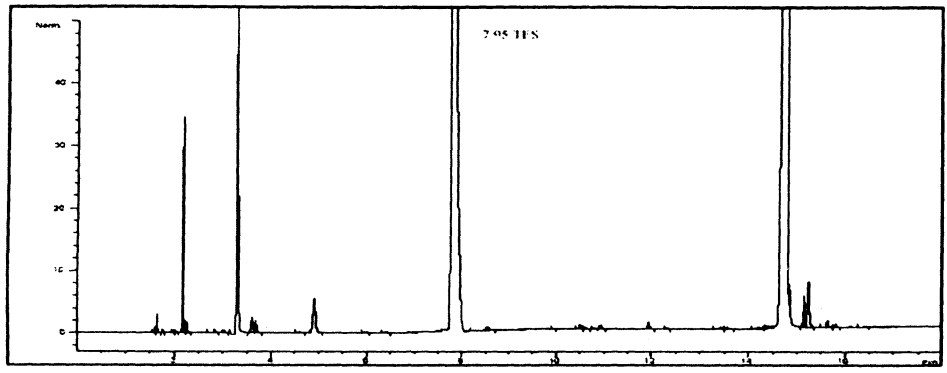


Fig. 4. Thymalfasin α-1 treated with 1% $H_2O_2$ for 30 min

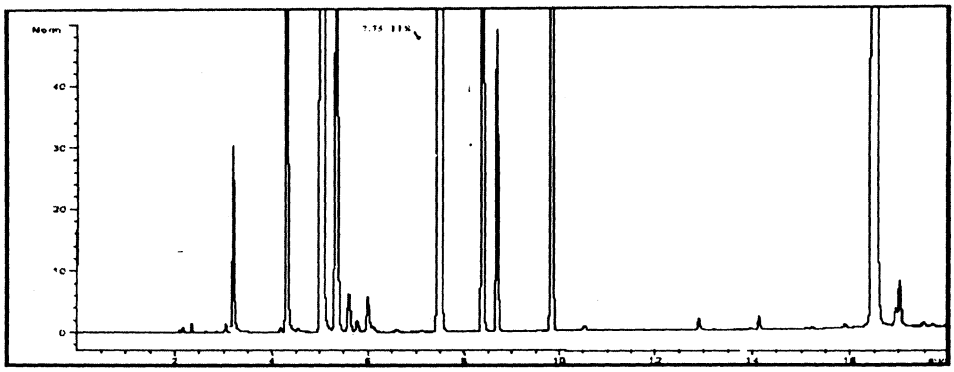


Fig. 5. Thymalfasin exposed to 765 W/m$^2$ for 16 h

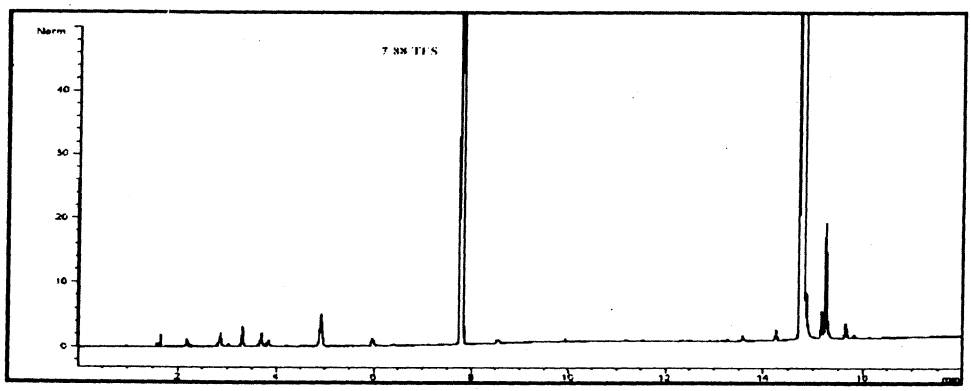


Fig. 6. Thymalfasin exposed to 80°C for 36 h

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