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Assessing the performance of new chromatographic technologies for the separation of peptide epimeric impurities: the case of Icatibant

Giulia Mazzoccanti [a], Simone Manetto [a], Michele Bassan [b], Marco Macis [b], Walter Cabri [c], Alessia Ciogli [a], Antonio Ricci [b,*], Francesco Gasparrini [a,*]

[a] Department of Drug Chemistry and Technology, "Sapienza" University of Rome, 00185 Rome, Italy
[b] Fresenius Kabi iPSUM, Via Roma 108, 20051 Cassina de Pecchi, Italy
[c] Department of Chemistry, Alma Mater Studiorum-University of Bologna, Via Gobetti 84, 40129 Bologna, Italy

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ABSTRACT

The biopharmaceutical industry faces the challenge of efficiently characterising impurity profiles of therapeutic peptides, also due to their complex polar and ionisable attributes. This research explores the potential of advanced chromatographic techniques to address this challenge. The study compares dynamic electrostatic repulsion reversed phase (d-ERRP) to its counterparts (static ERRP and ion pair reversed phase IP-RP) in analysing Icatibant and its elusive epimeric impurity, [L-Arg]^{1}-Icatibant and highlights its exceptional capabilities in generating symmetric peaks, mitigating the common tailing phenomenon, and serving as a steadfast guardian of column longevity. The result highlights d-ERRP as a pioneering tool in the domain of liquid chromatography, fostering its role as a reference technique for the analysis of therapeutic peptides.

1. Introduction

Peptides are increasingly used in therapeutic applications, and the improvement of peptide design allowed to overcome their limits. In particular, improving resistance to proteolysis, bioavailability, and heightened selectivity and potency (Cabri et al., 2021; Lau and Dunn, 2018). The potentiality of this class of products is demonstrated by the recent explosion of the glucagon-like peptides 1 (GLP-1) derivatives for the treatment of metabolic diseases, such as diabetes and obesity (Alavi et al., 2019; Kumar, 2019; Moon et al., 2012), achieving multi-billion sales (Ozempic Sales Up 58% As Drugmaker Novo Nordisk Nets Record Profits (forbes.com). According to the extent of the modification of the primary structure, they can be divided between *Analogs*, where a minimal part of the sequence is modified (e.g Liraglutide, Semaglutide), or *Heterologous*, where a relevant portion of the sequence is modified (e.g. Degarelix, Icatibant) (Cabri et al., 2021; Gentilucci et al., 2006; Qvit et al., 2017). Chemical synthesis has become a crucial method for manufacturing peptides, especially when non-native residues need to be introduced into their primary sequence. Solid-phase peptide synthesis (SPPS) is widely recognised as the most important approach for this purpose.(Abdildinova et al., 2021; Góngora-Benítez et al., 2013; Verlander, 2007).

The SPPS method involves the sequential coupling of the desired N-protected amino acid to the growing peptide chain linked to a solid (polymeric) support. The sequence of linking is usually performed by activating the carboxylic function of the N-protected amino acid (in solution) to the free amino function linked to the solid support. However, this activation is widely known to originate racemisation of the chiral carbon in the alpha position of the N-protected amino acid either via base extraction or through the oxazolone intermediate (Benoiton, 1996; Gryko and Jurczak, 2003). Racemisation turns a chiral molecule from being enantiomerically pure to a mix of two enantiomers. If only one chiral center loses its chirality in peptides with multiple chiral centers, it is called epimerisation. (Reist et al., 1995). Furthermore, the stereocenters present in the formed peptide can undergo epimerization during the purification (downstream) process, where basic mobile phases may be required for the separation of critical impurities. Lastly, the thermal stress procedure used for the sterilization of the peptide finished dosage form (FDF), such as the terminal sterilization, can facilitate the degradation of these products, passing also through the pathway of epimerization (De Luca et al., 2021).

Beyond the challenge of impurity formation, the identification, characterisation, and separation of these impurities pose significant challenges. Mass spectrometry (MS) fails to distinguish epimeric

* Corresponding authors.
E-mail addresses: antonio.ricci@fresenius-kabi.com (A. Ricci), francesco.gasparrini@uniroma1.it (F. Gasparrini).

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impurities, as they are isobaric in nature, indicating identical mass and mass-to-charge ratio. Consequently, the qualitative and quantitative determination of the final product and any associated impurities arising during the production process assumes paramount importance. High-performance liquid chromatography (HPLC) stands out as the most widely adopted technique for the analysis and purification of peptides containing such impurities. However, the stringent regulations imposed by the FDA necessitate the evaluation of impurity content at remarkably low levels (0.5% limit for identified impurities, 0.10% for unknown ones (Sesquile, 2018; U.S. Food and Drug Administration, 2021) and force the development of novel chromatographic techniques able to tackle the risk of impurities escaping detection, particularly when they co-elute or elute immediately following the primary peak.

Innovative strategies have emerged to separate epimeric impurities in peptides, reshaping the chromatographic landscape through novel stationary phase exploration. A significant breakthrough has been achieved by introducing charged surface hybrid (CSH) packing materials (Gritti and Guiochon, 2013; Iraneta et al., 2010; Kadlecová et al., 2021). These materials leverage hybrid particle technology, resulting in a reduction in the number of silanols, and are characterised by the presence of covalently bonded positive charges. Operating under acidic pH conditions, this approach capitalises on repulsion between the positive charges on the particle and those of protonated basic compounds. This unique interplay, involving a combination of hydrophobic interactions between the C18 chain and the analyte and repulsive interactions between protonated analytes and positive charges, is referred to as electrostatic repulsion reversed phase (ERRP), as elucidated by Gritti and Guiochon (Gritti and Guiochon, 2014). An innovative entry in this realm is the BEH C18 AX column, which functions as an anionic exchanger, incorporating both hydrophobic and anion exchange functionalities.

Both the BEH C18 AX and CSH columns operate as pH-dependent stationary ion exchange phases. The number of sites available for electrostatic interactions is contingent upon the pH of the mobile phase. Notably, the BEH C18 AX column, equipped with tertiary amine groups in addition to classic octadecyl groups, exhibits a positive surface charge at pH 8.5. In contrast, the CSH C18 column, featuring pyridine moieties, acquires a positive charge only at pH 5 (Davies et al., 2007; Waters, 2020; Wyndham, K. D.; H.W., Thomas; P.C., Iraneta; U.D., Neue; P. D., McDonald;D., Morrison; M., 2000).

As elucidated in a Waters Corporation Application Note, this novel RP/AX mixed-mode stationary phase offers exemplary reproducibility and remarkable stability, even under high pH conditions. When employed with mobile phases maintaining a pH below 8, it demonstrates enhanced retention of anions, such as ionised acids, and reduced retention of cations, including protonated bases, compared to conventional RP stationary phases (Waters, 2020). The selectivity of ionisable analytes can be adjusted by modulating the pH of the mobile phase. The BEH C18 AX column not only facilitates ion pair elution modes but also excels in ERRP applications. Notably, the introduction of surface charge engenders “static” ERRP, distinguishing this elution mode in the context of this work.

Furthermore, a compelling approach has been developed by incorporating tetra butyl ammonium (TBA) as a mobile phase additive to replicate the intermolecular interactions described previously, encompassing attractive-hydrophobic and repulsive-electrostatic forces. This innovative approach has been termed dynamic ERRP (d-ERRP) to distinguish it from chromatographic systems in which the positive charge remains fixed on the stationary phase(Manetto et al., 2020; Mazzoccanti et al., 2021b, 2020). While similar strategies involving ionic liquids (ILs) in the mobile phase have been explored, the significance of electrostatic repulsion, as emphasised here, sets this approach apart. Moreover, the advantages of using TBA in terms of transparency to UV and reduced conditioning time render it a more convenient choice than ILs (Mazzoccanti et al., 2021a).

The overarching objective of this study revolves around the comparative evaluation of the d-ERRP chromatographic technique, static ERRP, and the established gold standard of IP-RPLC (ion-pair reversed-phase liquid chromatography)(Felletti et al., 2020; Horvath et al., 1977; Shibue et al., 2005; Toll et al., 2005) in the analysis of isobaric epimeric impurities within pharmaceutical peptides. To this end, we leverage ultra-high-performance liquid chromatography (UHPLC) for our investigations, specifically focusing on Icatibant.

Icatibant, an active pharmaceutical ingredient (API), serves as a Bradykinin-receptor antagonist belonging to this peptide class (Ghazi and Andrew Grant, 2013; Straka et al., 2017). Firazyr® (Agenzia Italiana del Farmaco (AIFA), 2022), the brand name of Icatibant FDF, achieved European Commission approval in 2008 and received FDA approval in 2011 for treating hereditary angioedema attacks. It was chosen as a case study for the analytical comparison due to its rather complex impurity profile, probably generated by the terminal sterilization treatment performed on the Icatibant FDF solution. Notably, one of its epimeric impurities, [L-Arg]^1-Icatibant, was not reported in the Firazyr® samples despite its relatively high content (1.5%) and was identified only in 2019 in a dedicated study (Lajin et al., 2019). As emphasised by Lajin et al., a conformational alteration in a single amino acid is unlikely to induce substantial changes in physicochemical properties, rendering it susceptible to evading detection.

Moreover, Icatibant poses a particularly formidable challenge for chromatographic separation due to its basic nature, characterised by a pI of approximately 12.2. Consequently, it is an excellent testbed for evaluating both the d-ERRP and static ERRP as alternative elution modes. The mobile phase employed in the study of Lajin et al. encompasses a mobile phase A, prepared using diammonium hydrogen phosphate with pH adjusted to 1.2 via 85% phosphoric acid, and mobile phase B based on acetonitrile (ACN). It is essential to acknowledge that while this mobile phase configuration allows for separating the impurity from the principal peak with a relative retention time (RRT) of 0.99, it poses challenges for column longevity, necessitating frequent replacements.

In the context of this work, the d-ERRP elution mode emerges as the most favourable option, not only in terms of resolution and peak shape but also for ensuring the longevity of columns. Various columns featuring diverse geometries and stationary phases were employed to conduct this investigation. The gradient elution modes for each injection were meticulously optimised under conditions that promote superior resolution. The study further encompassed the evaluation of its thermal stability, assessing the potential formation of additional epimeric impurities when exposed to aqueous environments at varying pH ranges and elevated temperatures.

2. Materials and method

Peptides (Icatibant and [L-Arg]^1-Icatibant) were gently given by Fresenius Kabi IPSUM (Italy). HPLC quality H_2O and acetonitrile (ACN), tetrabutylammonium hydrogen sulfate (TBAHSO4) (>99% *w/w*), trifluoroacetic acid (TFA) (>99% *w/w*), and difluoroacetic acid (DFA) (>99% *w/w*), sodium carbonate decahydrate ($Na_2CO_3 \bullet 10\ H_2O$) and sodium bicarbonate ($NaHCO_3$), sodium hydrogen phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) and citric acid were purchased from Merck life-science (Darmstadt, Germany). All the solvents were filtered before use on a 0.2 μm filter. The commercial columns used are as follows:

- ACQUITY Premier Glycan BEH-95A C18 AX, 100 × 2.1 mm (L.xI.D.), 1.7 μm
- ACQUITY Premier Peptide BEH-95A C18, 150 × 2.1 mm (L.xI.D.), 1.7 μm
- HALO Peptide ES-C18 160A, 150 × 3.0 mm (L.xI.D.), 2.0 μm
- ACQUITY BEH Phenyl 160A, 100 × 3.0mm (L.xI.D.), 1.7 μm
- KINETEX Biphenyl 150 × 3.0 mm (L.xI.D.), 2.6 μm

When employing a gradient elution method, it is imperative to tailor

the gradient profile to the specific dimensions of the column in use. Modifying the column geometry, whether through adjustments in its length or diameter, necessitates the application of precise relationships to seamlessly transfer the gradient method from one column to another while ensuring a consistent linear velocity. In this study, we have employed the following equations for this purpose (Carsten Paul, Maria Grübner et al., 2022):

$$\varphi_1 \times d_1^2 = \varphi_2 \times d_2^2 \quad (1)$$

where φ represents the flow and d represents the diameter of the column.

$$t_{G_1} : t_{01} = t_{G_2} : t_{02} \quad (2)$$

where t_G represents the gradient time, and t_0 is the dead time for each column.

UPLC Acquity Waters (Milford, MA, USA) was used for the experiment. It consisted of a binary solvent manager capable of delivering a maximum flow rate of 2.0 mL/min, an auto-sampler with a 5 µL injection loop and a PDA detector with a 500 nL flow cell. The PDA detector had an acquisition rate of 80 Hz, a resolution of 4.8 nm, and no filter time constant was used. Empower 3 software was used for data acquisition, handling, and instrument control. A standard UPLC Acquity Waters column heater was used to maintain a constant temperature in still air conditions (the maximum temperature was set to 65°C). Additionally, Viper capillaries minimised the extra-column contribution to band broadening. The inlet Viper capillary had a dimension of 250 mm × 0.100 mm I.D., while the outlet Viper capillary was 350 mm × 0.100 mm I.D.

The stability of Icatibant was evaluated under the best elution conditions. The aim was to replicate any possible environment in which the sample might be found, and several aqueous buffers were prepared to solubilise the samples for analysis while recreating different pH environments.

To further stress the sample, it was stored at 50°C and analysed over time.

3. Results and discussion

3.1. Ion pair reversed-phase chromatography

The first technique used was ion-pair reversed-phase chromatography. Typical mobile phases are acetonitrile and water-based, and the separation mechanism is highly dependent on the composition of the eluent and the stationary phase, which, by influencing hydrophobic interactions, contributes to compound retention. Since an acid additive was required, a comparison was made by first enriching the mobile phase with 0.1% *v/v* DFA (difluoroacetic acid) and then 0.1% *v/v* TFA (trifluoroacetic acid) using commercially available chromatographic columns. In peptide and protein separation, DFA and TFA are both ionic pair agents. Still, the former is milder, modifying the hydrophobicity conferred on the analyte and causing a critical difference in retention times. The samples were analysed using columns with different stationary phases, from C18 through hybrid BEH C18 to phenyl and biphenyl selectors. The gradient method was adapted to the geometry of the column used. The first stationary phase screening was done using DFA as acidic additive. For the HALO Peptide ES-C18 160Å, 150 × 3.0 mm (L.xI.D.), 2.0 µm column, a gradient starting from 12% B to 19% B (were B is ACN+0.1% *v/v* DFA) was used in 45 minutes. The analysis was carried out by keeping the column at a constant temperature of 50°C.

The IP-RP chromatography technique does not provide a good result, with a highly asymmetric peak and an important tailing *(see* Fig. S1 in Supporting Information*)*.

For the Acquity Premier Peptide BEH C18 300Å, 150 × 2.1 mm (L.xI.D.), 1.7 µm, the same gradient conditions were used as the column length is the same as the previous one. The flow rate was modified using Eq. (1) from 0.5 mL/min to 0.2 mL/min. Using the hybrid silica particle BEH C18, which should ensure low activity of the residual silanol groups, was insufficient to improve the symmetry of the peak, which continues to appear somewhat asymmetric. The analyses were performed again by changing the stationary phase, but the symmetry of the peak does not seem to improve.

An ACQUITY BEH Phenyl 160Å, 100 × 3.0 mm (L.xI.D.), 1.7 µm column was used. As for the previous one, this column is formed by a stationary phase with BEH (*Bridged Ethylene Hybrid*) technology, but this time, there is a phenyl selector instead of the normal octadecyl. The gradient was optimised according to the column geometry by optimising the flow rate with Eq. (1) and the gradient time with Eq. (2). For this column, a gradient time of 30 minutes (from 15 % B to 22% B) and a flow rate of 0.5 mL/min were used.

Finally, a KINETEX Biphenyl 150 × 3.0 mm (L.xI.D.), 2.6 µm, whose selector is biphenyl was tested. The gradient was to go from 12% B to 19% B in a gradient time of 45 minutes.

The analyses were repeated using TFA as an acidic additive in the mobile phase. Still, there was no appreciable improvement in the symmetry of the peaks (see Fig. S2 in Supporting Information). The use of TFA promotes an increase in retention times. For this reason, a higher percentage of organic modifiers was used than predicted by the DFA analysis.

It is, therefore, important to underline, as shown in Fig. 1, how the poor efficiency does not make the IP-RP application useful because of the important asymmetry of the peaks and the evident tailing.

3.2. Static electrostatic repulsion reversed-phase

We switched to using the static ERRP technique after experiencing poor results in terms of efficiency and asymmetric peak shape with IP-RP. The method utilises a chromatographic column with a hydrophobic selector and a positive charge in the stationary phase at acidic pH. For this purpose, we used an Acquity Premier Glycan BEH-300A C18 AX, 100 × 2.1 mm (L.xI.D.), 1.7 µm column with a gradient elution. We optimised the gradient from 10% B to 20% B within 30 minutes. We compared two acidic additives, DFA and TFA, at a percentage of 0.1% v/v. A key point we want to emphasise through Fig. 2 is the notable enhancement in peak symmetry and the decrease in tailing when comparing the results obtained through two distinct chromatographic methods, both employing columns with identical particle technology but featuring different stationary phase chemistries.

Upon a comprehensive analysis of the initial two chromatographic methods, namely IP-RP and static ERRP, it becomes evident that the latter exhibits notable enhancements in efficiency. Subsequently, an effort was made to introduce the impurity [L-Arg]^1-Icatibant into the system to discern whether it could further enhance the resolution of the epimeric impurity. To clarify the results, we compared the chromatograms obtained from the analysis of the [L-Arg]^1-Icatibant impurity and two samples of spiked Icatibant with 2% and 6% of the [L-Arg]^1-Icatibant impurity, specifically Icatibant 1 and Icatibant 2.

Regrettably, the outcomes presented in Fig. 3 indicate that the

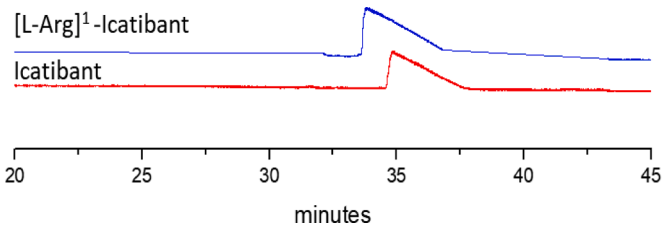

Fig. 1. Sample: Icatibant and [L-Arg]-Icatibant; MP: H_2O/ACN + TFA 0.1% v/v; Acquity Premier Peptide BEH C18 300Å, 150 × 2.1 mm (L.xI.D.), 1.7 µm, UV: 214 nm; Tcol: 50°C.

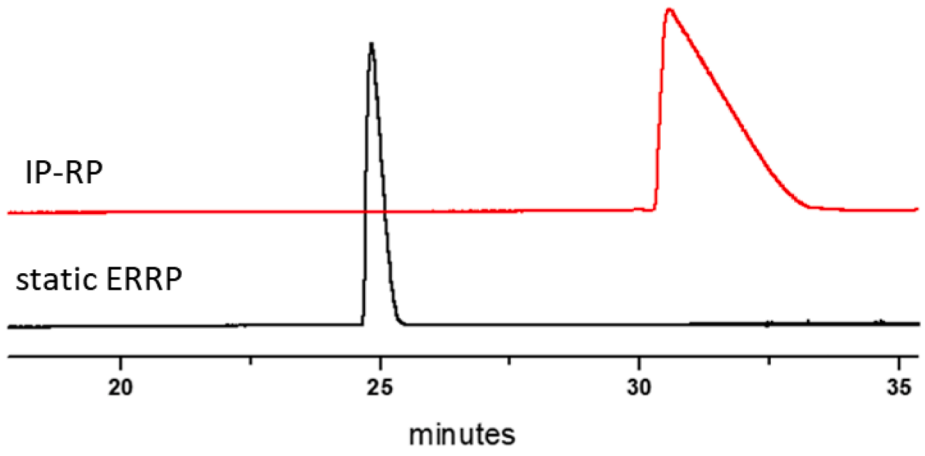

Fig. 2. Sample: Icatibant; MP: H_2O/ACN + TFA 0.1% v/v; Colonna: Acquity Premier Glycan BEH-300Å C18 AX, 100 × 2.1 mm (L.xI.D.), 1.7 µm (black line) VS Acquity Premier Peptide BEH C18 300Å, 150 × 2.1 mm (L.xI.D.), 1.7 µm (red line); UV: 214 nm; Tcol: 50°C.

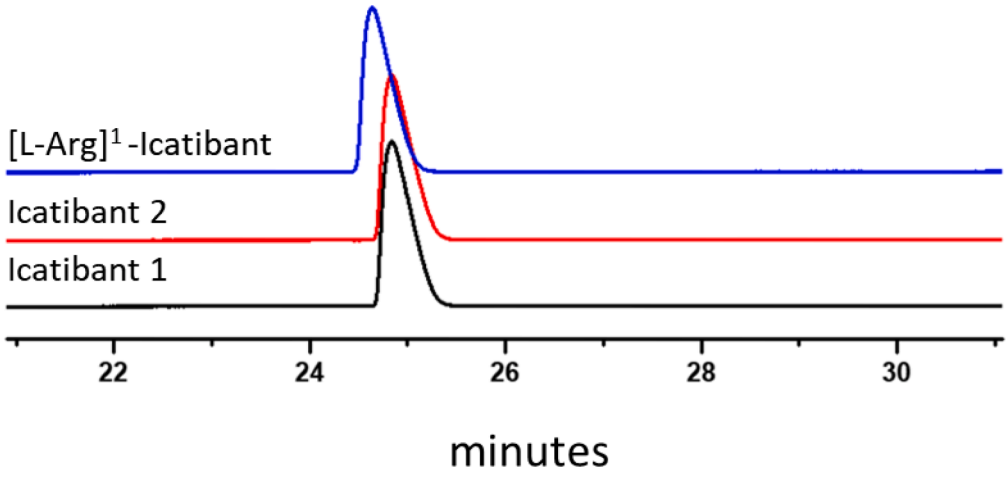

Fig. 3. Comparison. Sample: Icatibant 1, Icatibant 2 and [L-Arg]^1 Icatibant; MP: H_2O/ACN + TFA 0.1% v/v; Column: Acquity Premier Glycan BEH-300Å C18 AX, 100 × 2.1 mm (L.xI.D.), 1.7 µm; UV: 214 nm; Tcol: 50°C; flow rate: 0.2 mL/min.

method efficiency, even with incorporating the impurity [L-Arg]^1-Icatibant, still falls short of achieving the desired level necessary for successfully resolving this challenging epimeric impurity.

3.3. Dynamic electrostatic repulsion reversed-phase

Considering the suboptimal results obtained from the prior techniques, a transition was made to the d-ERRP strategy. A gradient elution was maintained throughout the experiments, with water serving as the mobile phase A and acetonitrile as the mobile phase B, enriched with 10 mM $TBAHSO_4$.

During the development of the d-ERRP technique, an evaluation of the same columns used in the previous methods was conducted. The Acquity Premier Glycan BEH-300A C18 AX column, which already possesses a positive charge on its surface, was excluded from this evaluation.

The outcomes revealed a marked enhancement in the peak shape, resulting in significantly more symmetrical chromatographic profiles. Most significantly, the d-ERRP method achieved excellent resolution of the isobaric epimeric impurity. The impact on the resolution of [L-Arg]^1 Icatibant was particularly striking when compared to the performance of the other methods. The resolution factor (Rs) values were notably higher for the d-ERRP approach: 2.26 for BEH C18 (RRT 0.95), 4.20 for Phenyl (RRT 0.91), and 3.07 for Biphenyl (RRT 0.93). These calculations underscore the substantial performance disparities among the columns employed, with the ACQUITY BEH Phenyl Column emerging as the most effective, as evidenced by its higher Rs value compared to the standard BEH C18.

Fig. 4 provides a comparative analysis of three samples analysed with the ACQUITY BEH Phenyl Column: Icatibant 1, Icatibant 2, and [L-Arg]1-Icatibant, underscoring the remarkable selectivity and efficiency achieved in resolving the epimeric impurity.

3.4. Evaluation of the chemical stability of icatibant

For the assessment of stability under basic pH conditions, a buffer solution at pH 10 was prepared by dissolving sodium carbonate decahydrate ($Na_2CO_3 \bullet 10\ H_2O$) and sodium bicarbonate ($NaHCO_3$) in water, resulting in solutions with a concentration of 0.1 M each. These solutions were subsequently combined to obtain the pH 10 buffer. The mobile phases employed in this assessment align with the d-ERRP methodology: mobile phase A consisted of water, while mobile phase B was composed of acetonitrile, with both phases enriched with 10 mM $TBAHSO_4$. The column utilised for this experiment was the ACQUITY BEH Phenyl, which was maintained at a controlled temperature of 50°C. A 45-minute elution gradient was employed, involving a variation in the organic modifier from 5% B to 12% B.

The evaluation of stability in a basic environment was carried out at a temperature of 50°C, with a heating plate that had a measurement error of 0.5°C. Sampling was conducted at specified time intervals.

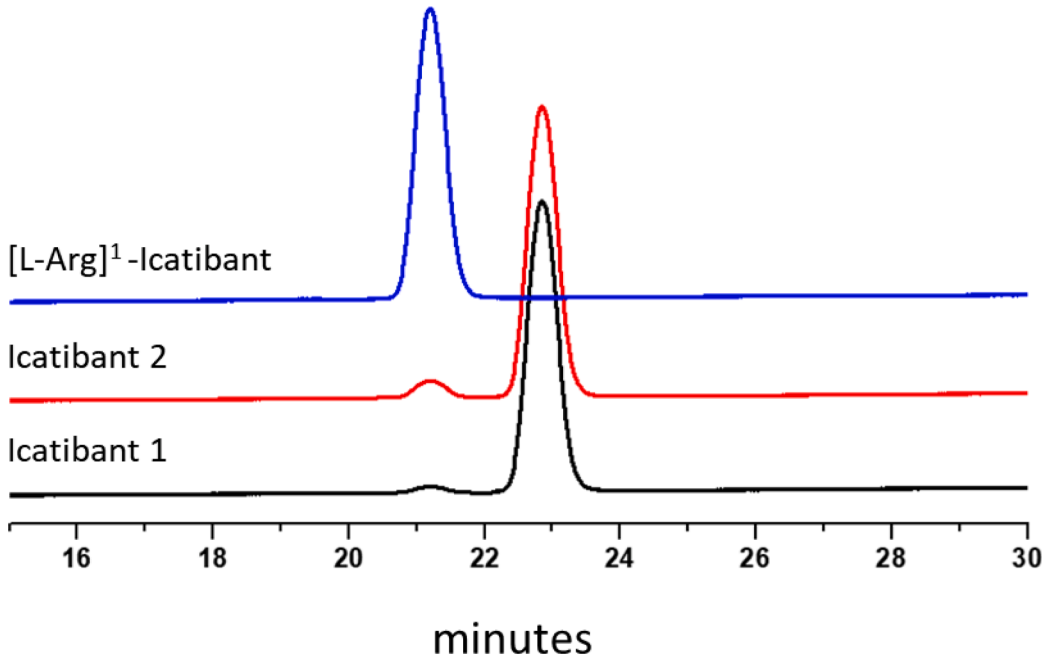

Fig. 4. Sample: Icatibant 1, Icatibant 2 and [L-Arg]^1 Icatibant; MP: H_2O/ACN + $TBAHSO_4$ 10 mM; Column: ACQUITY BEH Phenyl 160Å, 100 × 3.0 mm (L.xI.D.), 1.7 µm; UV: 214 nm; t_G: 30 min; Tcol: 50°C; Flow rate: 0.5 mL/min.

It's important to note that the pH value chosen for this assessment is conducive to the epimerisation reaction. In fact, the formation of the epimeric impurity [L-Arg]^1-Icatibant under these conditions appeared consistent with expectations.

The results illustrated in Fig. 5 reveal that the formation of the epimer occurs after 24 hours at 50°C, where an approximate percentage area of 3% is attributed to the epimeric peak in relation to the main peak.

For the evaluation of stability under neutral pH conditions, a pH 7 buffer was prepared by dissolving sodium hydrogen phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) in water. Both solutions were prepared at a concentration of 0.1 M and were subsequently mixed to create the pH 7 buffer.

In this analysis, the sample was directly weighed and solubilised in the buffer. No formation of impurities was observed under neutral pH conditions, as evidenced by the chromatographic profiles recorded over time (Fig. 6). The experiment was conducted at a temperature of approximately 50°C for an equivalent duration of around 30 hours, mirroring the analysis duration at basic pH.

Curiosity then led to an exploration of the sample behaviour in a purely aqueous environment. The sample was solubilised in water and injected following the same analytical procedure. The results remained

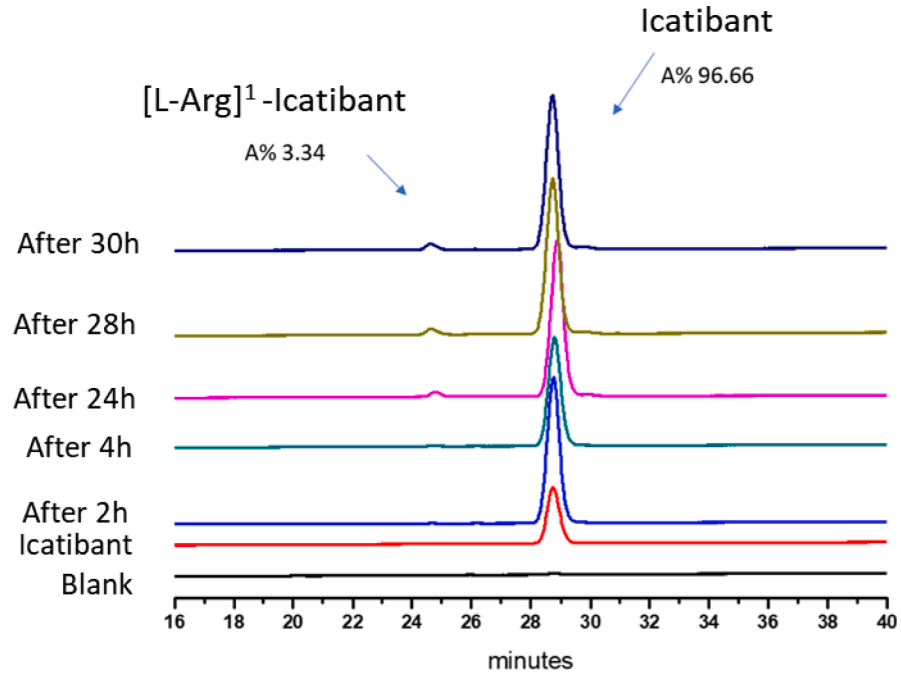

Fig. 5. Sample: Icatibant (buffer pH=10); MP: H_2O/ACN + TBAHSO4 10 mM; Column: ACQUITY BEH Phenyl 160Å, 100 × 3.0 mm (L.xI.D.), 1.7 µm; UV: 214 nm; t_G: 45 min; Tcol: 50°C; Flow rate: 0.5 mL/min.

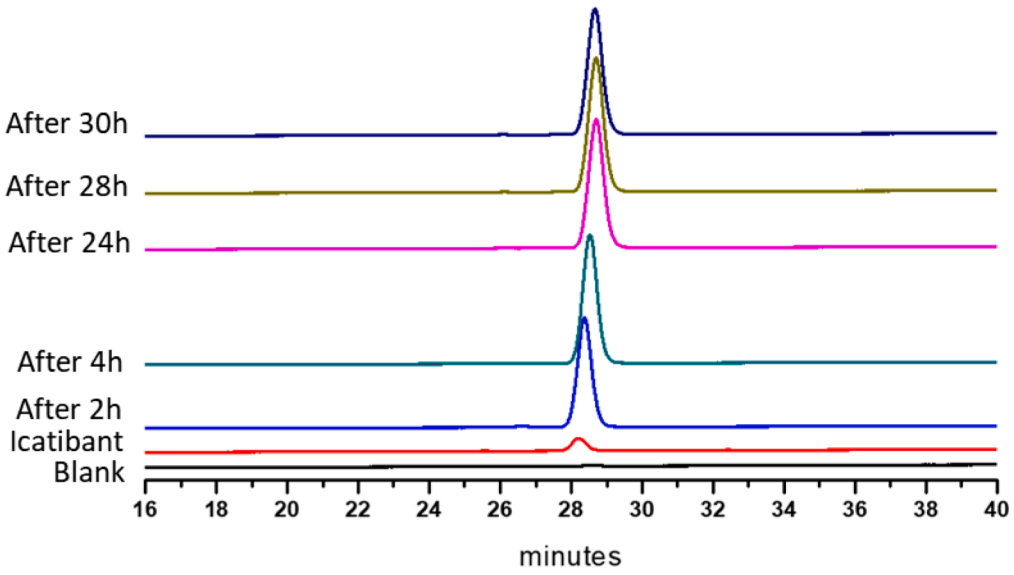

Fig. 6. Sample: Icatibant (buffer pH=7); MP: H_2O/ACN + $TBAHSO_4$ 10 mM; Column: ACQUITY BEH Phenyl 160Å, 100 × 3.0 mm (L.xI.D.), 1.7 µm; UV: 214 nm; t_G: 45 min; Tcol: 50°C; Flow rate: 0.5 mL/min.

consistent (Fig. 7), affirming the stability of the sample, both in a buffered neutral environment and in water, after 24 hours at 50°C. This observation underscores the robust stability of the sample even in the absence of buffering agents, providing valuable insights into its behaviour in different conditions.

To assess stability under acidic pH conditions, a pH 3 buffer was prepared. This buffer was created by dissolving citric acid in water to obtain a concentration of 0.1 M, and sodium dihydrogen phosphate (NaH_2PO_4) was dissolved to achieve a concentration of 0.2 M. These two solutions were subsequently combined to create the pH 3 buffer.

In this evaluation, the sample was directly weighed and dissolved in the prepared buffer. The solution was then placed on a heated plate at a constant temperature of 50°C. This experiment aimed to investigate the sample stability under acidic pH conditions, which can provide valuable insights into its behaviour in such an environment.

The results depicted in Fig. 8 reveal intriguing observations regarding the sample behaviour under acidic pH conditions. After approximately 24 hours, there is a noticeable deformation of the Icatibant peak, characterised by pronounced tailing. Following six additional hours (totalling 30 hours), two distinct, well-resolved peaks emerge. Notably, the retention time of the main peak does not align with that of Icatibant, suggesting that the elevated temperature and acidic environment may have induced the formation of two new chemical species. These findings underscore the dynamic nature of the sample under these conditions and warrant further investigation to elucidate the identity and characteristics of the newly formed species which have not been characterised in this work.

4. Conclusion

The intricate task of separating and quantifying pharmaceutical peptides, coupled with the complex challenge of addressing their elusive epimeric impurities, has assumed paramount importance within the realm of the biopharmaceutical industry. Peptides, characterised by their polar and ionisable attributes, introduce a unique set of complexities to the landscape of liquid chromatography, particularly when they coexist with isobaric epimeric impurities, effectively rendering their differentiation a formidable challenge when relying only on mass spectrometry coupling.

In this work, we have embarked on a comprehensive exploration of advanced chromatographic techniques, most notably d-ERRP and static ERRP. Our focus has been to decipher their potential in addressing the intricate analytical complexities posed by these compounds.

We have meticulously investigated the utility of d-ERRP, vis-à-vis its counterparts, static ERRP and IP-RP, within the context of analysing Icatibant along with its elusive epimeric impurity, [L-Arg]^1-Icatibant, originated from the epimerisation reaction of the amino acid residue Arginine at position 1.

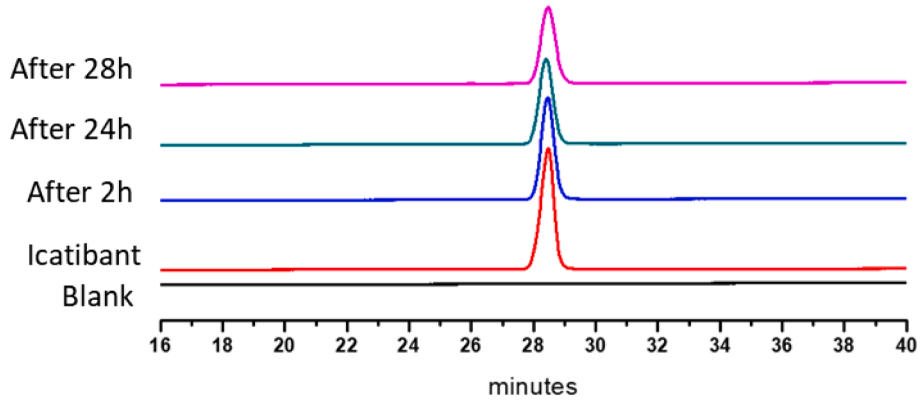

Fig. 7. Standard: Icatibant in H_2O; MP: H2O/ACN + TBAHSO4 10 mM; Column: ACQUITY BEH Phenyl 160Å, 100 × 3.0 mm (L.xI.D.), 1.7 µm; UV: 214 nm; t_G: 45 min; Tcol: 50°C; Flow rate: 0.5 mL/min.

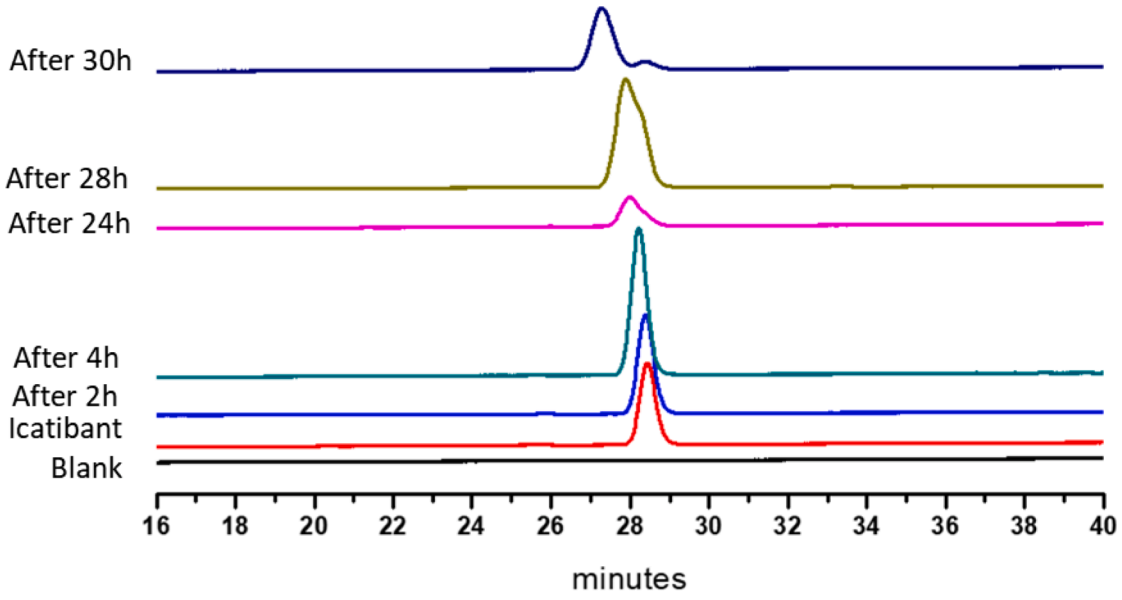

Fig. 8. Sample: Icatibant (buffer pH=3); MP: H_2O/ACN + $TBAHSO_4$ 10 mM; Column: ACQUITY BEH Phenyl 160Å, 100 × 3.0 mm (L.xI.D.), 1.7 µm; UV: 214 nm; t_G: 45 min; Tcol: 50°C; Flow rate: 0.5 mL/min.

The use of different methods to obtain chromatographic profiles for Icatibant reveals the exceptional abilities of the d-ERRP. This technique significantly produces symmetric peaks, thus eliminating the common tailing phenomenon that often occurs during the analysis of these compounds. Overall, the d-ERRP stands as an exemplary method for chromatographic analysis.

In the context of this work, it is essential to highlight that the d-ERRP elution mode emerges as the most auspicious and advanced option. It not only excels in terms of achieving exceptional resolution and impeccable peak shape but also stands as a steadfast guardian of column longevity. Consequently, improving the conventional methodologies found in the existing literature. This work underscores the transformative potential of d-ERRP and heralds its ascension as a pioneering tool in the realm of peptide analysis, poised to unlock new frontiers in the domain of pharmaceutical research and development.

CRediT authorship contribution statement

Giulia Mazzoccanti: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Simone Manetto:** Data curation, Investigation, Methodology, Validation, Visualization. **Michele Bassan:** Data curation, Methodology, Validation. **Marco Macis:** Data curation, Methodology, Validation. **Walter Cabri:** Funding acquisition, Project administration, Resources. **Alessia Ciogli:** Investigation, Methodology, Supervision. **Antonio Ricci:** Conceptualization, Funding acquisition, Resources, Writing – original draft, Writing – review & editing. **Francesco Gasparrini:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision.

Declaration of Competing Interest

There are no conflicts to declare.

Data availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2023.106682.

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