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Novel Peptide Impurity From Bivalirudin Formulation, Its Detection, Purification And Structure Elucidation Using Amino Acid Sequencing Along With1h And 13c Nmr Spectroscopy

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Abstract:
Bivalirudin is used as anticoagulant in patients with coronary artery disease undergoing percutaneous coronary intervention. Also it is safe and cost effective as compared to other anticoagulants which are in use. Bivalirudin is a peptide having 20 amino acids. Related impurities formed during the manufacturing process determine quality of the peptide drugs. These impurities may cause toxicity or change the desired efficacy. ICH guidelines established by regulatory authorities have provided identification threshold for related impurities to ensure purity of these peptide drugs. Identification, characterization and quantification of related impurities are called impurity profiling and regulatory authorities insist it to assure and control the quality. The stability studies sample of bivalirudin formulation received from pharmaceutical company Piramal Pharma Ltd shown one unknown impurity at RRT 0.54. New HPLC method with reverse phase C18 column having shorter run time than reported method was developed and also preparative HPLC method was developed for final purification of this impurity. Initial concentration of this impurity isaround 1.01% which after forced degradation increased to around 19.84%. Adsorption column chromatography was done to further enrich it to 65% and finally to 97.76% purity with preparative HPLC using reverse phase C18 column. Amino acid sequencing using High Resolution Mass Spectrometry was carried out on purified impurity to get amino acid sequence. Exact molecular weight found is 1683.6884 Da. The structure established by amino acid sequencing was further confirmed using 1H and 13C NMR data. The sequence of amino acids found was Glycine – Glycine – Glycine – Glycine – Asparagine – Glycine – Aspartic acid – Phenylalanine – Glutamic acid – Glutamic acid – Isoleucin – Proline – Glutamic acid – Glutamic acid – Tyrosine – Leucine. Any of the reported impurity from bivalirudin is not having this sequence as well as molecular weight. Therefore we conclude that we have detected, purified and structurally characterized novel impurity from bivalirudin formulation.

Keyword: *Bivalirudin, Mass, NMR Spectroscopy, HPLC, Amino acid sequencing, Anticoagulant*

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I. Introduction

Bivalirudin is anticoagulant drug, synthetic peptide having 20 amino acids and monoisotopic mass of 2178.986 Da. It isan analogue of Hirudin used during various coronary surgeries to prevent blood clotting and myocardial infarction.

Bivalirudin was first discovered by The Medicines Company from USA as an anticoagulant drug with brand name Angiomax. It has got amino acid sequence as (D-Phe-Pro- Arg-Pro-Gly-Gly-Gly-Gly-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu). Bivalirudin is marketed in USA as lyophilised powder comprising 250 mg and 100 mg per vial. It is to be reconstituted with water before injection with slightly acidic PH. Improper reconstitution fails to deliver optimal dose also process involves multiple steps and high manufacturing cost. Also peptide based drugs degrades in aqueous solution. To overcome these limitations there is a need to develop stable, ready to use and non aqueous formulation which can be injected directly. Pharmaceutical company, Piramal Pharma Ltd. had developed and patented such formulation. One unknown impurity was developed at RRT 0.54 during six months stability study of one such formulation. It exceeds the limit specified for unknown impurity. Therefore there arises the need to separate, purify and structurally characterize thisimpurity as per regulatory requirement.

As per literature reports, impurities from peptide drugs are isolated and purified by using reverse phase HPLC. The reported method used for separation and quantification of all impurities from bivalirudin uses

Phenomenex Kinetex 2.6µ XB-C18 (150x 4.6mm) column and a gradient of buffer and acetonitrile. The buffer used was 3.4 gm of Potassium dihdyrogen orthophosphate dissolved in 1L of water and PH was adjusted to 3.5 with ortho phosphoric acid. The gradient program (T/%Acetonitrile) was set as 0/20, 60/22, 62/20, 65/20. The buffer was filtered through nylon 0.45-mm membrane filter. The flow rate of mobile phase was kept at 0.6 ml/min and column temperature at 45^0C. The HPLC was monitored at 210nm. Injection volume was 5ul. The diluent for sample preparation was mixture of water and acetonitrile in the ratio of 1:1. Total five impurities reported from Bivalirudin formulation are BIVA (12-20), PLUS-GLY, DES- GLY, BETA-ASP9, ALPHA-ASP9 and under above reported HPLC method, they elutes at RRT 0.40, RRT 0.96, RRT 1.07, RRT 1.14 and RRT 1.19 respectively. New impurity generated elutes at RRT 0.54 under reported HPLC conditions.Therefore it is not matching with any of the known impurity from bivalirudin. The molecular weight of bivalirudin is 2180.29 Da and known impurities have molecular weights 1168.25 Da [BIVA (12-20)], 2237.34 Da [PLUS-GLY], 2123.23 Da [DES-GLY], 2181.27 Da [BETA-ASP9] and 2181.27 Da [ALPHA-ASP9]. Recently one more impurity reported which is bivalirudin isomer D-Asn Impurity having same molecular weight 2180.29 Da. New impurity at RRT 0.54 is having molecular weight 1683.6884 Da as per LC-HRMS. This is not matching with any of the reported impurity from bivalirudin. Hence we conclude that, we have identified a novel impurity from bivalirudin formulation. The advantage of HPLC method which we have developed is shorter run time of 10 min as compared to 65 min for reported method with resolution of all the above listed impurities. The Prep HPLC fractions can be monitored quickly with this new developed method. The original impurity at RRT 0.54 from stability study sample was increased to 19.84% with forced degradation by Piramal Pharma Ltd. We have developed method to remove the excipients from the formulation and enriched this impurity to around 65% with open column chromatography followed by preparative HPLC to isolate this impurity to purity 97.76% by area percentage. The LC-HRMS, amino acid sequencing and spectroscopic data like 1H NMR and 13C NMR was recorded on this purified impurity. This novel impurity was assigned, following structure after analyzing all the above data as reported in results and discussion section.

II. Literature Review

Jing Li, et al. (2022) reported safety and efficacy of Bivalirudin versus Heparin. Their study concluded that bivalirudin was significantly associated with lower rates of bleeding and mortality, compared with heparin monotherapy [1]

Kostromina M.A., et al. (2021) reported in their article that currently, synthetic bivalirudin (Angiomax, The MedicinesCompany, USA) is the most promising anticoagulant among hirudin-1 analogues [2].

Alessandra Basso, et al. (2019) reported in journal of chromatography Today, reverse phase HPLC method for thepurification of bivalirudin with > 99% purity and greater than 99% yield [3].

Nilini Ranbaduge, et al. (2018) demonstrated with examples how LC-HRMS can be used for peptide Impurity Profiling Using a Compliance-Ready LC-HRMS Workflow and MassLynx and ProMass Softwares from Waters [4].

Hua Yang, et al. (2018) reported various types of columns and mobile phases to be used for identification, separation andpurification of reported bivalirudin impurities [5].

Bondigalla. Ramachandra, et al. (2016) reported modern analytical techniques used for impurity profiling, particularly theUPLC, LC-MS, HRMS, GC-MS, HPTLC and NMR [6].

Vaillancourt K, et al. (2015) reported purification and characterization of novel peptide. The purification was carried outby cationic exchange and reversed-phase high-pressure liquid chromatography [7].

Kui Zeng, et al. (2015) reported LC-HRMS method for bivalirudin and its related impurities to determine amino acidcomposition [8].

Xiao-Jiao Li, et al. (2013) reported quantitation of bivalirudin in human plasma by LC-MS/MS with triple-quadrupolemass spectrometer, equipped with electro spray ionization (ESI) interface, and operated in the positive ion mode [9].

Van Dorpe S, et al. (2011) reported impurity profiling of peptide drugs with the emphasis on the related impurities. Regulatory authorities are insisting for impurity profiling of pharmaceutical drugs that includes identification, quantification and characterization in order to assure and control the quality [10].

Young Moon Choi, et al. (2009) reported that Angiomax injection having generic name Bivalirudin and launched by The Medicines Company is a direct thrombin inhibitor which is approved as an anticoagulant for coronary angioplasty [11].

Sonavaria vandana, et al. (2016) reported in international patent publication, a stable injectable composition of bivalirudinand process for its preparation [12].

Guodong Chen, et al. (2008) reported in their review article, current capabilities and future trends with respect to LC-MSfor protein characterization [13].

ICH, Q3B (R2) (2006): Reporting and identification threshold for impurities in new drug products were reported by FDA[14].

Carswell CI, et al. (2002) presented a review of Bivalirudin with its potential in the management of acute coronary syndromes, a complete profile of Bivalirudin and its importance over the other drugs from this class [15].

Ramaprasad S, et al. (1993) reported 1H and 13C NMR assignments of pentapeptide with similar types of amino acids asobserved in novel peptide which we have isolated and purified [16].

Guoliang Li, et al. (2012) reported UFLC-MS/MS method for quantification of bivalirudin. The acetonitrile and 0.2%formic acid (20:80, v/v) was used as mobile phase having 0.35 mL/min flow rate [17].

III. Research Methodology

Materials and Chemicals

Bivalirudin formulation degraded sample was procured from Piramal Pharma Ltd. LR grade solvents used for open column chromatography were procured from Fischer Scientific. Adsorption resin, Diaion HP-20 was from Sigma- Aldrich. All solvents and reagents used for HPLC were HPLC grade. Acetonitrile was procured from Qualigens (India).Trifluoro acetic acid was from Merck (India). MilliQ filtration unit, Millipore synergy (Millipore, France) was used for HPLC grade water.

Instrumentation

Analytical HPLC development was done on Waters Alliance HPLC system with 2996 diode array detector and Empower 2 software. Preparative HPLC was done on Waters Prep 2000 system with 2487 dual wavelength detector and fraction collector. Solvent evaporation was done on rotary evaporator from Buchi, model R-215. Lyophilizer used for freeze drying was from Thermo- super modulo model. Membrane filters of 0.22 µ were used for HPLC sample preparation. High resolution Mass Spectrometry on Synapt® G2-Si High Definition Mass Spectrometer (QToF) from Waters with dual-spray electro spray ionization (ESI) source was used for sequencing of amino acids. Acquisition software used was MassLynx and insilico analysis was done using BioLynx. Bruker 400 MHz spectrometer was used for NMR (1H and 13C) and solvent used was DMSO-d6.

Analytical Method for HPLC

The reported method used for separation and quantification of all impurities from bivalirudin uses Phenomenex Kinetex 2.6µ XB-C18 (150x 4.6mm) column and a gradient of buffer and acetonitrile. The buffer used was 3.4 gm of Potassium dihdyrogen orthophosphate dissolved in 1L of water and PH was adjusted to 3.5 with ortho phosphoric acid. The gradient program (T/%Acetonitrile) was set as 0/20, 60/22, 62/20, 65/20. The buffer was filtered through nylon 0.45-mm membrane filter. The flow rate of mobile phase was kept at 0.6 ml/min and column temperature at 45^0C. The HPLC was monitored at 210nm. Injection volume was 5ul. The diluent for sample preparation was mixture of water and acetonitrile in the ratio of 1:1. The new impurity elutes at 19.9 min under above reported HPLC method. New HPLC method was developed using volatile buffers as they are more preferable for preparative HPLC purification and shorter run time is better for monitoring the fractions of column purification. Developed method had shown similar HPLC profile and separation of all the impurity peaks as reported method.

The new HPLC method was developed using Phenomenex C18, 2.5µ (50 x 4.6mm) column with an isocratic program containing solvent A and B. Solvent A is acetonitrile and solvent B is 0.1% aqueous trifluoroacetic acid. The isocratic mobile phase was set as 22%Acetonitrile in 0.1% aqueous trifluoroacetic acid. Flow rate was 1ml/min. The column temperature was maintained at 45^0C and monitoring wavelength was kept at 210nm in a PDA system set at 205nm to 400nm. Acetonitrile and water in the ratio of 1:1 was used as diluent. The new impurity elutes at retention time 2.4 – 2.5min under new developed method.

Excipients removal and enrichment of impurity

The patented formulation procured from pharmaceutical company, Piramal Pharma Ltd. has excipients like polyol, non aqueous solvents and PH adjustment agents. These excipients were removed using adsorption column chromatography with resin Diaion HP-20. Formulation was diluted with water five times and loaded on adsorption resin Diaion HP-20. After collecting spent, water wash was given to the column (2 bed volumes) and then elution was done using step gradient of acetonitrile in water. The elution started with 2% acetonitrile in water with increment of 2%, each with two bed volumes. The desired impurity got eluted in 20% acetonitrile in water as observed by HPLC with around 65% purity. The off-white powder was obtained when enriched fractions having desired impurity were pooled and lyophilized.

Preparative Method of HPLC

After initial load optimization and column selection, final preparative HPLC method was developed using Knauer eurosphere C18, 250 x 32 mm, 10 µ column with step gradient of acetonitrile in 0.1% aqueous

trifluroacetic acid (TFA). Elution was done starting with 2% acetonitrile in 0.1% TFA and increment of 2% each. The prep HPLC was monitored at210 nm with flow rate, 40ml/min and column temperature 45^0C. Sample loading was done by dry charged and peaks werecollected using fraction collector. Fractions were monitored by analytical HPLC using developed method as described in section 3.3. Fractions having > 95% purity of the desired impurity by HPLC were pooled and lyophilized to get impurity with 97.76% purity. Amino acid sequencing and spectral data was recorded on this sample.

Sequencing of amino acids using High Resolution Mass Spectrometry

The molecular weight of the impurity, fragmentation pattern of peptide by MS-MS study and tentative molecular formula was determined by this study. CID based fragmentation study has been performed on the impurity and the MS/MS fragment spectra with annotation of b and y ions was done. The amino acid sequence generated using the annotated b and y ions and the tentative molecular formula has been provided. The instrument used is hybrid quadrupole/orthogonal acceleration, time-of-flight (oa-TOF) mass spectrometer having high sensitivity and speed. Ion mobility is the useful technique to study the peptides. MassLynx software was used for acquisition and BioLynx for insilico analysis.

MassLynx is a application that controls Waters mass spectrometer. BioLynx contain Protein/Peptide Editor tool, which isused to analyze peptide sequences so as to facilitate the interpretation of data obtained from mass spectrometry. Various matching routines allow data to be automatically matched against known protein or peptide structures. The sequence of peptide was determined using Peptide Sequencing program.

IV. Results And Discussion

Analytical HPLC Method development

The aim of the HPLC method development was to get the method having the same HPLC profile as observed in reported method with regards to all the impurities but having shorter run time and uses volatile buffers which can be removed very easily during purification. Since reported HPLC methods for bivalirudin formulation are either having long run time or having buffers which are very difficult to remove during purification process. The method which we have developed is having run time of 10 min as against 65 min for reported method and uses 0.1% Trifluoroacetic acid as volatile buffer.

Various types of columns with reverse phases and different buffers with combination of solvents like methanol and acetonitrile were tried for optimization. Finally method development was done which had fulfilled all the above criteria and preparative HPLC purification can be done using the same method. The new HPLC method was developed using Phenomenex C18, 2.5µ (50 x 4.6mm) column with an isocratic program containing solvent A and B. Solvent A is acetonitrile and solvent B is 0.1% aqueous trifluoroacetic acid. The isocratic mobile phase was set as 22%Acetonitrile in 0.1% aqueous trifluoroacetic acid. Flow rate was 1ml/min. The column temperature was maintained at 45^0C and monitoring wavelength was kept at 210nm in a PDA system set at 205nm to 400nm. Acetonitrile and water in the ratio of 1:1 was used as diluent. The new impurity elutes at retention time 2.4 – 2.5 min whereas in reported method it was around 19.9 min.

Impurity Enrichment

Diaion HP-20 adsorption resin was used for removal of excipients and enrichment of impurity. 1L of formulation was diluted with 4L of water and loaded on 1L of Diaion HP-20 resin packed in 4.5 x 30 cm glass column. Spent collection was done and then sufficient water wash was given (2 to 3 bed volume) to remove water soluble excipients. Elution was carried out using step gradient of acetonitrile in water. Elution started with 2% acetonitrile in water with increment of 2% after every step. 1L size fractions were collected and monitored on analytical HPLC using the method which we have developed for presence of desired impurity of RRT 0.54 as described in section 4.1. The desired impurity was observed in 22% acetonitrile in water elutes. 22% acetonitrile in water fraction no. 3 to 20 having purity around 65% by HPLC were pooled and lyophilized to get 2.1 gm of impurity as off white compound.

Purification using Preparative HPLC

Knauer eurosphere C18, 250 x 32 mm, 10 µ column was used for preparative HPLC method development as reported in section 3.5. Above enriched impurity of 2.1 gm was dry charged on C18 resin and packed in pre column. Elution was carried out using step gradient of acetonitrile in 0.1% Aq.TFA. The step gradient was set from 2% acetonitrile in 0.1% aq. Trifluoroacetic acid with 2% increment after every 10 min. Fractions of 50 ml size were collected for all the peaks from preparative HPLC. All these fractions were monitored using analytical method which we have developed as reported in earlier section. Fractions no. 73 to 95 having purity of more than 95% by HPLC method were pooled and lyophilized to get 530 mg of impurity as white compound with purity of 97.76% by HPLC area percentage method.

Sequencing of Amino acids using High Resolution Mass Spectrometry

Bivalirudin is a polypeptide having molecular weight 2180 Da having 20 amino acids as per literature. The amino acid sequence reported for bivalirudin is D-Phe–Pro–Arg–Pro–Gly–Gly–Gly–Gly– Asn–Gly–Asp–Phe–Glu–Glu–Ile–Pro– Glu–Glu–Tyr–Leu. Here the abbreviations are Phe is phenylalanine, Pro for proline, Arg is arginine, Gly for glycine, Asnis asparagine, Asp for aspartic acid, Glu is glutamic acid, Ile for isoleucin, Tyr is tyrosine and Leu for leucine. Molecular weight of the impurity found as per deconvoluted mass spectra using maximum entropy algorithm is 1683.6884 Da.

Annotated MS/MS fragment spectra of b and y ions of impurity obtained with its respective amino acid sequence is [GGGGNGDFEEIPEEYL]. Here abbreviations are G for glycine, N for asparagine, D for aspartic acid, F for phenylalanine, E for glutamic acid, I for isoleucin, P for proline, Y for tyrosine and L for leucine. Therefore the sequenceof amino acids for this impurity is Glycine – Glycine – Glycine – Glycine – Asparagine – Glycine – Aspartic acid – Phenylalanine – Glutamic acid – Glutamic acid – Isoleucin – Proline – Glutamic acid – Glutamic acid – Tyrosine – Leucine. These amino acids were connected by amide linkages between acid and amine group to get the structure of this novel impurity as shown in figure 1. The high resolution mass spectrometry method has provided the elemental composition for this impurity as $C_{73}H_{103}N_{17}O_{29}$. The calculated molecular formula and formula weight for the structure shown in figure 1 matches with Elemental composition and molecular weight provided by deconvoluted mass spectra.

Therefore this data supported the derived structure of this novel impurity. This structure was also confirmed by 1H and13C NMR experiments as shown in section 4.5 and 4.6.

V. Conclusion

We conclude that, we have detected, purified and structurally characterized novel impurity using amino acid sequencingand 1H, 13C NMR spectroscopy from bivalirudin formulation.

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