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Chemical characterization of biological peptide using modern analytical techniques

Rajan Mukund Panshikar[1], Jayshree Parikh[2], Ramesh Yamgar[3], Dattatraya Desai[4]

[2] Research Guide, Department of Chemistry, Shri JJT University, Jhunjhunu, Rajasthan, India
[3]Research Co-Guide, Department of Chemistry, C.S.'s Patkar - Varde college, Mumbai University, India
[4] Technical Director, UltraPure Analytics (I) Pvt. Ltd.
Corresponding Author: Rajan Mukund Panshikar

Abstract:
Bivalirudin is a peptide medication derived from natural peptide, Hirudin. One unknown peptide impurity was observed in stability studies sample provided by pharmaceutical company Piramal Pharma Ltd at RRT 0.44. It was purified using open column chromatography and preparative HPLC to 98.03% for chemical characterization. High Resolution Mass Spectrometry based Amino Acid Sequencing was carried out to get amino acid sequence. It has provided sequence of total 18 amino acids as Arginine –Proline – Glycine – Glycine – Glycine – Glycine – Asparagine – Glycine – Aspartic acid – Phenylalanine – Glutamic acid – Glutamic acid – Isoleucin – Proline – Glutamic acid – Glutamic acid – Tyrosine – Leucine. These amino acids after connecting through amide linkages had provided the structure of this novel impurity. Molecular weight of the impurity obtained is 1935.8921 Da. The elemental composition provided by high resolution mass spectrometry method as $C_{84}H_{122}N_{22}O_{31}$. Elemental composition and molecular weight matches with calculated molecular formula and formula weight for the derived structure. This derived structure was further confirmed by 1H and 13C NMR and 2D NMR experiments like COSY, DEPT90, DEPT135, HSQC and HMBC respectively. This sequence and molecular weight was not matching with any of the reported peptide. Therefore we conclude that we have chemically characterized novel peptide impurity from bivalirudin formulation.
Keyword: *Bivalirudin, COSY, DEPT, HSQC, HMBC*

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I. Introduction

Bivalirudin is the most promising anticoagulant among all anti-thrombin drugs reported so far and it is a peptide. It is a 20 amino acid peptide, with a monoisotopic mass of 2178.986 Da. The amino acid sequence reported for bivalirudin is D-Phenylalanine–Proline–Arginine–Proline–Glycine–Glycine–Glycine–Glycine–Asparagine–Glycine–Aspartic acid–Phenylalanine–Glutamic acid–Glutamic acid–Isoleucin–Proline–Glutamic acid–Glutamic acid–Tyrosine–Leucine. Pharmaceutical company, Piramal Pharma Ltd. has developed and patented ready to use formulation of bivalirudin using non aqueous solvent system. The stability study of this formulation have developed one unknown peptide impurity at RRT 0.44 which exceeds the limit specified in ICH guidelines. Therefore the aim of my study is isolation, purification and chemical characterization of this unknown peptide impurity from bivalirudin formulation with the help of various spectroscopic techniques like LC-HRMS and amino acid sequencing. The structure derived from amino acid sequencing method was further confirmed by NMR spectroscopy. The experiments carried out are 1H NMR, 13C NMR and other two dimensional NMR experiments like COSY, DEPT135, DEPT90, HSQC and HMBC. The Dictionary of Natural Products data base search was done to establish the novelty of this peptide. This novel impurity was assigned, following structure after analyzing all the above data as reported in results and discussion section.

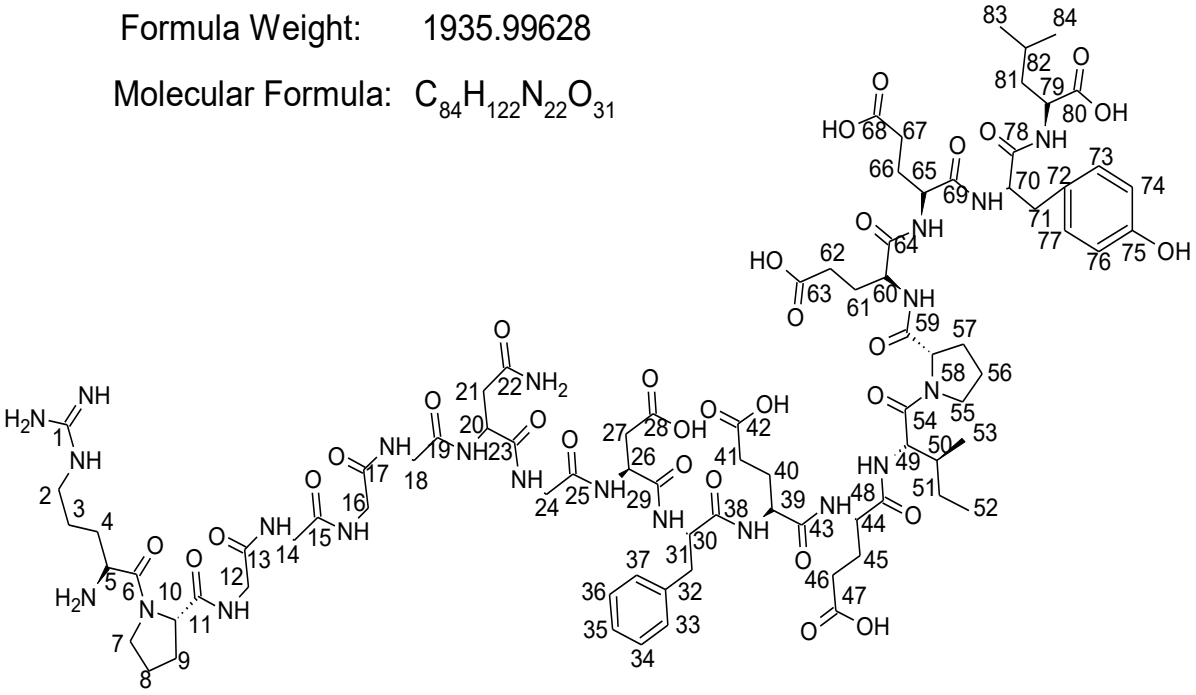

Fig.1 Chemical Structure of novel Impurity at RRT 0.44
(Arbitrary system for labeling protons and carbons for the purpose of making NMR assignments)

II. Literature Review

Carlos A. Valdes, et al. (2023) reportad that Bivalirudin is most promising, safe and cost effective among all anticoagulants reported due to its better pharmacological and pharmacokinetic properties [1].
Vyatkina K, et al.(2022) reported peptide sequencing using collection of high-resolution bottom-up MS/MS spectra and concept of tag convolution [2].
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 [3].
Kostromina M.A., et al. (2021) reported in their article that currently, synthetic bivalirudin (Angiomax, The Medicines Company, USA) is the most promising anticoagulant among hirudin-1 analogues [4].

Alessandra Basso, et al. (2019) reported in journal of chromatography Today, reverse phase HPLC method for the purification of bivalirudin with > 99% purity and greater than 99% yield [5].

K.Y. Kiran Kumar, et al. (2019) reported the development of a reverse-phase ultra-high-performance liquid chromatography (RP-UHPLC) method for analyzing the related substances of the therapeutic peptide bivalirudin with separation of all twelve reported impurities [6].

Wen-Xin Xiang, et al. (2019) reported characterization of peptides using 1D and 2D NMR spectroscopy [7].
Delphine Vincent, et al. (2018) showcased the use of Top-down sequencing through in-source collision-induced dissociation on the Maxis quadrupole Time-of Flight mass spectrometer [8].

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 [9].

Hua Yang, et al. (2018) reported various types of columns and mobile phases to be used for identification, separation and purification of reported bivalirudin impurities [10].
Bondigalla. Ramachandra, et al. (2016) reported modern analytical techniques used for impurity profiling, particularly the UPLC, LC-MS, HRMS, GC-MS, HPTLC and NMR [11].

Sonavaria Vandana, et al. (2016) reported in their international patent filing about stable, ready to use injectable formulation of bivalirudin and its preparation process and observed unknown impurities from bivalirudin [12].

Kui Zeng, et al. (2015) reported LC-HRMS method for bivalirudin and its related impurities to determine amino acid composition [13].
Xiao-Jiao Li, et al. (2013) reported quantitation of bivalirudin in human plasma by LC-MS/MS with triple-quadrupole mass spectrometer, equipped with electro spray ionization (ESI) interface, and operated in the positive ion mode [14].
JoséC. del Río, et al.(2012) described the structural characterization using 2D NMR spectroscopy, with a particular focus on HSQC and HMBC experiments [15].
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 [16].

Guodong Chen, et al. (2008) reported in their review article, current capabilities and future trends with respect to LC-MS for protein characterization [17].

Guodong Chen, et al. (2007) reported the use of LC-MS in drug discovery for the structural elucidation of small molecules and proteins. It measures the molecular weight (MW) of compounds and delivers structural insights through fragmentation studies using MS/MS [18].

ICH, Q3B (R2) (2006): Reporting and identification threshold for impurities in new drug products were reported by FDA [19].

III. Research Methodology

3.1 Materials and Chemicals

Bivalirudin formulation stability study sample was procured from Piramal Pharma Ltd. 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.

3.2 Instrumentation

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. 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 and solvent used was DMSO-d6.

3.3 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 at 210 nm with flow rate, 40ml/min and column temperature 45^0C. Sample loading was done by dry charged and peaks were collected using fraction collector. Fractions were monitored by analytical HPLC. Fractions having > 95% purity of the desired peptide by HPLC were pooled and lyophilized. Amino acid sequencing and spectral data was recorded on this sample.

3.4 Sequencing of amino acids using High Resolution Mass Spectrometry

Waters High Resolution Mass Spectrometry and MassLynx and BioLynx softwares were used for amino acid sequencing of this peptide impurity. The instrument is a hybrid quadrupole/orthogonal acceleration, time-of-flight (oa-TOF) mass spectrometer which gives high sensitivity, selectivity and speed. Here the objective is, to find out the molecular weight of the impurity, to find out the fragmentation pattern of peptide by MS-MS study and to find out tentative molecular formula.

3.5 1H NMR Experiment

Peptides are made up of amino acids which form amide linkage among them. Each proton based on its surrounding appears at different ppm (ð values) in 1H NMR spectrum. Since many peptides have similar amino acids, many signals get overlapped to give multiplates.

3.6 D_2O Exchange NMR Experiment

This NMR experiment is used to identify exchangeable protons in the compound. Thus protons attached to nitrogen or oxygen can be identified by using this technique.

3.7 13C NMR Experiment

It provides information about type of carbon within peptide. Carbon from free acid group (-COOH) from amino acids like leucine, glutamic acid and terminal acid group from peptide usually appears at ð 167 – 168 ppm and amide carbon appears anywhere between ð 169 – 174 ppm. Aromatic carbons from amino acids like tyrosine and phenylalanine appears in-between ð 110 – 136 ppm.

3.8 DEPT NMR Experiment

Distortionless Enhancement by Polarization Transfer (DEPT) is a technique used to determine multiplicity of carbon atoms within the molecule. It distinguished between methyl (CH_3), methylene (CH_2), methine (CH) and quaternary carbon present in the molecule.

3.9 COSY NMR Experiment

This technique determines correlations between protons within the sample. COSY experiment shows which protons are coupled with each other. Cross peaks observed in COSY spectra are between the protons that are coupled.

3.10 HSQC NMR Experiment

It couples 1H nuclei with other NMR active nuclei like 13C that are directly attached to each other. Cross peaks in the spectra represents which proton is attached to which carbon. It is used to determine proton, carbon coupling in peptide chemistry.

3.11 HMBC NMR Experiment

This experiment provides information about protons that are connected to carbons which are two, three or sometimes four bonds apart. It is used to determine structure of complex molecules like peptides. Here even quaternary carbon atoms can be detected.

IV. Results and Discussion

4.1 Purification using Preparative HPLC

The final purification of the enriched peptide was achieved using preparative HPLC. The lyophilized peptide obtained was 100 mg as white powder with purity of 98.03% by HPLC area percentage method.

4.2 Sequencing of Amino acids using High Resolution Mass Spectrometry

Molecular weight of the impurity obtained as per deconvoluted mass spectra using maximum entropy algorithm is 1935.8921 Da. Based on the fragment spectra, the sequence of amino acids from the impurity was derived as Arginine –Proline – Glycine – Glycine – Glycine – Glycine – Asparagine – Glycine – Aspartic acid – Phenylalanine – Glutamic acid – Glutamic acid – Isoleucin – Proline – Glutamic acid – Glutamic acid – Tyrosine – Leucine. The elemental composition provided by high resolution mass spectrometry method as $C_{84}H_{122}N_{22}O_{31}$. These amino acids by connecting through amide linkages have given the structure of this peptide. The molecular weight and molecular formula obtained from LC-HRMS experiment matches with the derived structure of this peptide. This structure was further confirmed by 1D and 2D NMR experiments as follows

4.3 ^{1}H NMR and its interpretation

Table 4.1 1H NMR Chemical shifts and assignments of proton signals

Chemical shift ð (H), ppm	No of Protons	H Atom No.	Assignments
12.0 – 12.3 (s)	5	-COOH (28) -COOH (42, 47, 63, 68)	Aspartic acid Glutamic acids
9.1 (s)	1	-COOH (80)	Leucine
8.3 – 8.4 (t)	1	-NH (2)	Arginine -NH
8.0 – 8.2 (m)	4 1 1 1 4 1 1 1	-NH (11, 13, 15, 17) -NH (19) -NH (25) -NH (29) -NH (38, 43, 59, 64) -NH (48) -NH (69) -NH (78)	Glycine (4 units) Asparagine Aspartic acid Phenylalanine Glutamic acid (4 units) Isoleucin Tyrosine Leucine
7.9 – 8.0 (m)	2	NH_2 (5)	Arginine
7.8 (bs)	2	NH_2 (22)	Asparagine
7.5 (t)	1	-NH (23)	Glycine
7.4 (s)	1	-OH (75)	Tyrosine
7.1 – 7.3 (m)	2 5	$-NH_2$ (1) H33, H34, H35, H36, H37	Arginine $-NH_2$ Aromatic –CH, Phenylalanine

7.0 (d)	2	H73, H77	Aromatic –CH, Tyrosine
6.9 (bs)	1	-NH (1)	Arginine
6.6 (d)	2	H74, H76	Aromatic –CH, Tyrosine
4.5 – 4.6 (m)	1 1	H49 H70	-CH, Isoleucin -CH, Tyrosine
4.4 – 4.5 (m)	2 1	H10, H58 H79	-CH, Proline (2 units) -CH, Leucine
4.1 – 4.4 (m)	2 5 1	H7e, H55e H12e, H14e, H16e, H18e, H24e H5	$-CH_2$ (H-equ), Proline (2 units) $-CH_2$ (H-equ), Glycine (5 units) -CH, Arginine
3.65 – 3.85 (m)	1 1 2 5 1	H31e H71e H7a, H55a H12a, H14a, H16a, H18a, H24a H21e	$-CH_2$ (H-equ), Phenylalanine $-CH_2$ (H-equ), Tyrosine $-CH_2$ (H-axi), Proline $-CH_2$ (H-axi), Glycine (5 units) $-CH_2$ (H-equ), Asparagine
3.5 – 3.6 (m)	4	H41e, H46e, H62e, H67e	$-CH_2$ (H-equ), Glutamic acid
3.05 – 3.15 (m)	1 1	H30 H71a	-CH, Phenylalanine $-CH_2$ (H-axi), Tyrosine
2.95 – 3.0 (m)	1	H81e	$-CH_2$ (H-equ), Leucine
2.85 – 2.95 (m)	1	H4e	$-CH_2$ (H-equ), Arginine
2.75 – 2.85 (m)	1	H4a	$-CH_2$ (H-axi), Arginine
2.55 – 2.70 (m)	2	H39, H44	-CH, Glutamic acid (2 units)
2.4 – 2.5 (m)	2	H60, H65	-CH, Glutamic acid (2 units)
2.1 – 2.3 (m)	1 1 1 1 4 1	H27e H20 H31a H21a H41a, H46a, H62a, H67a H26	$-CH_2$ (H-equ), Aspartic acid -CH, Asparagine $-CH_2$ (H-axi), Phenylalanine $-CH_2$ (H-axi), Asparagine $-CH_2$ (H-axi), Glutamic acid -CH, Aspartic acid
1.9 – 2.0 (m)	1 1	H27a H50	$-CH_2$ (H-axi), Aspartic acid -CH, Isoleucin
1.8 – 1.9 (m)	4 1 1 1 1	H40e, H45e, H61e, H66e H81a H8e H9e H51e	$-CH_2$ (H-equ), Glutamic acid $-CH_2$ (H-axi), Leucine $-CH_2$ (H-equ), Proline $-CH_2$ (H-equ), Proline $-CH_2$ (H-equ), Isoleucin
1.6 – 1.75 (m)	4 2 2	H40a, H45a, H61a, H66a H56e, H57e H3e, H2e	$-CH_2$ (H-axi), Glutamic acid $-CH_2$ (H-equ), Proline (2 units) $-CH_2$ (H-equ), Arginine
1.4 – 1.7 (m)	4 2 1	H8a, H9a, H56a, H57a H3a, H2a H51a	$-CH_2$ (H-axi), Proline (2 units) $-CH_2$ (H-axi), Arginine $-CH_2$ (H-axi), Isoleucin
1.0 – 1.1 (m)	1	H82	-CH, Leucine
0.8 – 0.9 (t to m)	3 3	H52 H83	$-CH_3$, Isoleucin $-CH_3$, Leucine
0.7 – 0.8 (t to m)	3 3	H53 H84	$-CH_3$, Isoleucin $-CH_3$, Leucine

4.4 ^{13}C NMR and its interpretation

Table 4.2 ^{13}C NMR Chemical shifts and assignments of carbon signals

Chemical shift ð (C), ppm	Assignments	DEPT135/DEPT90 Data	C Atom No.
173.955	$-CONH_2$, Asparagine -CONH-, Glycine	Quaternary Quaternary	C22 C13
173.806	-CONH-, Glycine	Quaternary	C15, C17
171.681	-CONH-, Glycine	Quaternary	C19, C25
171.544	-CONH, Asparagine -CONH-, Aspartic acid	Quaternary Quaternary	C23 C29
171.329	-CONH-, Phenylalanine	Quaternary	C38
170.950	-CONH-, Glutamic acids	Quaternary	C43, C48
170.841	-CONH-, Isoleucin -CONH-, Proline	Quaternary Quaternary	C54 C59
170.586	-CONH-, Arginine -CONH-, Proline	Quaternary Quaternary	C6 C11
170.334	-CONH-, Glutamic acid	Quaternary	C64
169.978	-CONH-, Glutamic acid	Quaternary	C69
169.109	-CONH-, Tyrosine	Quaternary	C78
168.745	-COOH, Leucine	Quaternary	C80
167.109	-COOH, Glutamic acids	Quaternary	C42, C47
158.595	N-C=NH, Arginine	Quaternary	C1

158.270	-COOH, Aspartic acid	Quaternary	C28
156.850	-COOH, Glutamic acids	Quaternary	C63, C68
155.740	Phenolic –C-OH, Tyrosine	Quaternary	C75
137.545	Aromatic quaternary C, Tyrosine	Quaternary	C72
129.956	Aromatic –CH, Tyrosine	-CH	C73, C77
129.224	Aromatic –CH, Phenylalanine	-CH	C33, C37
128.011	Aromatic –CH, Phenylalanine	-CH	C34, C36
127.619	Aromatic C, Phenylalanine	Quaternary	C32
126.211	Aromatic –CH, Phenylalanine	-CH	C35
114.842	Aromatic –CH, Tyrosine	-CH	C74, C76
59.751	-CO-CH-NH_2, Arginine	-CH	C5
59.193	"-CO-CH-NH-", Leucine	-CH	C79
54.602	"-CO-CH-NH-", Tyrosine	-CH	C70
53.797	"-CO-CH-NH-", Isoleucin	-CH	C49
52.178	"-CO-CH-N-", Proline	-CH	C58
51.765	"-CO-CH-NH-", Glutamic acids	-CH	C60, C65
51.549	-CO-CH-N-, Proline	-CH	C10
50.534	"-CO-CH-NH-", Glutamic acid	-CH	C44
50.191	"-CO-CH-NH-, Glutamic acid"	-CH	C39
49.786	"-CO-CH-NH-", Aspartic acid	-CH	C26
49.460	"-CO-CH-NH-", Asparagine	-CH	C20
47.026	CH_2-N-, Proline (2)	$-CH_2$	C7, C55
47.184	CH_2-NH-, Arginine	$-CH_2$	C2
42.161	$-CH_2$, Glycine	$-CH_2$	C24
41.964	$-CH_2$, Glycine	$-CH_2$	C12, C14, C16, C18
40.219	$-CH_2$, Proline (2 units)	$-CH_2$	C9, C57
39.950	$-CH_2$, Isoleucin	$-CH_2$	C51
39.727	-CO-CH-NH-, Phenylalanine	-CH	C30
37.110	$-CH_2$, Arginine	$-CH_2$	C4
36.401	$-CH_2$, Tyrosine	$-CH_2$	C71
36.132	-CH, Isoleucin	-CH	C50
35.796	$-CH_2$, Leucine	$-CH_2$	C81
30.094	-CH_2-COOH, Glutamic acids	$-CH_2$	C46, C62
30.220(DEPT135)	-CH_2-COOH, Glutamic acids	$-CH_2$	C67
29.847	-CH_2-COOH, Glutamic acids	$-CH_2$	C41
29.148	$-CH_2$, Arginine	$-CH_2$	C3
28.974	$-CH_2$, Phenylalanine	$-CH_2$	C31
27.484	$-CH_2$, Asparagine $-CH_2$, Aspartic acid	$-CH_2$ $-CH_2$	C21 C27
27.035	$-CH_2$, Proline (2 units)	$-CH_2$	C8, C56
24.648	$-CH_2$, Glutamic acid	$-CH_2$	C66
24.478	$-CH_2$, Glutamic acid	$-CH_2$	C61
24.204	$-CH_2$, Glutamic acid	$-CH_2$	C45
24.163	-CH, Leucine	-CH	C82
23.420	$-CH_3$, Isoleucin	$-CH_3$	C52
22.829	$-CH_2$, Glutamic acid	$-CH_2$	C40
21.252	$-CH_3$, Isoleucin	$-CH_3$	C53
14.869	$-CH_3$, Leucine	$-CH_3$	C83
10.699	$-CH_3$, Leucine	$-CH_3$	C84

4.5 2D-HSQC NMR and its interpretation

Table 4.3 "Assignments of the ^{13}C- ^{1}H correlation peaks in the 2D HSQC spectra"

ðC/ ðH (ppm)	C Atom No/H Atom No	Assignment
126/7.16	C35/H35	Aromatic –CH, Phenylalanine
128/7.23	C34/H34, C36/H36	Aromatic –CH, Phenylalanine
129/7.18	C33/H33, C37/H37	Aromatic –CH, Phenylalanine
130/7.0	C73/H73, C77/H77	Aromatic –CH, Tyrosine
115/6.6	C74/H74, C76/H76	Aromatic –CH, Tyrosine
60/4.35	C5/H5	-CH, Arginine
55/4.5	C70/H70	-CO-CH-NH-, Tyrosine
54/4.45	C49/H49	-CO-CH-NH-, Isoleucin
52/4.4	C58/H58	-CO-CH-N-, Proline
51/4.2	C65/H65, C60/H60	-CO-CH-NH-, Glutamic acids
50/4.55	C44/H44, C39/H39	-CO-CH-NH-, Glutamic acids
47/3.7	C7/H7a	-CH_2-N-, Proline
47/3.5	C55/H55a	-CH_2-N-, Proline
42/3.75	C12/H12a, C14/H14a, C16/H16a, C18/H18a, C24/H24a	$-CH_2$, Glycine
40/3.1	C30/H30	-CO-CH-NH-, Phenylalanine
40/1.5	C51/H51a	$-CH_2$, Isoleucin
37/2.9-3.0	C4/H4e	$-CH_2$, Arginine

37/2.8	C4/H4a	-CH_2, Arginine
30/2.0-2.2	C62/H62a, C46/H46a, C67/H67a	-CH_2 (H-axi), Glutamic acids
27.5/1.7-1.85	C27/H27a	-CH_2 (H-axi), Aspartic acid
25/1.85	C66/H66e	-CH_2 (H-equ), Glutamic acid
23.5/1.6	C40/H40a	-CH_2 (H-axi), Glutamic acid
23.5/0.85	C52/H52	-CH_3, Isoleucin
21.5/0.8	C53/H53	-CH_3, Isoleucin
15/0.85	C83/H83	-CH_3, Leucine
11/0.8	C84/H84	-CH_3, Leucine

4.6 2D-HMBC NMR and its interpretation

Table 4.4 "Assignments of the ^{13}C- ^{1}H long range correlation peaks in the 2D HMBC spectra"

δC/ δH (ppm)	**C Atom No/H Atom No**	**Assignment**
170/8.39	C11/NH (11) C59/NH (59)	Proline – Glycine Proline carbon with -NH
170/8.05-8.2	C64/NH (64), C48/NH (48), C43/NH(43)	Glutamic acids
170/7.95	C6/NH_2 (5)	Arginine
171/7.8	C23/NH_2 (22)	Asparagine
158/7.0	C1/NH (1)	Arginine
155/6.6	C75/H74 C75/H76	Tyrosine
138/7.23	C72/H73	Tyrosine
128/7.1-7.3	C34/H33, C36/H37	Phenylalanine
129.9/7.0	C73/H77, C77/H73	Tyrosine
129/6.6	C73/H74, C77/H76	Tyrosine
115/7.0	C74/H73, C76/H77	Tyrosine
38/7.2	C4/-NH_2 (5)	Arginine
38/7.0	C71/H73	Tyrosine
170/4.4-4.55	C48/H49 C54/H58 C59/H58	Glutamic acid – Isoleucin Isoleucin – Proline Proline
170/4.1-4.3	C54/H55e C6/H5 C11/H12e	Isoleucin – Proline Arginine Proline - Glycine
170/3.6-3.85	C54/H55a C6/H7a C11/H12a C43/H41e, C48/H46e, C64/H62e, C69/H67e	Isoleucin – Proline Arginine – Proline Proline – Glycine Glutamic acids (4 units)
172-175/2.4-2.7	C38/H39 C43/H44	Phenylalanine – Glutamic acid Glutamic acid – Glutamic acid
172-175/2.1-2.3	C22/H21a C29/H31a C19/H20 C29/H27e	Asparagine Aspartic acid – Phenylalanine Glycine – Asparagine Aspartic acid
55/2.6-2.9	C70/H71a	Tyrosine
59/0.85	C79/H83 C79/H84	Leucine
50/2.1-2.65	C44/H46a C26/H27e	Glutamic acid Aspartic acid
50/1.5-1.75	C44/H45a C39/H40a	Glutamic acid Glutamic acid
40/4.2	C9/H7e C57/H55e	Proline Proline
40/2.5	C57/H60	Proline – Glutamic acid
40/0.8	C51/H52 C51/H53	Isoleucin Isoleucin
28/2.1-2.3	C21/H20	Asparagine
23-25/0.8	C82/H83 C82/H84 C52/H53	Leucine Leucine Isoleucin
14-15/0.9-1.0	C83/H82	Leucine

4.7 2D-COSY NMR and its interpretation

Table 4.5 "Assignments of the ^{1}H- ^{1}H correlation peaks in the 2D COSY spectra"

ðH/ ðH (ppm)	H Atom No/H Atom No	Assignment
8.0-8.4/3.6-3.8	NH (11)/H12a NH (13)/H14a NH (15)/H16a NH (17)/H18a NH (23)/H24a	-NH with adjacent Glycine axial protons
8.0-8.2/4.1-4.3	NH (11)/H12e NH (13)/H14e NH (15)/H16e NH (17)/H18e NH (23)/H24e	-NH with adjacent Glycine equatorial protons
8.0-8.2/4.4-4.5	NH (11)/H10 NH (59)/H58	Proline proton with Glycine –NH Proline proton with Glutamic acid -NH
7.75-8.0/4.1-4.5	-NH_2(5)/H5	Arginine –CH with –NH_2
4.2-4.5/2.5-3.0	H5/H4a,e	Arginine adjacent protons
1.5-1.8/0.75-0.85	H51e/H52 H81a/H83	Isoleucin adjacent protons Leucine adjacent protons

V. Conclusion

The peptide impurity was purified by preparative HPLC with purity 98.03% by HPLC. Molecular weight was found to be 1935.8921 Da. The amino acid sequence was found to be Arginine –Proline – Glycine – Glycine – Glycine – Glycine – Asparagine – Glycine – Aspartic acid – Phenylalanine – Glutamic acid – Glutamic acid – Isoleucin – Proline – Glutamic acid – Glutamic acid – Tyrosine – Leucine. The elemental composition provided by high resolution mass spectrometry method is $C_{84}H_{122}N_{22}O_{31}$. Based on this the final structure was assigned and confirmed by 1D and 2D NMR spectroscopy. Dictionary of Natural Products (DNP) Search with respect to molecular weight, molecular formula and Amino acid composition has provided no hit. Therefore we conclude that novel peptide from bivalirudin formulation not reported from any other source is isolated and chemically characterized.

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References

[1]. Carlos A. Valdes, Sharaf OM, Bleiweis MS, Jacobs JP, Mumtaz M, Sharaf RM, Jeng EI and Peek GJ (2023), Heparin-based versus bivalirudin-based anticoagulation in pediatric extracorporeal membrane oxygenation: A systematic review, Front. Med, 10:1137134

[2]. Vyatkina K., (2022) Validation of De Novo Peptide Sequences with Bottom-Up Tag Convolution, Proteomes 2022, 10, 1, https:// doi.org/10.3390/proteomes10010001

[3]. Jing Li, Sanbao Chen, Sicong Ma, Mingque Yang, Zizhao Qi, Kun Na, Miaohan Qiu, Yi Li, and Yaling Han, (2022), Safety and Efficacy of Bivalirudin versus Unfractionated Heparin Monotherapy in Patients with CAD and DM Undergoing PCI: A Retrospective Observational Study, Cardiovascular Therapeutics, Volume 2022, Article ID 5352087, 10 pages

[4]. Kostromina M.A., Tukhovskaya E.A., Shaykhutdinova E.R., Slashcheva G.A., Ismailova A.M., Palikov V.A., Palikova Y.A., Dyachenko I.A., Kravchenko I.N., Sadovnikova E.S., (2021), Screening of the Promising Direct Thrombin Inhibitors from Haematophagous Organisms. Part I: Recombinant Analogues and Their Antithrombotic Activity In Vitro, Biomedicines 2022, 10, 11

[5]. Alessandra Basso, (2019), Development of a novel combined IEX-RP chromatographic process for the purification of bivalirudin, CHROMATOGRAPHY TODAY, August / September 2019

[6]. K. Y. Kiran Kumar, et al. (2019), A simple, sensitive, high-resolution, customized, reverse phase ultra-high performance liquid chromatographic method for related substances of a therapeutic peptide (bivalirudin trifluoroacetate) using the quality by design approach, Analytical Methods, The Royal Society of Chemistry, 2019, DOI: 10.1039/c9ay01998g

[7]. Wen-Xin Xiang, (2019) Four pairs of proline-containing cyclic dipeptides from Nocardiopsis sp. HT88, an endophytic bacterium of Mallotusnudiflorus L, Natural Product Research, DOI: 10.1080/14786419.2019.1577834

[8]. Delphine Vincent, et al (2018), Optimisation of Milk Protein Top-Down Sequencing Using In-Source Collision-Induced Dissociation in the Maxis Quadrupole Time-of-Flight Mass Spectrometer, Molecules 2018, 23, 2777

[9]. Nilini Ranbaduge, Ying Qing Yu, (2018), Synthetic Peptide Characterization and Impurity Profiling Using a Compliance-Ready LC-HRMS Workflow, Waters, 720006367, August 2018

[10]. Hua Yang, Stephan M. Koza, William J. Warren, Weibin Chen, (2018), Synthetic Peptide Impurity Analysis on Waters Reversed-Phase Columns, Waters, 720006244, April 2018

[11]. Bondigalla Ramachandra, (2016), Development of impurity profiling methods using modern analytical techniques, Critical Reviews in Analytical Chemistry, DOI: 10.1080/10408347.2016.1169913

[12]. Sonavaria Vandana, Banerjee Shashwat, Upadhyay Kamal Kumar, (2016), Stable Injectable Composition of Bivalirudin and Process for its Preparation, PCT/IB2015/057921, International Publication Number WO 2016/059588 AI

[13]. Kui Zeng, Ilan Geerlof-Vidavisky, Ashley Gucinski, Xiaohui Jiang, Michael T. BoyneII, (2015), Liquid Chromatography-High Resolution Mass Spectrometry for Peptide Drug Quality Control, The AAPS Journal (# 2015) DOI: 10.1208/s12248-015-9730-z

[14]. Xiao-Jiao Li, Yan-Tong Sun, Lei Yin , Xue-Ju Zhang , Yan Yang, J. Paul Fawcett , Yi-Min Cui, Jing-Kai Gu, (2013), Quantitation of bivalirudin, a novel anticoagulant peptide, in human plasma by LC–MS/MS: Method development, validation and application to pharmacokinetics, Journal of Pharmaceutical Analysis, 3, 1, 1-8
[15]. JoséC. del Río, Jorge Rencoret, Pepijn Prinsen, Á ngel T. Martínez, John Ralph and Ana Gutierrez´, (2012), Structural Characterization of Wheat Straw Lignin as Revealed by Analytical Pyrolysis, 2D-NMR, and Reductive Cleavage Methods, J. Agriculture and Food Chemistry, 2012, 60, 5922−5935
[16]. Van Dorpe S, Verbeken M, Wynendaele E, De Spiegeleer B (2011) Purity profiling of Peptide Drugs. J Bioanalysis & Biomedicine S6: 003
[17]. Guodong Chen & Birendra N Pramanik, (2008), LC-MS for protein characterization: current capabilities and future trends, Expert Review of Proteomics, 5:3, 435-444
[18]. Guodong Chen, Birendra N. Pramanik, Yan-Hui Liu, Urooj A. Mirza, (2007), Applications of LC/MS in structure identifications of small molecules and proteins in drug discovery, Journal of Mass Spectrometry, 42: 279–287
[19]. International Conference on Harmonization, Step 4 Version dated 25 October 2006, Impurities in New Drug Products Q3B (R2)