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Analytical Method Development and Validation of Stability Indicating RP-HPLC Method Including Isolation and Estimation of Some Degradation Product of Enoxaparin Sodium API.

BhavyaMehta, Dr. Ankit Chaudhary, Ms. Neetu Dharu
Saraswati Institute Of Pharmaceutical Science,

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ABSTRACT:
The present work describes a validated reverse phase high performance liquid chromatographic method for simultaneous estimation of Enoxaparin Sodium API and Degradant product. Chromatography was performed on C8 (250 mm x 4.6 mm i.d., 5 µm particle size) column with mobile phase containing 0.1% Trifluoroacetic acid: Acetonitrile: (40:60). The flow rate was 1.0 ml/min and the eluent was monitored at 230 nm. The selected chromatographic conditions were found to effectively separate Enoxaparin Sodium (RT- 3.412min) and Degradant product (RT- 6.514 min). Linearity for Enoxaparin Sodium and Degradant product was found in the range of 50- 150µg/ml and 12.5-37.5µg/ml respectively. The proposed method was found to be fast, accurate
precise, and reproducible and can be used for simultaneous estimation of API.
KEYWORDS: Enoxaparin Sodium, Degradant Product, Reversed-phase HPLC

I. INTRODUCTION

Anticoagulants are the drug's use to prevent clotting and thus prevent thrombosis Blood clotting define as that blood been converted from a liquid to a solid state. IT is also called as thrombus formation, which prevent blood clot is called as anticoagulants.

Blood coagulation or clotting is a possess by which the blood transformation from a liquid to a gel- like substance and forms a blood clot. The blood clotting process result in homeostasis and lead to repair of damage blood vessels after cessation of blood loss. The blood clotting process occurs with the use of platelets and fibrin.
So, anticoagulants are inhibition of this process results in decreased thrombin and ultimately the prevention of fibrin clot formation.

MechanismofActionAnticoagulants

The oral anticoagulants are antagonist of vitamin K. coagulation factor ii,vii,ix,x anticoagulant proteins C and S synthesized mainly in the liver and are biologically inactive unless 9 to 13 of the amino terminal glutamate residues are carboxylated to form the ca2+ binding r-carboxyglutamate residues. This reaction of the descaboxy precurosor protein requires carbon dioxide, molecular oxygen and reduced vitamin k, and is catalyzed by r-glutamyl carboxylase in the rough endoplasmic reticulum carboxylation is directly coupled to the oxidation of vitamin k to its corresponding exoxide. The enzyme that catalyzed this vitamin k epoxide reductase is inhibited by pharmacokinetics coumarins.

Introduction to HPLC

HPLC is the most widely used of all of the analytical separation techniques. It is a physical separation technique conducted in the liquid phase in which a sample is separated into its constituent components (or analytes) by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column). An online detector monitors the concentration of each separated component in the column effluent and generates chromatogram.

HPLC is presently used in pharmaceutical research and development in following areas: Purification of synthetic or natural products, in pharmacokinetic and pharmaco dynamics studies, in characterization of metabolites, Assay of active ingredients, impurities, degradation products and in dissolution studies.

Typical HPLC system consists of the following main components:
Solvent Reservoirs: For storage of sufficient amount of HPLC solvents for continuous operation of the system. A degasser is needed to remove dissolved air and other gases from the solvent.
Pump: This provides the constant and continuous flow of the mobile phase through the system; most

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modern pumps allow controlled mixing of different solvents from different reservoirs. Its performance directly affects retention time, reproducibility and detector sin captivity. Three main types of pumps are: Displacement, Reciprocating and Pneumatic or constant pressure pump.

Injector: This allows injection of the analytes mixture into the stream of the mobile phase is for eitenters the column. Most modern injectors are auto samplers, which allow programmed injections of different volumes of samples. Three ways of introducing the sample into injection port: Loop injection, Valve injection and on column injection.

Column: This is the heart of HPLC system. The column is usually made up of heavy glass or stain less steel tubing to with stand high pressure .The columns are usually10- 30cm long and 4-10mm in side diameter .Columns with an internal diameter of 5mm give good results because of compromise between efficiency, sample capacity, and the amount of packing and solvent required.

Column packing: It consists of small, rigid particles having an arrow particle size distribution. Three main types of column packing are: Porouspolymeic beds, Porouslayer beds, Totally Porous silica particles (diameter <10µm).

Detector: This is advice for continuous registration of specific physical (sometimes chemical) properties of the column effluent. The most common detector used in pharmaceutical analysis is UV. They are usually of two types:

- Bulk property detectors-Refractive index, Dielectric constant or Density.
- Solute property detectors-UV (Diode Array Detectors), Fluorescence or Diffusion current.

Data Acquisition and Control System: Computer-based system that controls all parameters of HPLC instrument and acquires data from the detector and monitors system performance.

Introduction to LC-MS

❖ **Mobile Phase:-**

– The mobile phase is the solvent that moves the solute throughout column.
– General requirements:-
(1) Low cost, UV transparency, high purity.
(2) Lowv is cosity, low toxicity, non flammability.
(3) Non corrosive to LC system component.
– Solvent strength and selectivity: - It is the ability of solvent to elute solutes from a column.

❖ **Column:-**

– Column type
– Specialized mode
– The use of di-functional or tri-functional silanes to create bonded groups withtwo or three attachement points leading to phases with higher stability in lowor higher pH and lower bleed for LCMS
– Most widely used columns for LCMS are:-
(1) Fast LC column.The use of short column (15-50mm)
(2) Micro LC column.The use of large column.(20-150mm)

❖ **Ionization and Interface:-**

– It is difficult to interface a liquid chromatography to a mass-spectrometer cause of the necessity to remove the solvent.
– The commonly used interface are:-
(1) Electro spray ionization(ESI)
(2) Thermo spray ionization(TSI)
(3) Atmospheric pressure chemical ionization(APCI)
(4) Atmospheric pressure photo ionization(APPI)
(5) Partial beamionization.

❖ **Electron Spray Ionization:-**

Generate analyte ions in solution before the analyte reaches the mass spectrometer.
– The LC eluent is sprayed (nebulizer) into a chamber at atmospheric pressure in the presence of a strong electrostatic field and heated drying gas.
The electrostatic field causes further dissociation of the analyte molecules.

II. EXPERIMENTATION

6.1 PreliminaryAnalysisofEnoxaparinSodium

1) Description

The sample of Enoxaparin Sodium was observed for its color and texture.

2) Melting point

The sample of Enoxaparin Sodium was taken in capillary and place into the melting point apparatus.

3) Identification Test

IR Spectra of Enoxaparin Sodium was observed in the region of 4000–500cm -1 in FTIR.

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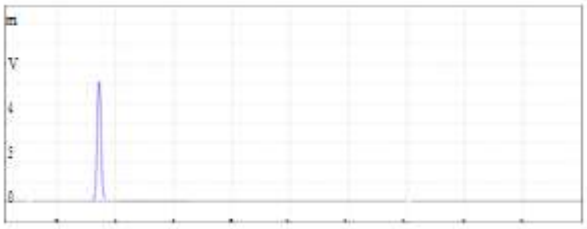
4) Solubility: The sample of Enoxaparin Sodium was taken in test tubes and observed for solubility in various solvents like water, methanol, Acetonitrile.

Instruments and Apparatus

Sr.no.	NameofEquipment /Instrument	Manufacturer
1	UVSpectrophotometer	LabMan
2	HPLC	Model: Spectra series HPLCColumn:C8(250mmx4.6mmx5µm)column Injector: 20µL "fixed loop"Detector: UV DetectorSoftware:PeakABCSolution
3	Analyticalbalance	ElectronicBalance(Shimadzu AUX220)
4	pH Meter	LabMan
5	MeltingPointApparatus	LabMan
6	Ultrasonicator	Lab-India
7	FT-IR	Lab-India

Selection of Wavelength
Standard solution of Enoxaparin Sodium (10 µg/mL) was scanned between 200-400 nm using UV-visible spectrophotometer.
Selected wavelength 230 nm.



HPLC Chromatogram of Enoxaparin Sodium 100 µg/ml in 0.1% TFA buffer, pH 2.5: Acetonitrile (40:60)
Force Degradation

Acid Degradation

Acid decomposition studies were performed by transferring 2 ml of stock solution in to 20 ml of volumetric flask. 2 ml of 0.1 N HCl solutions was added and mixed well and solution was heated for 60 min for acid hydrolysis. Then the solution was neutralized with 0.1 N NaOH and then volume was made up to the mark with diluent to get 100 µg/ml and filtered through 0.45 µm membrane filter paper and injected in HPLC system. Similarly solution wearer prepared for respective conditions mentioned for acidic degradation optimization and injected in to HPLC to observe the peak.

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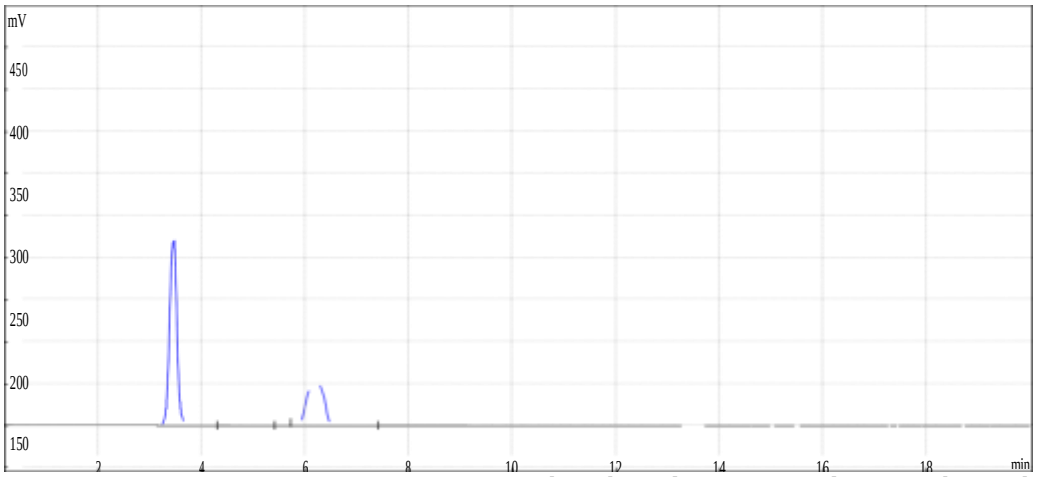
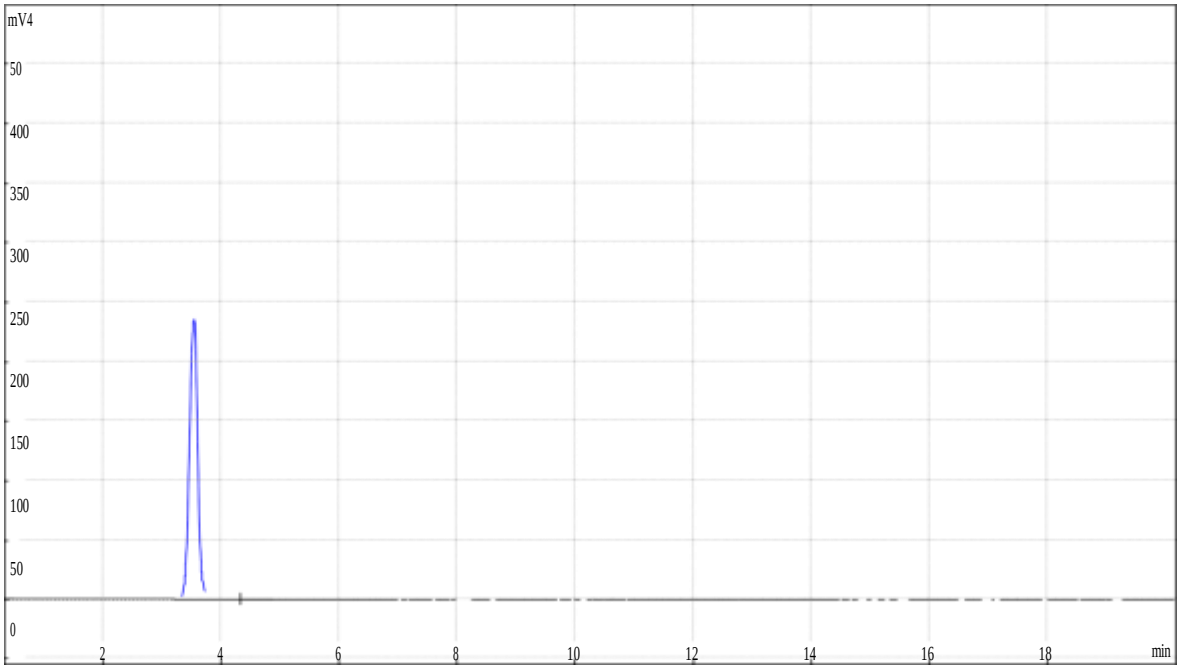


Photo Degradation

Photo decomposition studies were performed by transferring 2 ml of stock solution in to 20 ml of volumetric flask. Make up with diluents transfer in to the vial and kept under UV chamber for 2 hrs then the volume was made up to the mark with diluent to get 100 µg/ml and filtered through 0.45 µm membrane filter paper and injected in HPLC system to observe the peak.



III. OBSERVATION

Validation Parameters

Sr. No.	Parameter	EnoxaparinSodium	DegradantProduct
1	Specificity	Specific	

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2	Linearity/Range(µg/ml)		50 -150µg/ml	12.5 -37.5µg/ml
3	Regressionequation		y=24924x +34982	y=28661 –6819
4	Correlationco-efficient (r)		0.9991	0.9993
5	Precision(%RSD)	Repeatability	0.763	0.667
		Intraday	0.409-0.767	0.420–1.03
		Interday	0.402-0.710	0.391–0.728
6	Accuracy (% recovery)	50%	100.157	-
		100%	101.006	-
		150%	100.000	-
7	LOD		0.939µg/ml	2.847µg/ml
8	LOQ		1.127µg/ml	3.417µg/ml
9	Robustness		TheMethodwasFoundRobust	

IV. CONCLUSION

The quality and safety of the drug product not only depends on the adopted manufacturing procedure and toxicological properties of an active substance but also depends on the Degradant Product that it contains. Hence, a thorough examination of Degradant Product plays a significant role in controlling the quality of a drug product. In the current research work, a simple, sensitive, specific, and accurate HPLC method was developed for separation and determination of Enoxaparin Sodium in the presence of its Degradant Product. The developed method was validated as per the ICH guidelines and it was found to be precise, linear, and robust. Hence, the present method can be adapted to separate, identify, and quantify the Degradant Product of Enoxaparin Sodium Active Pharmaceutical Indigent.

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