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Novel Peptide Impurity From Bivalirudin Formulation, Its Detection,PurificationAndStructureElucidationUsingAminoAcidSequencingAlongWith1hAnd13cNmrSpectroscopy

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Abstract:
Bivalirudin is used as anticoagulant in patients with coronary artery disease undergoing percutaneous coronaryintervention. Also it is safe and cost effective as compared to other anticoagulants which are in use. Bivalirudin is apeptide having 20 amino acids. Related impurities formed during the manufacturing process determine quality of thepeptide drugs. These impurities may cause toxicity or change the desired efficacy. ICH guidelines established byregulatory authorities have provided identification threshold for related impurities to ensure purity of these peptidedrugs. Identification, characterization and quantification of related impurities are called impurity profiling andregulatory authorities insist it to assure and control the quality. The stability studies sample of bivalirudin formulationreceived from pharmaceutical company Piramal Pharma Ltd shown one unknown impurity at RRT 0.54. New HPLCmethod with reverse phase C18 column having shorter run time than reported method was developed and alsopreparative 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 wasdone 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 aminoacid sequence. Exact molecular weight found is 1683.6884 Da. The structure established by amino acid sequencingwas further confirmed using 1H and 13C NMR data. The sequence of amino acids found was Glycine – Glycine –Glycine –Glycine–Asparagine–Glycine–Asparticacid–Phenylalanine–Glutamicacid–Glutamicacid –Isoleucin – Proline – Glutamic acid – Glutamic acid – Tyrosine – Leucine.Any of the reported impurity frombivalirudin is not having this sequence as well as molecular weight. Therefore we conclude that we have detected,purifiedandstructurallycharacterizednovelimpurityfrombivalirudinformulation.

Keyword:*Bivalirudin,Mass, NMRSpectroscopy,HPLC,Aminoacidsequencing,Anticoagulant*

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I. Introduction

Bivalirudinis anticoagulantdrug, syntheticpeptidehaving20aminoacidsandmonoisotopicmassof2178.986Da. ItisananalogueofHirudinused duringvarious coronarysurgeriestopreventbloodclottingandmyocardialinfarction.

Bivalirudin was first discovered by The Medicines Company from USA as an anticoagulant drug with brand nameAngiomax. 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 tobe reconstituted with water before injection with slightly acidic PH. Improper reconstitution fails to deliver optimal dosealso process involves multiple steps and high manufacturing cost. Also peptide based drugs degrades in aqueous solution.Toovercometheselimitationsthereisaneedtodevelopstable,readytouseandnonaqueousformulationwhichcanbeinjected directly. Pharmaceutical company, Piramal Pharma Ltd. had developed and patented such formulation. Oneunknown impurity was developed at RRT 0.54 during six months stability study of one such formulation. It exceeds thelimit specifiedforunknown impurity.Thereforetherearises theneedtoseparate,purifyandstructurallycharacterizethisimpurityasper regulatoryrequirement.

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

Phenomenex Kinetex 2.6µXB-C18 (150x 4.6mm) column and a gradient of buffer and acetonitrile. The buffer used was 3.4 gm of Potassiumdihdyrogen orthophosphate dissolved in 1L of water and PH was adjusted to 3.5 with ortho phosphoric acid. The gradientprogram (T/%Acetonitrile) was set as 0/20, 60/22, 62/20, 65/20. The buffer was filtered through nylon 0.45-mmmembrane filter. The flow rate of mobile phase was kept at 0.6 ml/min and column temperature at 45^{0}C. The HPLC wasmonitored at 210nm. Injection volume was 5ul. The diluent for sample preparation was mixture of water and acetonitrilein 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, RRT1.07,RRT1.14andRRT1.19respectively.Newimpurity generated elutesat RRT0.54underreportedHPLCconditions.Therefore it is not matching with any of the known impurity from bivalirudin. The molecular weight of bivalirudin is2180.29 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 impurityreportedwhichisbivalirudinisomerD-AsnImpurityhavingsamemolecularweight2180.29Da.NewimpurityatRRT0.54 is having molecular weight 1683.6884 Da as per LC-HRMS. This is not matching with any of the reported impurityfrom bivalirudin. Hence we conclude that, we have identified a novel impurity from bivalirudin formulation. Theadvantage of HPLC method which we have developed is shorter run time of 10 min as compared to 65 min for reportedmethod with resolution of all the above listed impurities. The Prep HPLC fractions can be monitored quickly with thisnew developed method. The original impurity at RRT 0.54 from stability study sample was increased to 19.84% withforced degradation by Piramal Pharma Ltd. We have developed method to remove the excipients from the formulationand enriched this impurity to around 65% with open column chromatography followed by preparative HPLC to isolatethis impuritytopurity97.76%byareapercentage.TheLC-HRMS,aminoacidsequencingandspectroscopicdatalike1HNMR and 13C NMR was recorded on this purified impurity. This novel impurity was assigned, following structure afteranalyzingalltheabove data asreportedinresultsanddiscussionsection.

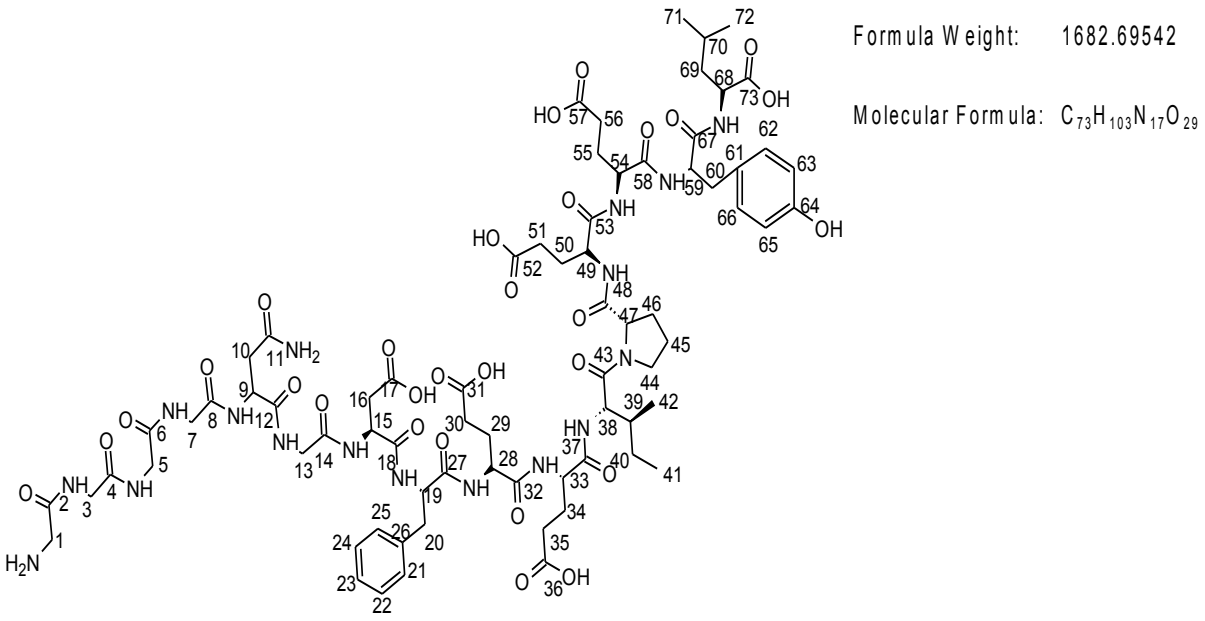

Fig.1 Chemical Structure of novel Impurity at RRT 0.54
(Arbitrary system for labeling protons and carbons for the purpose of making NMR assignments)

II. LiteratureReview

JingLi,etal.(2022)reportedsafetyandefficacyofBivalirudinversusHeparin.Theirstudyconcludedthatbivalirudinwas significantlyassociatedwithlowerratesofbleedingandmortality,comparedwithheparin monotherapy[1]

Kostromina M.A., etal. (2021)reportedintheirarticlethatcurrently, syntheticbivalirudin(Angiomax, TheMedicinesCompany,USA)isthe mostpromisinganticoagulantamonghirudin-1analogues[2].

Alessandra Basso, et al. (2019) reported in journal of chromatography Today, reverse phase HPLC method for thepurificationofbivalirudinwith>99% purityandgreaterthan99%yield[3].

NiliniRanbaduge,et al. (2018) demonstratedwith exampleshowLC-HRMScanbeusedforpeptideImpurityProfilingUsingaCompliance-ReadyLC-HRMSWorkflowandMassLynxandProMassSoftwaresfromWaters[4].

HuaYang,etal.(2018)reportedvarioustypesofcolumnsandmobilephasestobeused foridentification,separationandpurificationof reportedbivalirudinimpurities[5].

Bondigalla. Ramachandra, etal.(2016)reportedmodernanalyticaltechniquesusedforimpurityprofiling, particularlytheUPLC,LC-MS,HRMS,GC-MS,HPTLCandNMR[6].

VaillancourtK,etal.(2015)reportedpurificationandcharacterizationofnovelpeptide.Thepurificationwascarriedoutbycationicexchangeandreversed-phasehigh-pressureliquidchromatography[7].

KuiZeng,etal.(2015) reported LC-HRMSmethod forbivalirudin and itsrelated impuritiesto determine amino acidcomposition[8].

Xiao-JiaoLi,etal.(2013)reportedquantitationofbivalirudininhumanplasmabyLC-MS/MSwithtriple-quadrupolemassspectrometer,equippedwith electrosprayionization(ESI)interface,and operatedinthepositiveion mode[9].

VanDorpeS,etal.(2011)reportedimpurityprofiling ofpeptidedrugswiththeemphasisontherelatedimpurities.Regulatory authorities are insisting for impurity profiling of pharmaceutical drugs that includes identification,quantificationandcharacterizationinordertoassureandcontrolthequality[10].

YoungMoonChoi,etal.(2009)reportedthatAngiomax injectionhavinggenericnameBivalirudinandlaunchedbyTheMedicines Companyis adirect thrombininhibitorwhichis approvedasananticoagulantforcoronaryangioplasty[11].

Sonavariavandana, etal. (2016)reportedininternationalpatentpublication,astableinjectablecompositionofbivalirudinandprocessfo ritspreparation[12].

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

ICH,Q3B(R2)(2006):Reportingandidentificationthreshold forimpurities innewdrugproducts werereportedbyFDA[14].

CarswellCI,etal.(2002)presentedareviewofBivalirudinwithitspotentialinthemanagementofacutecoronarysyndromes,acompleteprofileofBivalirudinanditsimportanceovertheotherdrugsfromthisclass[15].

Ramaprasad S, et al. (1993) reported 1H and 13C NMR assignments of pentapeptide with similar types of amino acids asobservedinnovelpeptide whichwe have isolatedandpurified[16].

Guoliang Li, et al. (2012) reported UFLC-MS/MS method for quantification of bivalirudin. The acetonitrile and 0.2%formicacid(20:80,v/v)wasusedasmobilephasehaving0.35mL/minflowrate[17].

III. ResearchMethodology

MaterialsandChemicals

Bivalirudin formulation degraded sample was procured from Piramal Pharma Ltd. LR grade solvents used for opencolumn chromatography were procured from Fischer Scientific. Adsorption resin, Diaion HP-20 was from Sigma-Aldrich. AllsolventsandreagentsusedforHPLCwereHPLCgrade.AcetonitrilewasprocuredfromQualigens(India).Trifluoro acetic acid was from Merck (India). MilliQ filtration unit, Millipore synergy (Millipore, France) was used forHPLCgrade water.

Instrumentation

AnalyticalHPLC developmentwasdoneonWaters AllianceHPLC systemwith2996 diodearraydetectorand Empower2 software.PreparativeHPLCwasdoneonWatersPrep 2000systemwith2487 dualwavelengthdetectorandfractioncollector. Solvent evaporation was done on rotary evaporator from Buchi, model R-215. Lyophilizer used for freezedrying 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 withdual-spray electro spray ionization (ESI) source was used for sequencing of amino acids. Acquisition software used wasMassLynx and insilico analysis was done using BioLynx. Bruker 400 MHz spectrometer was used for NMR (1H and13C)andsolventusedwasDMSO-d6.

AnalyticalMethodforHPLC

The reported method used for separation and quantification of all impurities from bivalirudin uses Phenomenex Kinetex2.6µ XB-C18 (150x 4.6mm) column and a gradient of buffer and acetonitrile. The buffer used was 3.4 gm of Potassiumdihdyrogenorthophosphatedissolvedin1LofwaterandPHwasadjustedto 3.5 withorthophosphoricacid.Thegradientprogram (T/%Acetonitrile) was set as 0/20, 60/22, 62/20, 65/20. The

buffer was filtered through nylon 0.45-mmmembrane filter. The flow rate of mobile phase was kept at 0.6 ml/min and column temperature at 45^{0}C. The HPLC wasmonitored at 210nm. Injection volume was 5ul. The diluent for sample preparation was mixture of water and acetonitrilein the ratio of 1:1. The new impurity elutes at 19.9 min under above reported HPLC method. New HPLC method wasdeveloped using volatile buffers as they are more preferable for preparative HPLC purification and shorter run time isbetter for monitoring the fractions of column purification. Developed method had shown similar HPLC profile andseparationof allthe impurity peaksasreportedmethod.

The new HPLC method was developed using Phenomenex C18, 2.5µ (50 x 4.6mm) column with an isocratic programcontaining solvent A and B. Solvent A is acetonitrile and solvent B is 0.1% aqueous trifluoroacetic acid. The isocraticmobile phase was set as 22%Acetonitrile in 0.1% aqueous trifluoroacetic acid. Flow rate was 1ml/min. The columntemperature was maintained at 45^{0}C and monitoring wavelength was kept at 210nm in a PDA system set at 205nm to400nm. Acetonitrile and water in the ratio of 1:1 was used as diluent. The new impurity elutes at retention time 2.4 – 2.5minundernewdevelopedmethod.

Excipientsremovalandenrichmentofimpurity

The patented formulation procured from pharmaceutical company, Piramal Pharma Ltd. has excipients like polyol, nonaqueous solvents and PH adjustment agents. These excipients were removed using adsorption column chromatographywith resin Diaion HP-20. Formulation was diluted with water five times and loaded on adsorption resin Diaion HP-20.Aftercollectingspent,waterwash was giventothecolumn(2bedvolumes)andthen elutionwas doneusing stepgradientof acetonitrile in water. The elution started with 2% acetonitrile in water with increment of 2%, each with two bedvolumes. The desired impurity got eluted in 20% acetonitrile in water as observed by HPLC with around 65% purity. Theoff-whitepowderwas obtainedwhenenrichedfractionshavingdesiredimpuritywerepooledandlyophilized.

PreparativeMethodofHPLC

After initial load optimization and column selection, final preparative HPLC method was developed using Knauereurosphere 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^{0}C. Sample loading was done by dry charged and peaks werecollected using fraction collector. Fractions were monitored by analytical HPLC using developed method as described insection 3.3. Fractions having > 95% purity of the desired impurity by HPLC were pooled and lyophilized to get impuritywith97.76% purity.Aminoacidsequencingandspectraldata wasrecordedonthissample.

SequencingofaminoacidsusingHighResolutionMassSpectrometry

Themolecularweight oftheimpurity,fragmentationpatternofpeptidebyMS-MSstudyandtentativemolecularformulawas determined by this study. CID based fragmentation study has been performed on the impurity and the MS/MSfragment spectrawithannotation ofbandy ionswas done.Theaminoacidsequencegeneratedusingtheannotatedb andy ions and the tentative molecular formula has been provided. The instrument used is hybrid quadrupole/orthogonalacceleration, time-of-flight (oa-TOF) mass spectrometer having high sensitivity and speed. Ion mobility is the usefultechniquetostudythepeptides.MassLynxsoftwarewasusedforacquisitionandBioLynxforinsilicoanalysis.

MassLynxisaapplicationthatcontrolsWatersmassspectrometer.BioLynxcontain Protein/PeptideEditortool,whichisused to analyze peptide sequences so as to facilitate the interpretation of data obtained from mass spectrometry. Variousmatching routines allow data to be automatically matched against known protein or peptide structures. The sequence ofpeptidewasdeterminedusingPeptideSequencingprogram.

IV. ResultsAndDiscussion

AnalyticalHPLCMethoddevelopment

The aim of the HPLC method development was to get the method having the same HPLC profile as observed in reportedmethod withregardstoalltheimpuritiesbuthavingshorterruntimeandusesvolatilebufferswhichcanberemoved veryeasily during purification. Since reported HPLC methods for bivalirudin formulation are either having long run time orhaving buffers which are very difficult to remove during purification process. The method which we have developed ishavingruntimeof10minasagainst65 minforreported methodanduses 0.1%Trifluoroaceticacidas volatilebuffer.

Various types of columns with reverse phases and different buffers with combination of solvents like methanol andacetonitrile were tried for optimization. Finally method development was done which had fulfilled all the above criteriaand preparative HPLC purification can be done using the same method. The new HPLC

method was developed usingPhenomenex C18, 2.5µ (50 x 4.6mm) column with an isocratic program containing solvent A and B. Solvent A isacetonitrile and solvent B is 0.1% aqueous trifluoroacetic acid. The isocratic mobile phase was set as 22%Acetonitrile in0.1% aqueous trifluoroacetic acid. Flow rate was 1ml/min. The column temperature was maintained at 45^{o}C andmonitoring wavelength was kept at 210nm in a PDA system set at 205nm to 400nm. Acetonitrile and water in the ratio of1:1was usedas diluent.Thenewimpurityelutesatretentiontime2.4 – 2.5minwhereas inreportedmethod itwasaround19.9min.

ImpurityEnrichment

Diaion HP-20 adsorption resin was used for removal of excipients and enrichment of impurity. 1L of formulation wasdiluted with 4L of water and loaded on 1L of Diaion HP-20 resin packed in 4.5 x 30 cm glass column. Spent collectionwas done and then sufficient water wash was given (2 to 3 bed volume) to remove water soluble excipients. Elution wascarried 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 havedeveloped for presence of desired impurity of RRT 0.54 as described in section 4.1. The desired impurity was observed in22% acetonitrile in water elutes. 22% acetonitrile in water fraction no. 3 to 20 having purity around 65% by HPLC werepooledandlyophilizedtoget2.1gmof impurityasoffwhitecompound.

PurificationusingPreparativeHPLC

Knauer eurosphere C18, 250 x 32 mm, 10 µ column was used for preparative HPLC method development as reported insection 3.5. Above enriched impurity of 2.1 gm was dry charged on C18 resin and packed in pre column. Elution wascarried 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 frompreparative HPLC. All these fractions were monitored using analytical method which we have developed as reported inearlier section. Fractions no. 73 to 95 having purity of more than 95% by HPLC method were pooled and lyophilized toget530mgofimpurityaswhitecompoundwithpurityof 97.76% byHPLCareapercentage 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 acidsequence 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. Molecularweightoftheimpurityfoundas perdeconvoluted massspectrausing maximumentropyalgorithmis 1683.6884Da.

Annotated MS/MS fragment spectra of b and y ions of impurity obtained with its respective amino acid sequence is[GGGGNGDFEEIPEEYL].Hereabbreviations areGforglycine,Nforasparagine,Dforasparticacid,Fforphenylalanine, 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 thisnovel impurity as shown in figure 1. The high resolution mass spectrometry method has provided the elementalcomposition for this impurity as C73H103N17O29. The calculated molecular formula and formula weight for the structureshowninfigure1 matches with Elementalcompositionand molecularweightprovidedbydeconvolutedmassspectra.

Therefore this data supported the derived structure of this novel impurity. This structure was also confirmed by 1H and13CNMRexperimentsasshowninsection4.5and4.6.

4.5 ^{1}H NMR Assignments

Table 1 ^{1}H NMR Chemical shifts and assignments of proton signals

Chemical shift ð (H), ppm	No of Protons	H Atom No.	Assignments
12.2 (s)	5	-COO**H** (17) -COO**H** (31, 36, 52, 57)	Aspartic acid Glutamic acids

9.15 (s)	1	-COO**H** (73)	Leucine
8.6 (t)	1	-N**H** (2)	Glycine
8.3 (t)	1	-N**H** (4)	Glycine
8.0 – 8.2 (m)	8	-N**H$_2$** (11)	Asparagine
		-N**H** (8, 14, 18)	Asparagine, Aspartic acid, Phenyl alanine
		-N**H** (27, 32. 37)	Glutamic acid, Glutamic acid, Isoleucin
7.9 – 8.0 (m)	4	-N**H** (48, 53, 58, 67)	Glutamic acids (2), Tyrosine, Leucine
7.8 (t)	2	-N**H** (6, 12)	Glycine, Glycine
7.42 (s)	1	-O**H** (64)	Tyrosine
7.1 – 7.3 (m)	5	H22, H23, H24, H63, H65	Aromatic –CH, Phenyl alanine & Tyrosine
7.0 (d)	2	H62, H66	Aromatic –CH, Tyrosine
6.6 (d)	2	H21, H25	Aromatic –CH, Phenyl alanine
4.55 (m)	2	H47, H38	-CH, Proline, -CH, Isoleucine
4.45 (m)	2	H59, H68	-CH, Tyrosine, -CH, Leucine
4.1 – 4.35 (m)	6	H44e	-CH_2 (H-equ), Proline
		H1e, H3e, H5e, H7e, H13e	-CH_2 (H-equ), Glycine
3.85 (d)	2	H20e	-CH_2 (H-equ), Phenylalanine
		H60e	-CH_2 (H-equ), Tyrosine
3.65 – 3.80 (d)	8	H44a	-CH_2 (H-axi), Proline
		H1a, H3a, H5a, H7a, H13a	-CH_2 (H-axi), Glycine
		H20a	-CH_2 (H-axi), Phenylalanine
		H60a	-CH_2 (H-axi), Tyrosine
3.5 – 3.65 (m)	6	H10e	-CH_2 (H-equ), Asparagine
		H30e, H35e, H51e, H56e	-CH_2 (H-equ), Glutamic acid
		H69e	-CH2 (H-equ), Leucine
3.0 (d)	1	H16e	-CH2 (H-equ), Aspartic acid
2.9(d)	1	H16a	-CH2 (H-axi), Aspartic acid
2.8 (t)	1	H9	-CH, Asparagine
2.6 – 2.7 (t)	2	H28, H33	-CH, Glutamic acid
2.5 – 2.6 (t)	1	H19	-CH, Phenylalanine
2.4 – 2.5 (t)	2	H49, H54	-CH, Glutamic acid
2.1 – 2.35 (m)	7	H10a	-CH_2 (H-axi), Asparagine
		H30a, H35a, H51a, H56a	-CH_2 (H-axi), Glutamic acid
		H69a	-CH_2 (H-axi), Leucine
		H15	-CH, Aspartic acid
2.05 (s)	1	-NH_2 (1)	-NH_2, Glycine
1.95 – 2.03 (m)	1	H39	-CH, Isoleucin
1.6 – 1.9 (m)	12	H29ae, H34ae, H50ae, H55ae	-CH_2, Glutamic acid
		H45e	-CH_2 (H-equ) Proline
		H40e	-CH_2 (H-equ), Isoleucin
		H46ae	-CH_2, Proline
1.45 – 1.55 (m)	3	H45a	-CH_2 (H-axi), Proline
		H70	-CH, Leuine
		H40a	-CH_2 (H-axi), Isoleucin
1.0 – 1.1 (bs)	1	NH_2 (1)	-NH_2- Glycine
0.85 – 0.9 (m)	6	H41, H42	-CH_3, Isoleucin
0.75 – 0.85 (m)	6	H71, H72	-CH_3, Leucine

a , (axi) – Axial, e, (equ) - Equatorial

NMR signals in the region 7.10 – 7.30 ppm are aromatic protons indicating presence of aromatic amino acids, tyrosine and phenylalanine in this peptide impurity.

4.6 ^{13}C NMR Assignments

Table 2 ^{13}C NMR Chemical shifts and assignments of carbon signals

Chemical shift ð C, (ppm)	C Atom No	Assignments
173.994	C11	-$CONH_2$, Asparagine
173.939	C4, C6	-CONH-, Glycine
173.786	C2	-CONH-, Glycine
171.663	C8, C14	-CONH-, Glycine
171.617	C12	-CONH-, Asparagine
171.308	C18	-CONH-, Aspartic acid
171.016	C27	-CONH-, Phenylalanine
170.929	C32	-CONH-, Glutamic acid
170.821	C37	-CONH-, Glutamic acid
170.781	C43	-CONH-, Isoleucine
170.570	C48	-CONH-, Proline
170.310	C53	-CONH-, Glutamic acid
169.961	C58	-CONH-, Glutamic acid
169.013	C67	-CONH-, Tyrosine
168.728	C73	-COOH, Leucine
168.674	C17	-COOH, Aspartic acid
166.302	C36	-COOH, Glutamic acid
158.206	C31	-COOH, Glutamic acid
157.877	C52	-COOH, Glutamic acid
157.544	C57	-COOH, Glutamic acid
155.722	C64	Phenolic –C-OH, Tyrosine
137.526	C61	Aromatic quaternary C, Tyrosine
129.941	C62, C66	Aromatic –CH, Tyrosine
129.210	C21, C25	Aromatic –CH, Phenyl alanine
127.994	C22, C24	Aromatic –CH, Phenyl alanine
127.606	C26	Aromatic quaternary C, Phenylalanine
126.202	C23	Aromatic –CH, Phenyl alanine
114.828	C63, C65	Aromatic –CH, Tyrosine
59.178	C68	-CO-CH-NH-, Leucine
54.590	C59	-CO-CH-NH-, Tyrosine
53.841	C38	-CO-CH-NH-, Isoleucin
53.775	C47	-CO-CH-N-, Proline
52.151	C54	-CO-CH-NH-, Glutamic acid
51.830	C49	-CO-CH-NH-, Glutamic acid
51.747	C33	-CO-CH-NH-, Glutamic acid
51.526	C28	-CO-CH-NH-, Glutamic acid
50.180	C19	-CO-CH-NH-, Phenyl alanine
49.758	C15	-CO-CH-NH-, Aspartic acid
49.458	C9	-CO-CH-NH-, Asparagine
47.192	C44	-CH_2-N-, Proline
42.188	C1	-CH_2, Glycine
41.942	C3, C5	-CH_2, Glycine
37.214	C7	-CH_2, Glycine
37.090	C13	-CH_2, Glycine
36.397	C60	-CH_2, Tyrosine
36.127	C39	-CH, Isoleucin
35.786	C69	-CH_2, Leucine
30.211	C56	**-CH_2**-COOH, Glutamic acid
30.079	C35, C51	**-CH_2**-COOH, Glutamic acid
29.832	C30	**-CH_2**-COOH, Glutamic acid
28.955	C20	-CH_2, Phenylalanine
27.477	C10	-CH_2, Asparagine
	C16	-CH_2, Aspartic acid
27.019	C40	-CH_2, Isoleucin
24.462	C70	-CH, Leucine
24.153	C50, C55	-CH_2, Glutamic acid
24.812	C29, C34	-CH_2, Glutamic acid
21.244	C45, C46	-CH_2, Proline
14.858	C41, C42	-CH_3, Isoleucin
10.685	C71, C72	-CH_3, Leucine

V. Figures

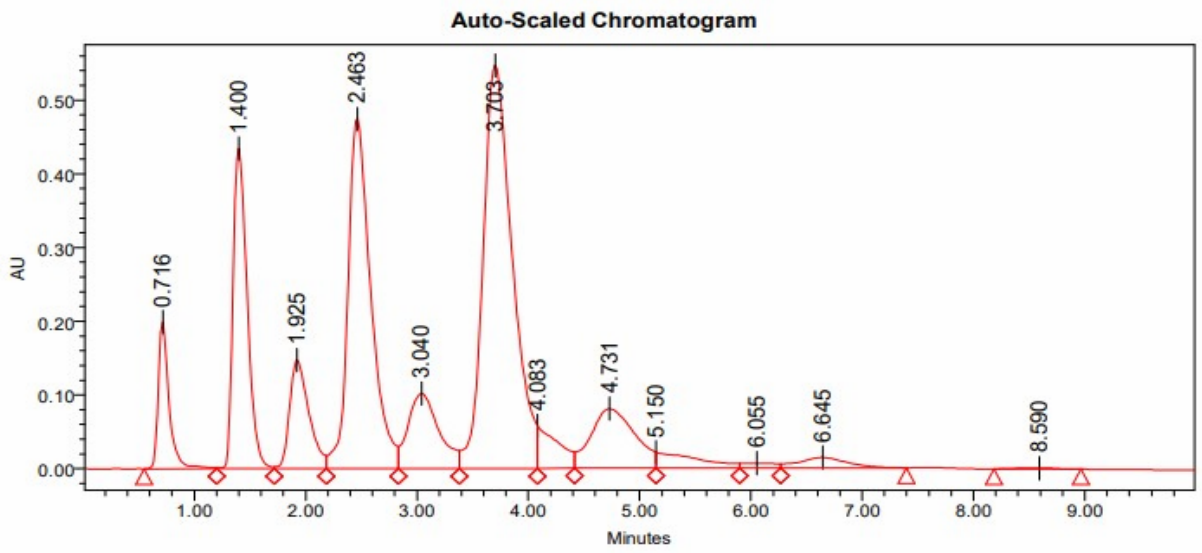

Figure 2. HPLC of the degraded formulation

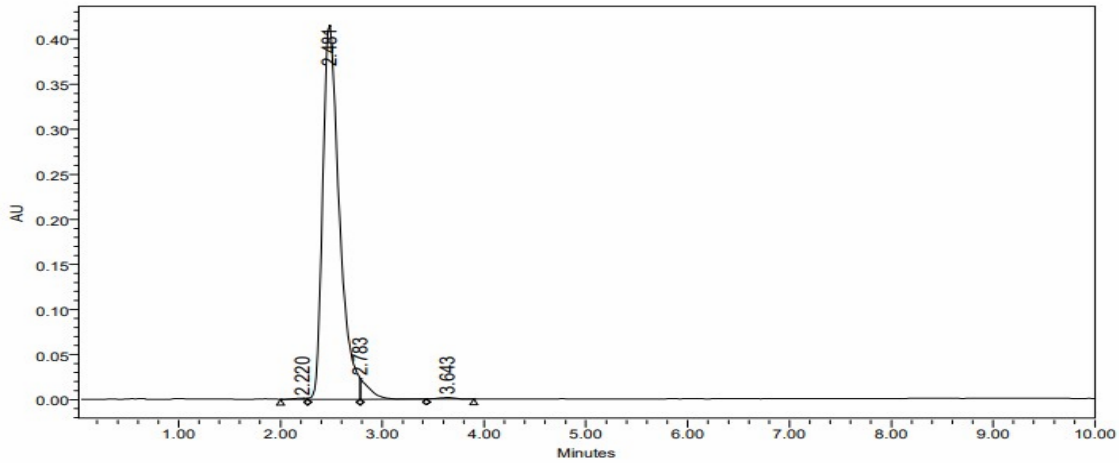

Figure 3. HPLC of the purified impurity

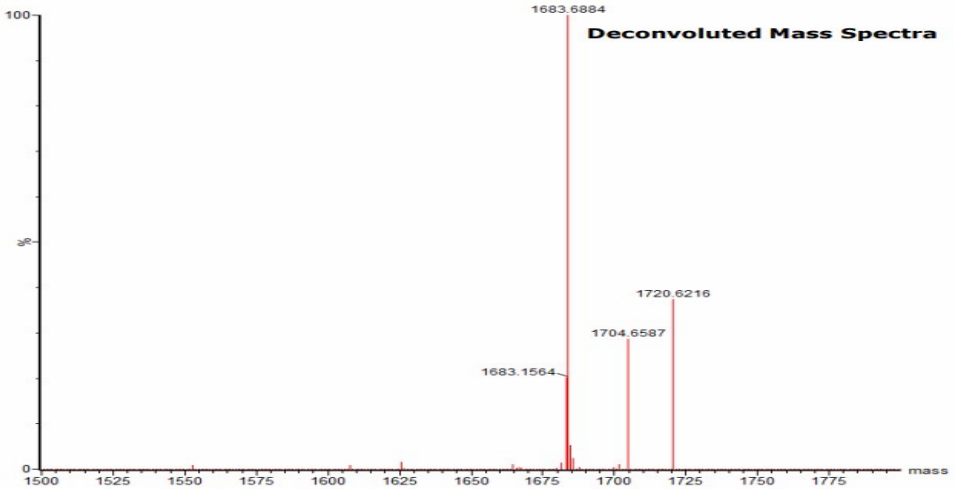

Figure 4. LCHRMS Spectra of the impurity

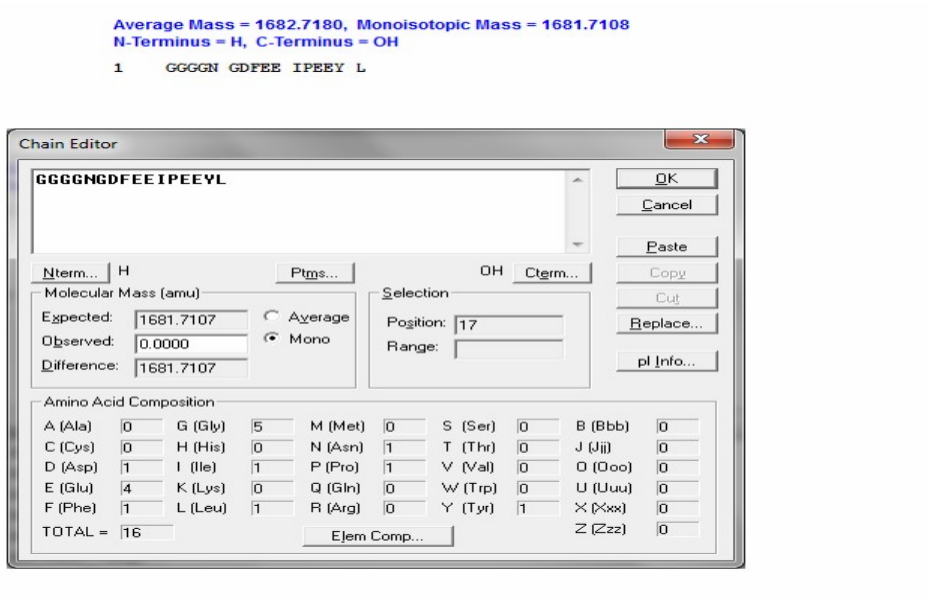

Figure 5. The sequence of amino acids from the impurity

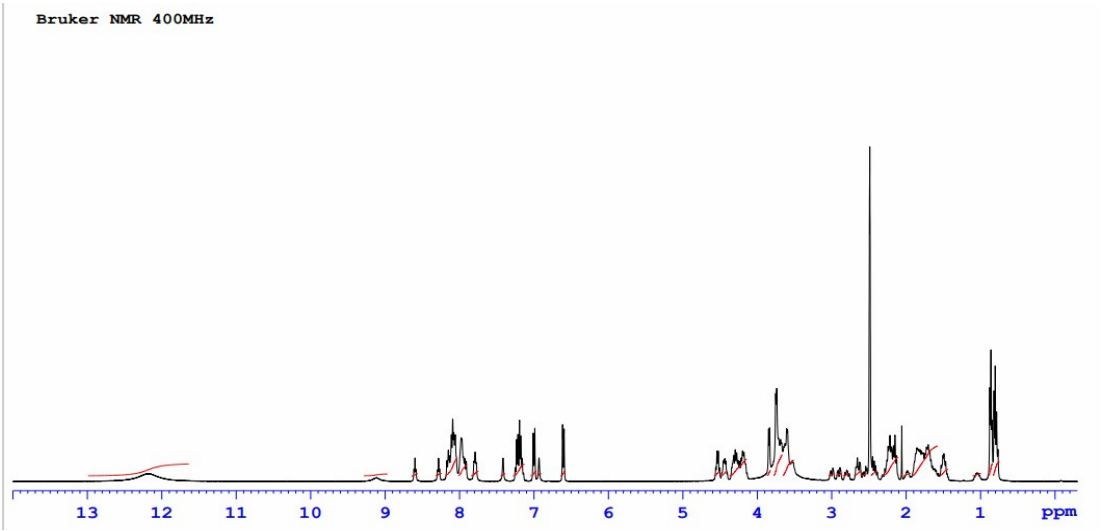

Figure 6. 1H NMR of the impurity

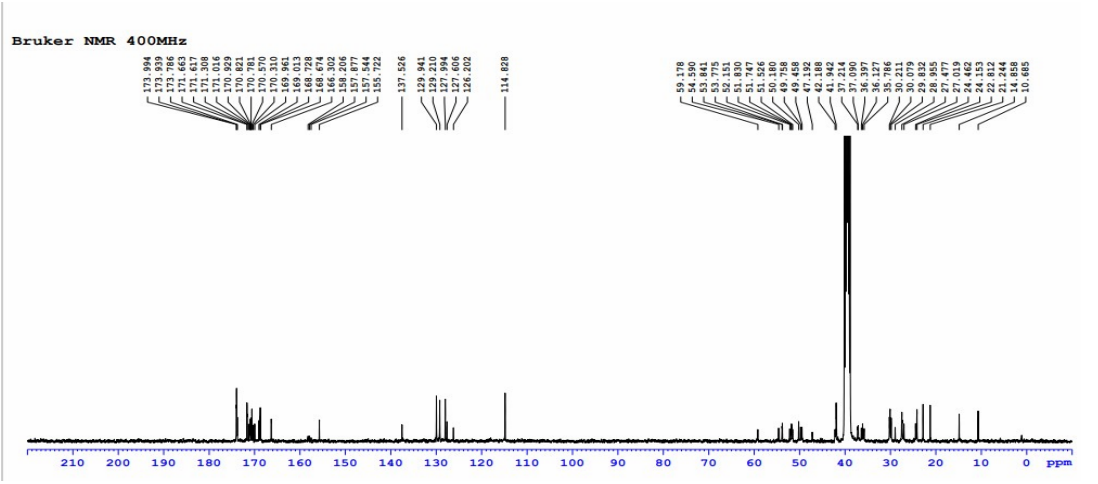

Figure 7. 13C NMR of the impurity

VI. Conclusion

Weconcludethat,wehavedetected,purifiedandstructurallycharacterizednovelimpurityusingaminoacidsequencinga nd1H,13CNMRspectroscopyfrombivalirudinformulation.

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