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The development and optimisation of an HPLC-based in vitro serum stability assay for a calcitonin gene-related peptide receptor antagonist peptide

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Evaluation of the stability of peptide drug candidates in biological fluids, such as blood serum, is of high importance during the lead optimisation phase. Here, we describe the optimisation and validation of a method for the evaluation of the stability of a lead calcitonin gene-related peptide antagonist peptide (P006) in blood serum. After initially determining appropriate peptide and human serum concentrations and selection of the quenching reagent, the HPLC method optimisation used two experimental designs, Plackett–Burman design and Taguchi design. The analytical method was validated as complying with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use guidelines. The optimised method allowed the successful resolution of the parent peptide from its metabolites using RP-HPLC and identification of the major metabolites of P006 by mass spectrometry. This paradigm may be widely adopted as a robust early-stage platform for screening peptide stability to rule out candidates with low in vitro stability, which would likely translate into poor in vivo pharmacokinetics.

KEYWORDS
CGRP, design of experiments, HPLC, migraine, serum stability

1 | INTRODUCTION

As of 2022, over 110 peptide-based therapeutics have been granted clinical approval in a number of disease areas, for example, metabolic disorders, cancers, cardiovascular, and infectious diseases.[1] For peptide candidates, the path to successful translation from lab to clinic is often challenging, particularly due to poor oral bioavailability, low permeability, low metabolic stability, short half-life, and inadequate residence time in tissues.[2] Notably, many peptides showing promising in vitro pharmacological activities fail to show suitable in vivo effects. One main reason for this is their typically short half-life due to low metabolic stability, becoming a major chokepoint to overcome in the lead-optimisation (or earlier) phase of a peptide drug candidate.[2,3] On the other hand, a very long half-life may cause systemic toxicity.[4] Thus, optimisation of the stability and indeed of the half-life is key in the early-stage development of peptide candidates.

Literature reporting of peptide stability is based on a range of different assays, e.g., in vitro stability assays in different media (i.e., plasma/serum, simulated gastric or intestine fluids, and microsomes), in vivo half-life measured in animal models, in vivo half-life measured in healthy volunteers or in patients with particular disease of interest.[2] This results in challenges when comparing different datasets and difficulties in benchmarking compounds. The scientific literature is full of examples of assays to measure in vitro stability of small molecules. However, these methods have more rarely been applied to peptide candidates,[3,5–14] or are often poorly described.

The drug candidate is typically incubated at 37°C in blood serum, blood plasma or in a medium containing one or more isolated enzymes. After incubation of the compound for specified time intervals, the reaction is generally stopped with a quenching reagent, normally an aqueous acidic solution or an organic solvent, that denatures and precipitates larger serum proteins. The supernatant solution containing intact peptide and metabolites is then analysed by reversed-phase liquid chromatography (RP-HPLC) and/or mass spectrometry (LC-MS, LC-MS/MS or MALDI-TOF).[3,5,7–14] The use of LC-MS/MS is becoming increasingly common to quantify compounds in biological fluids, but this needs expensive equipment, highly skilled analysts, and robust techniques,[15] while RP-HPLC, being more common and less expensive, is accessible to a greater number of scientists.

The 37-amino acid neuropeptide calcitonin gene-related peptide (CGRP) is considered a potent vasodilator and a mediator of pain transmission and has been demonstrated to be a key player in migraine pathophysiology,[16,17] exerting its function as the natural agonist of the CGRP receptor complex (CGRP-R).[18,19] The involvement of CGRP in migraine pathophysiology is supported by a number of studies.[20–26] Various research groups have worked on the identification of peptide-based CGRP-R antagonists based on truncations of the natural CGRP.[27–32] Recently, our research group reported on P006, a truncated and modified version of CGRP that showed strong binding affinity to the receptor ($pK_i = 7.8$), functioning as a potent antagonist ($pK_b = 7.9$), which reduced CGRP-induced cutaneous vasodilation in a mouse model assessed by Laser Speckle Contrast Imaging to measure blood perfusion.

To estimate the apparent half-life of our lead candidate P006, we developed and optimised an in vitro assay to test proteolytic resistance, specifically tailored for peptide drug candidates. After selection of the appropriate concentration of peptide, human serum, and quenching reagent, we performed the optimisation of the chromatographic method for quantification of the intact peptide and identification of the metabolites, and finally the validation of the analytical method following the ICH guidelines. The approach to method optimisation and the methodology itself could become a benchmark procedure to use in drug discovery to rank compounds for biological stability and exclude the ones with unacceptable values within a library.

2 | MATERIALS AND METHODS

2.1 | Materials

All Fmoc L-amino acids, Rink Amide ProTide resin (100–200 mesh, 0.56 mmol/g), and Oxyma Pure™ were purchased from CEM Microwave Technologies (Buckinghamshire, UK). *N*,*N*′-Diisopropylcarbodiimide (DIC), piperidine, trifluoroacetic acid (TFA), triisopropylsilane (TIPS), serum from human male AB plasma (USA origin, sterile-filtered, H4522, Lot#SLCF0252), trichloroacetic acid (TCA) and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich (Gillingham, UK). *N*,*N*-Dimethylformamide (DMF) and phosphate buffered saline (PBS) tablets (tablet formulation: sodium chloride 137 mM, phosphate buffer 10 mM, potassium chloride 2.7 mM) were purchased from Fisher Scientific (Leicestershire, UK). Solvents for liquid chromatography (HPLC-grade acetonitrile (MeCN), methanol (MeOH)) were purchased from Fisher Scientific (Leicestershire, UK). All reagents and solvents were used as supplied.

2.2 | Synthesis of P006 and its main metabolite (LJMU025)

Peptides were prepared using automated Fmoc-SPPS methods on a Liberty Blue microwave-assisted peptide synthesiser (CEM). Solid phase synthesis was conducted at 0.1 mmol scale using Rink amide ProTide resin (179 mg, 0.56 mmol/g loading). Amino acid coupling reactions were performed employing the required Fmoc-amino acids (0.2 M in DMF, 5 eq.) with DIC (1 M in DMF; 10 eq.) and Oxyma Pure™ (1 M in DMF, 5 eq.), and Fmoc deprotection was performed with piperidine (20% *v*/*v* in DMF, 4 mL). Standard coupling procedures employed single coupling of each amino acid (2.5 min, 90°C). After completion of the on-resin synthesis, the resin was shrunk in diethyl ether (Et_2O) and the compound was cleaved from the resin as the C-terminal amide using a cleavage cocktail (3 mL; comprising TFA, TIPS, and water [8:1:1 *v*/*v*]) with regular shaking (Eppendorf Thermomixer comfort) at room temperature for 3 h. Following the cleavage step, the crude peptides were precipitated in cold Et_2O, kept in the freezer for 30 min, then the suspension was centrifuged (3500 rpm, 5 min). The isolated pellets were resuspended in Et_2O and centrifuged again to remove residual TFA. Finally, the isolated solids were dissolved in water, flash-frozen using liquid N_2, and lyophilised.

Crude peptides (10 mg/mL) were purified using an Agilent Infinity 1260 equipped with a Waters XBridge Peptide BEH C18 Prep 130 Å column (5 µm particle size, 10 × 150 mm). A gradient elution method used MeOH/H_2O (30 min: from 30% to 99% MeOH, with 0.1% TFA) at a flow rate of 8 mL/min and 50 to 800 µL injection volumes. The column was kept at room temperature (~25°C), and signals were recorded by their absorption at a wavelength of 215 nm. The isolated pure compound solutions were concentrated in vacuo to remove residual organic solvents. The resulting aqueous solutions were flash frozen with liquid N_2, lyophilised and stored at −20°C as the TFA salt of the peptide. Peptide identity and purity were analysed by RP-HPLC and LC-MS.

2.3 | Initial HPLC analysis

Samples were analysed using an Agilent 1100 HPLC system equipped with a Phenomenex Aeris PEPTIDE XB-C18 LC Column (150 × 4.6 mm, pore size: 3.6 µm) and diode array detector (absorbance measured at 215 nm), employing a binary eluent system of H_2O/0.1% TFA, and MeCN/0.1% TFA. A gradient method from 99% H_2O/0.1% TFA to 99% MeCN/0.1% TFA was employed, with a run time of 22 min at a flow rate of 1 mL/min, injection volume of 10 µL,

and a temperature in the column compartment of 25°C. Finally, the column was equilibrated back to initial conditions for 2 min. Operating pressures were in the range of 140–180 bar.

2.4 | Optimisation of HPLC method—Design of experiments

To optimise the HPLC conditions, two design of experiments (DoE), a Plackett–Burman design and a Taguchi design, were used. In each design, the resolution (R_s, Equation 1) between the two peaks was the response measured.[34]

$$R_s = \frac{2 * (RT_2 - RT_1)}{W_1 + W_2} \quad (1)$$

In the Plackett–Burman design, seven factors were studied, as listed in Table 1. The HPLC system (Agilent 1100), sample concentration (0.2 mg/mL), wavelength (215 nm, band width 4 nm), injection volume (10 µL), and preparation of sample mixture (P006 0.2 mg/mL + LJMU025 0.2 mg/mL) were kept constant; 36 randomised runs (12 runs, three replicates) were suggested from Minitab (version 12.2020.2.0).

A Taguchi design of experiment was constructed with Minitab (version 12.2020.2.0) by choosing the L9 3^3 array (three factors evaluated at three levels). Factors under evaluation in the Taguchi design are described in Table 2. Parameters kept constant during the investigation included the HPLC instrument (Agilent 1100), injection volume (10 µL), sample concentration (0.2 mg/mL), column (Phenomenex Aeris, 3.6 mm XB-C18 LC-Column 100 Å 150 × 4.6 mm), diode-array detection wavelength (215 nm, band width 4 nm), gradient time (10 min), % of organic modifier at start of the gradient (i.e., 20% MeCN), % of organic modifier at end of the gradient (i.e., 60% MeCN).

TABLE 1 Factors under evaluation in the Plackett–Burman design.

Factor under evaluation	Level 1	Level 2
Column particle size (µm)[a]	3.6	5
Initial gradient (% of MeCN)	5	20
Final gradient (% of MeCN)	50	60
Gradient time (min)	10	30
Flow rate (mL/min)	0.5	1
Column temperature (°C)	25	65
TFA in mobile phases (%)	0.05	1

[a]Two HPLC columns used: Phenomenex Aeris PEPTIDE XC-C18 LC column 150 × 4.6 mm C18 particle size 3.6 µm and YMC 150 × 4.6 mm C18 particle size 5 µm.

TABLE 2 Factors under evaluation in the Taguchi design (L9 3^3 array) and levels at which the factors were evaluated.

Factor under evaluation	Level 1	Level 2	Level 3
Column temperature (°C)	35	50	65
Flow rate (mL/min)	0.5	0.8	1
TFA in mobile phases (%)	0.05	0.08	0.1

2.5 | Optimised HPLC method for stability study

For the stability study, the supernatant solutions derived from pelleting the serum proteins were analysed by RP-HPLC (Agilent 1100, fitted with a Phenomenex Aeris C18 Column; particle size 3.6 µm peptide XB-C18 100 Å LC Column 150 × 4.6 mm) using a linear gradient from 80% solution A (H_2O, 0.05% TFA) to 60% solution B (MeCN, 0.05% TFA) over 10 min. A flow rate of 1 mL/min was used, the absorbance was measured using diode array detection (DAD) at a wavelength of 215 nm and the analysis was performed at 65°C. The amount of the intact peptide was quantified by measuring the peak areas against the appropriate blank and the standard curves in blank serum supernatant ($r^2 > 0.999$; see Supporting Information). The amount of compound at time 0 min was used as 100%, and the remaining intact peptide at various time points was calculated as a percentage of the initial concentration, using Equation (2):

$$\%Remaining\ intact\ compound = \frac{Concentration\ at\ time\ T * 100}{Concentration\ at\ time\ zero} \quad (2)$$

2.6 | Validation of optimised HPLC method

The developed HPLC method was validated according to the ICH guidelines.[35] Linearity, range, intraday and interday precision, sensitivity, specificity, robustness, and stability were evaluated.[35,36] Linearity was evaluated in triplicate by calculating the regression line from the graphical plot of the area derived from the chromatographic peak (y) versus the peptide concentration (x) of 11 standard solutions (5, 7.5, 10, 25, 75, 500, 750, 1000 µg/mL), obtaining a calibration curve. This analysis was followed by the corresponding statistical study using a linear least-squares regression method and by analysis of the respective response factors. The specified range was derived from linearity studies.

For investigation of precision, the repeatability was calculated on three levels (50, 100, and 250 µg/mL), as the standard deviation of three replicates on the calibration curve on the same day (intraday precision). Interday variation was calculated by determination of the same samples at three concentration levels (50, 100, 250 µg/mL) in the analysis series on three different days. Repeatability of injection was assessed by the analysis of three standard solutions (50, 100, 250 µg/mL) injected five times in the same day. Sensitivity was evaluated through the values of the lower limit of detection (LLOD) and lower limit of quantification (LLOQ) using Equations (3) and (4):

$$LLOD = 3.3 * \frac{S_Y}{S} \quad (3)$$

$$LLOQ = 10 * \frac{S_Y}{S} \quad (4)$$

where S_Y is the standard error derived from the regression analysis and S is the slope of the calibration curve.

Specificity was evaluated by analysing P006 in deionised H_2O, in H_2O + DMSO (5% *v/v*), in H_2O + TCA (2.5% *w/v*), and in serum supernatant solutions. To assess the robustness of the method, flow rate (±0.1 mL/min), wavelength (±2 nm), and column compartment temperature (±2°C) were changed during analysis. A sample solution of 250 μg/mL was prepared and injected for every condition and read-out was retention time and RSD% calculated using the equation:

$$RSD\% = \frac{Standard\ Deviation\ of\ the\ three\ areas\ under\ the\ peak}{Average\ of\ the\ three\ areas\ under\ the\ peak} * 100 \quad (5)$$

Storage stability at 25°C ± 2°C was evaluated at different time intervals up to 72 h.

2.7 | Stability of peptides over 24 h at 37°C

P006 solutions (200 μL in H_2O, 5% DMSO) at different concentrations (520, 260, 100, and 50 μg/mL) were incubated at 37°C for 24 h while shaking at 200 rpm. The solutions were then centrifuged (5 min at 13,200 rpm) and the supernatant was analysed by RP-HPLC using the method described in Section 2.5. The resulting area under the peak of interest was analysed against a calibration curve (serial dilutions of peptide in PBS, 5% DMSO). Retention time of the peak of interest was used to check possible peptide degradation. The experiment was performed in duplicate.

2.8 | Stability of peptides in acidic conditions

A peptide solution (200 μL, 130 μg/mL, in PBS, 5% DMSO) was incubated at 37°C for 15 min with shaking at 200 rpm. After addition of aq. TCA (40 μL, 15% *w/v*), the peptide solution was cooled on ice for 15 min and then centrifuged (13,200 rpm for 5 min). The resulting supernatant was analysed by RP-HPLC using the method described in Section 2.5. Control samples were prepared under the same conditions but adding H_2O instead of TCA aq. solution and taken as 100% against the TCA samples. The retention time of the peak of interest was checked to exclude the possibility of peptide degradation in acidic conditions. The experiment was performed in duplicate.

2.9 | Stability of peptides in acidic conditions at elevated temperature

A peptide solution (200 μL, 0.1 mg/mL in water, 0.25% DMSO, 0.05% TFA, pH 2) was incubated at 65°C and shaken at 500 rpm, using a temperature-controlled thermal shaker, at known time intervals (0, 2.5, 5, 7.5, 10 min). After the given time frame, the peptide solution was cooled on ice for 5 min and then brought to room temperature. The peptide solution was analysed by RP-HPLC using the method described in Section 2.5. HPLC analysis was performed at 25°C. The experiment was performed in triplicate.

2.10 | Stability of peptides in human serum

Aqueous pooled human serum (25% *v/v* in PBS buffer), temperature-equilibrated at 37°C for 15 min, was spiked with the compound under evaluation to a final concentration of 100 μg/mL and final volume of 200 μL (five samples in total). Samples were mixed in a sample mixer (20 rpm) at 37°C. At known time intervals (i.e., 0, 15, 30, 45, 60 min), the reaction was stopped by adding aq. TCA (40 μL, 15% *w/v*). The cloudy reaction samples were cooled on ice (>15 min) to precipitate serum proteins, before centrifugation at 13,200 rpm for 2 min to pellet the precipitated proteins. The resulting supernatant was analysed by RP-HPLC using the method optimised and validated for the stability study (see Section 2.5). The concentration of intact peptide at time zero was taken as 100% and concentrations were measured against a calibration curve (Figure S7). A blank control experiment was carried out under the same conditions but adding deionised H_2O in place of peptide stock solution. The experiment was performed in triplicate.

2.11 | Mass spectrometry analysis and identification of metabolites of P006

Peptide identity and metabolite identification were performed with an oa-TOF mass spectrometer (Waters LCT), using an XBridge (Waters) C18 analytical column (5 μm particle size, 4.6 × 150 mm) with a binary eluent system comprising MeCN/H_2O (20 min gradient: from 90% H_2O/10% MeCN to 100% MeCN with 0.1% formic acid) as mobile phase. Operating pressures were in the range of 2000–3000 PSI. Electrospray ionisation mass spectrometry was conducted in positive ion mode (*m/z* range: 600–1700) using a cone voltage of 50 V, desolvation temperature of 300°C and source temperature of 100°C. Exact mass measurements of the products were based on the protonated molecules $[M+H]^+$.

2.12 | In vitro half-life estimation

Estimation of in vitro half-life in case of intravenous administration of P006 was obtained using the degradation data collected. For first-order reactions, half-life can be calculated as follows:

$$\ln [A]_t - \ln [A]_0 = -kt \quad (6)$$

where $\ln[A]_t$ is the natural logarithm of the concentration of the analyte at time t, $\ln[A]_0$ is the natural logarithm of the concentration of the analyte at time zero, while k is the rate constant, and t is the time. This equation can be simplified as:

$$[A]_t = [A]_0 * e^{-kt} \quad (7)$$

From the definition of half-life ($t_{½}$), $[A]_t = [A]_0/2$. Hence, Equation (7) can be rearranged as follows (Equation 8) where 0.693 is the natural logarithm of 2 and k (or k_{el}) is the constant of elimination:

$$t_{\frac{1}{2}} = \frac{0.693}{k} \quad (8)$$

2.13 | Data analysis and statistical analysis

Results were reported and evaluated with Microsoft Excel (version 16.58) or GraphPad Prism (version 9.3.1).

3 | RESULTS AND DISCUSSION

As part of our drug discovery programme towards the identification of novel peptide-based antagonists of the CGRP-R for migraine management, we sought to develop a method to evaluate the in vitro stability of peptides to identify candidates with suitable in vivo half-life. In particular, we chose to study the lead compound P006, which showed relatively high potency in vitro and modest activity in vivo (mouse), which was consistent with antagonism of CGRP-R.[33] Unfortunately, in vivo PK analysis was unsuccessful following *i.v.* administration in a larger sheep model, indicating that it was broken down rapidly in blood serum (unpublished data).

3.1 | Peptide synthesis and solution stability

The lead antagonist peptide P006 and its major metabolite LJMU025 (lacking the last two amino acids at the N-terminus) were synthesised with a CEM Liberty Blue peptide synthesiser, purified by preparative HPLC, flash-frozen and lyophilised. RP-HPLC and LC-MS confirmed their purity (>96%) and identity, respectively, as shown in Table 3 and in Figures S1 and S2 and Figures S3 and S4, respectively, in the Supporting Information.

Serum stability experiments require conditions that may cause peptide chemical instability (e.g., incubation at 37°C over 24 h and acidic conditions). Hence, solutions of P006 at various concentrations (520, 260, 100, 50 μg/mL, in PBS buffer, 5% DMSO) were tested for stability at 37°C over 24 h by measuring the area under the peak from HPLC analysis. P006 was stable and remained intact and in solution at each concentration over 24 h (Figure 1). P006 was also stable to the acidic and relatively high temperature conditions used in the HPLC analysis (Figures S5 and S6). To test whether the addition of TCA (40 μL, 15% *w/v*) to the peptide solution caused instability and/or precipitation, P006 (130 μg/mL) was analysed before and after addition of the acidic solution. Peptide recovery after addition of TCA, was ~94%, demonstrating that TCA does not cause significant peptide precipitation and is a valuable option to precipitate serum proteins without significantly affecting peptide concentration in solution.

3.2 | Stability of P006 in human serum

To investigate the blood serum stability of P006, the presence of intact peptide was quantified after incubation with diluted serum. Notably, it has been reported that in vivo peptide stability can be modelled by in vitro stability in human serum or plasma with a certain level of accuracy, even though, in some cases, the stability in serum or plasma may be underestimated compared to whole blood.[5,37] Incubation times were selected between 15 and 60 min, using 0 min as the reference sample (100% of intact peptide). Incubation times shorter than 15 min resulted in highly variable results, while incubation times longer than 60 min failed to detect intact peptide for P006—presumed to be due to complete degradation.

The use of diluted serum has previously been explored by various research groups.[5,8,10,13,38] In this study, by using diluted human serum (25% *v/v*, in H_2O), it was possible to detect changes in peptide concentration with a higher grade of precision due to the longer time needed for the peptide to degrade, eventually yielding more accurate stability profiles over 60 min. The initial concentration of peptide in diluted serum was fixed at 100 μg/mL as per literature precedent.[8]

TABLE 3 P006 and LJMU025 peptide sequences.

Entry	Sequence	Exact mass	Calculated *m/z* $[M+H]^+$	Observed *m/z* $[M+H]^{+a}$	Purity by UV (%)[b]	HPLC RT (min)
P006	VPTDVGPFAF-NH_2	1047.540	1048.5400	1048.55055	96.6	5.661 ± 0.075
LJMU025	TDVGPFAF-NH_2	852.400	853.4000	852.5260	96.0	5.073 ± 0.020

[a]Data obtained with LC-MS/MS system (Agilent 1260 Infinity II LC system and Agilent 6530 Accurate-Mass QToF spectrometer).
[b]By measuring the area under the peak from the HPLC chromatogram (wavelength at 215 nm, band width 4 nm). RT = retention time when peptide solutions in deionised H_2O (125 μg/mL) were analysed with optimised HPLC method described in Section 2.5. The RT data are expressed as average ± standard deviation. With a confidence level of 95%, P006 RT is 5.64–5.69, while LJMU025 RT is 5.05–5.1.

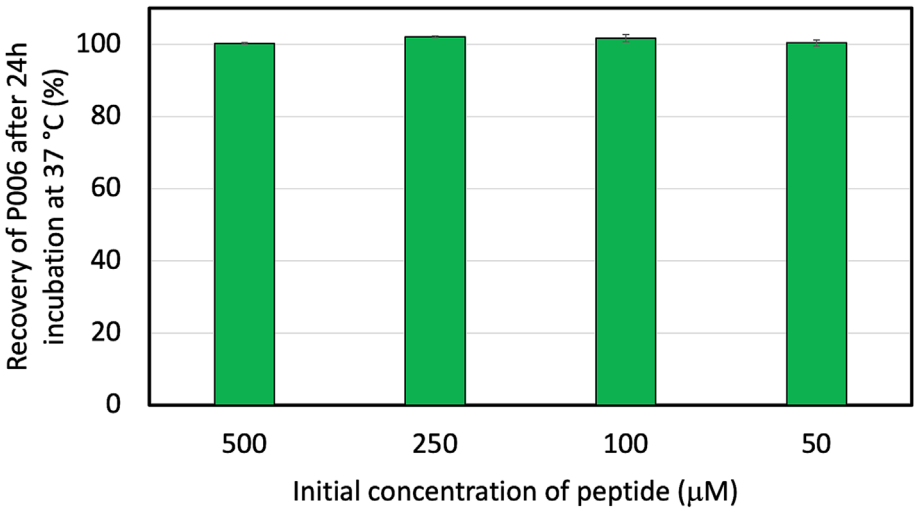

FIGURE 1 Thermal stability of P006 over 24 h at 37°C with shaking, expressed as percentage of recovery compared to a standard solution.

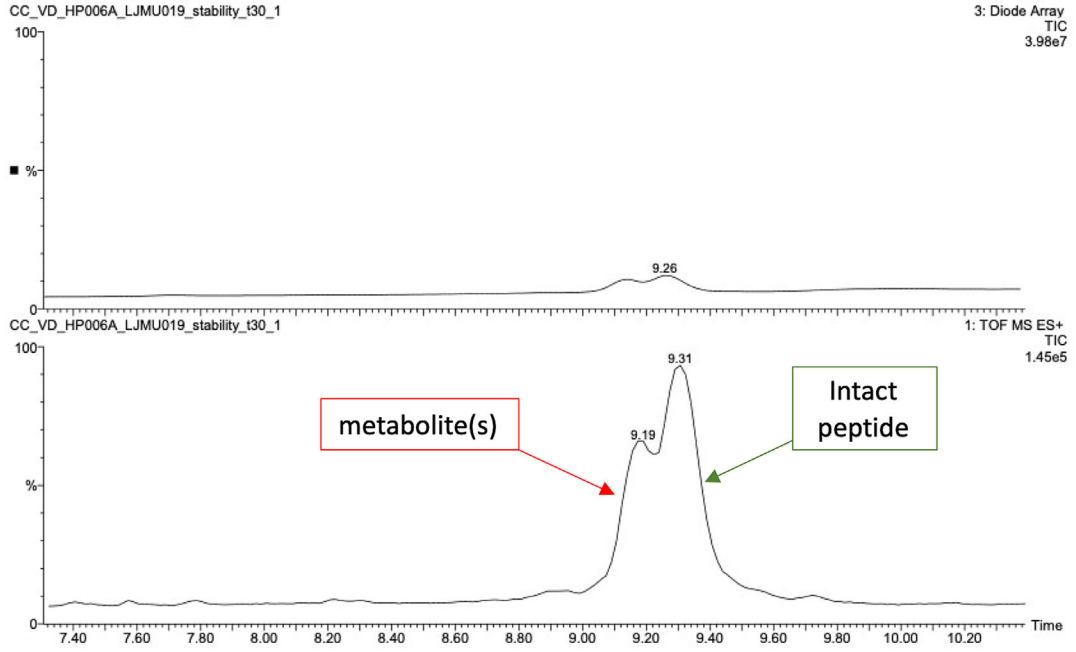

FIGURE 2 Example LCMS chromatogram and total ion chromatogram (TIC) spectra of the peptide after 30 min incubation in diluted human serum.

Denaturation of serum proteins was performed by adding aq. TCA solution as quenching reagent. Initially, 6% *w/v* aq. TCA solution was added to the samples in a *ratio* 1:1 (200 µL), as reported in the literature.[5] However, this resulted in a superfluous dilution of the starting concentration of intact peptide yielding a higher detection limit by RP-HPLC and, for this reason, protein denaturation was finally obtained by adding a lower volume of a more concentrated solution of TCA (15% *w/v*, 40 µL), as previously explored by Cudic et al. and Nguyen et al.[10,13]

3.3 | HPLC method optimisation—Plackett–Burman design and Taguchi design

Quantification of intact peptide at various time points following incubation in serum was performed by RP-HPLC (Agilent 1100 equipped with a Phenomenex Aeris PEPTIDE XB-C18 LC Column, 150 × 4.6 mm, 3.6 µm) using a standard HPLC gradient method over 22 min at room temperature. However, this method was unable to separate P006 from its degradation product(s) (Figure 2) and the main

identified metabolite (RT 9.19 min, *m/z* 852.4575) eluted very close to the intact peptide (RT 9.31 min, *m/z* 1048.6028). Therefore, to enable the accurate quantification of the intact compound, further chromatographic optimisation was required to achieve full resolution of the two peaks.

As a model system with which to optimise the chromatographic separation, a 1:1 solution of P006 (VPTDVGPFAF-NH_2) and its major assumed metabolite (i.e., LJMU025, TDVGPFAF-NH_2) was prepared at concentration 0.1 mg/mL and analysed by HPLC changing numerous parameters. The development of an optimised method potentially requires a large number of experiments that increases exponentially with the number of independent influencing factors under evaluation. To decrease the number of experiments required, a preliminary screening study was performed to exclude independent variables that had no significant effect on the outcome. Hence, RP-HPLC method optimisation was carried out using two experimental designs: an initial Plackett–Burman design to identify the factors that mainly influenced the resolution of the two peaks, followed by a Taguchi design to investigate the most important factors at different levels and to define the final factor levels to use.

The quantifiable response was the resolution (R_s) between the two peaks deriving from elution of LJMU025 and P006.[14,39] Notably, R_s is influenced by efficiency, retention, and separation. Therefore, it was decided to increase R_s through improvement in efficiency (by studying column particle size and temperature) and selectivity (by changing mobile phases and mobile phases pH).

Plackett–Burman design was selected as screening tool to identify the important factors that influenced the process output (i.e., R_s) and reduce the number of factors to study. The most important factors when optimising HPLC methods were identified (i.e., column pore size, volume of organic modifier at start and at end of the gradient, gradient time, flow rate, column temperature, and TFA concentration in mobile phases) and were studied at the minimum and maximum levels (Table S1). The experiments were performed in triplicate.

In the standardised main effect Pareto chart (Figure 3), obtained from the Plackett–Burman design study results, the length of the bar is proportional to the weight of the effects of the variables and this determines if the coefficient is significant or not for R_s. Hence, the factors that most influence the resolution of the peaks were found to be column compartment temperature (**F**), flow rate (**E**) and TFA % in the mobile phases (**G**). Other significant variables were column particle size (**A**), the % of organic modifier at the beginning of the gradient (initial gradient, **B**), and gradient time (**D**), while the % of organic modifier at the end of the gradient (final gradient, **C**) had no significant influence on the outcome.

Consequently, the three factors that had the greatest significance were further investigated using a fractional design. Taguchi design with three factors (i.e., column temperature, flow rate, and TFA % in the mobile phases) evaluated at three levels (L9, 3^3) was chosen (Table 4).[40,41]

The analysis of the Taguchi design outcome was obtained using the criterion "the larger the better": the greater the resolution of the two peaks, the better for our purpose of separating P006 from its metabolite(s). By visually inspecting the main effect plot of means (Figure 4), it was clear that resolution increased linearly with an increase in temperature (Figure 4A) and flow rate (Figure 4B). No such clear linear relationship was observed for the TFA percentage in the mobile phases, which had a very subtle effect on the outcome (Figure 4C). Hence, the parameters that yielded the greater resolution of the two peaks were column compartment temperature at 65°C and flow rate at 1 mL/min, while TFA % in mobile phases had almost no influence on the outcome and, for this reason, was chosen as 0.05%.

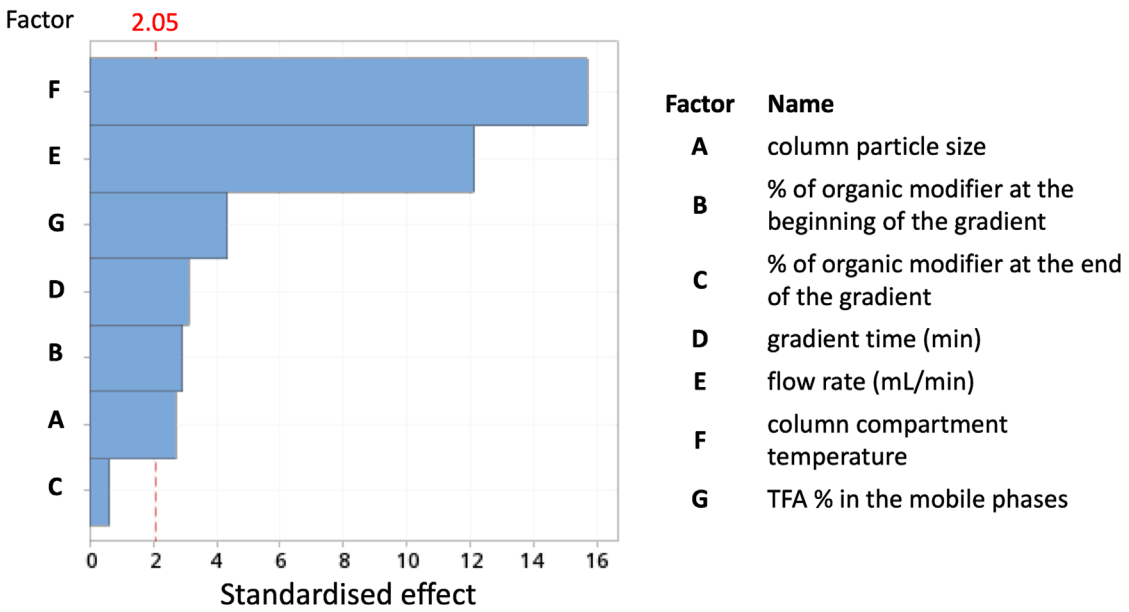

FIGURE 3 Standardised main effect Pareto chart for the Plackett–Burman design of experiment. The vertical red line in the chart defines the 95% confidence level; the bars that cross the reference line are statistically significant.

Run number	Column temperature (°C)	Flow rate (mL/min)	TFA concentration (%)
1	35	0.5	0.05
2	35	0.8	0.08
3	35	1	0.1
4	50	0.5	0.08
5	50	0.8	0.1
6	50	1	0.05
7	65	0.5	0.1
8	65	0.8	0.05
9	65	1	0.08

TABLE 4 Taguchi design matrix (L9, 3^3) to study the effect of column temperature, flow rate, TFA concentration.

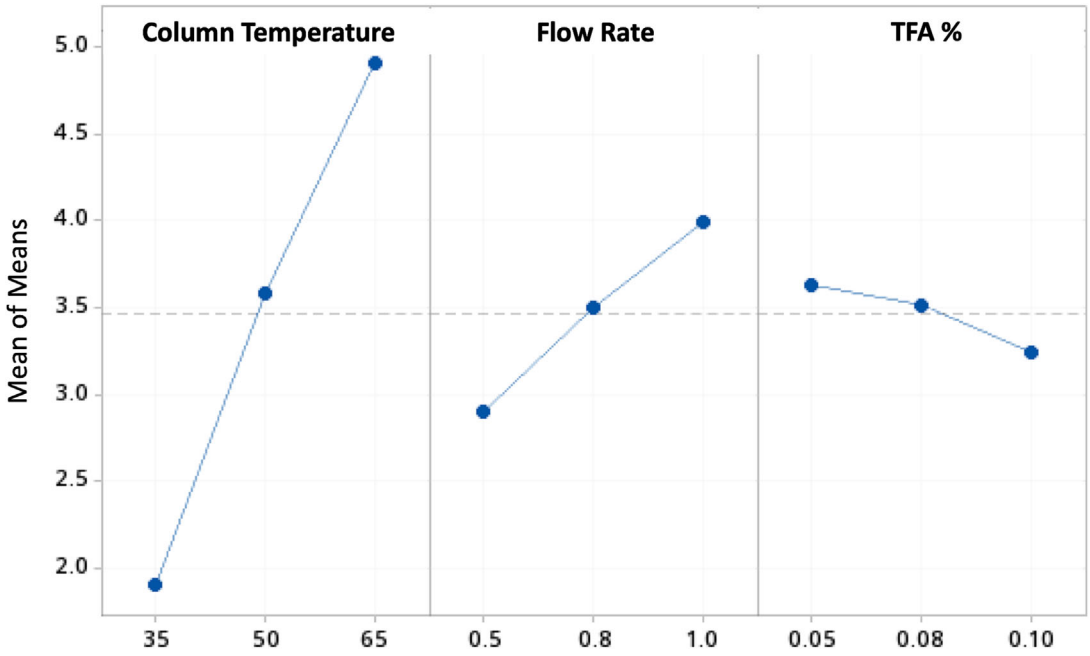

FIGURE 4 Main effect plots for signal-to-noise ratios from Taguchi design. (A) Column compartment temperature, (B) flow rate, (C) TFA percentage in the mobile phases.

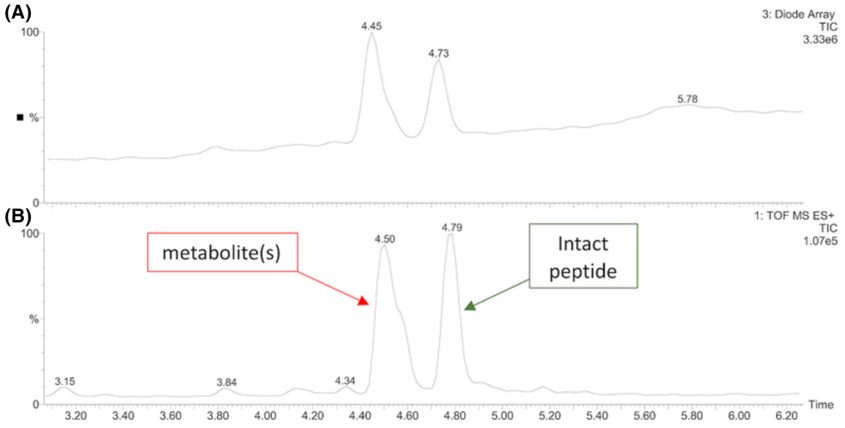

FIGURE 5 LCMS analysis of supernatant solutions: (A) diode-array detection (DAD) and (B) total ion chromatogram (TIC) of the peptide after 30 min incubation in diluted serum. Note: LCMS analysis used a method adapted from the optimised HPLC method (see Section 2.11), therefore, there are slight differences in retention times as compared to HPLC.

As shown in Figure 5, the success of the HPLC optimisation study was demonstrated by analysis of a sample obtained from incubation of P006 in diluted serum for 30 min. Here, a notable fivefold increase in the R_s of the peaks between parent peptide and its main metabolite was obtained (from 1.13 to 5.65). The aim of this optimisation was to fully resolve the peak of the parent peptide from its metabolites, with

$R_s = 1.5$ being sufficient for baseline separation. However, an $R_s > 1.5$ allowed the full resolution from other secondary metabolites, that may have otherwise co-eluted with the parent peptide in smaller quantity.

3.4 | HPLC method validation

The optimised analytical technique was validated in agreement with the ICH guidelines to prove its adequacy for the planned purpose.[35,42] In the validation of the analytical method, linearity, range, sensitivity, specificity, and robustness were evaluated.

The linearity study verified that the samples were in a concentration range in which the analyte response was linearly proportional to the concentrations. A standard curve (Figure 6) was obtained by plotting the mean peak area ($n = 3$) against the concentration of the peptide. The slope, y-intercept, and linearity of the curve were determined by linear regression analysis. The peak area of standard curve samples was a linear function of the peptide concentration over the concentration range of 5–1000 μg/mL. The regression coefficient (R^2) was 0.9999 and the y-intercept was not significantly different from zero.

The specified range (5–1000 μg/mL) was derived from linearity studies and confirmed that the analytical procedure provided an acceptable degree of linearity, accuracy, and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. The precision of the analytical method was confirmed through intraday repeatability testing, assessed using nine determinations (three concentrations/three replicates each), and interday repeatability (assessed in three different days), as shown in Table 5. The repeatability of injection was demonstrated for the same concentrations and repeatedly injecting each sample five times (Table 6).

Method sensitivity was studied through two parameters: LLOQ and LLOD. These could be particularly affected by small changes in concentration, and, for this reason, their variability should be assessed. The LLOD (Equation 3) was found to be 13.2357 μg/mL, while the LLOQ (Equation 4) was 40.1083 μg/mL. The specificity test proved that, at the retention time of P006, there were no interferences between the peptide and other impurities (i.e., deionised H_2O

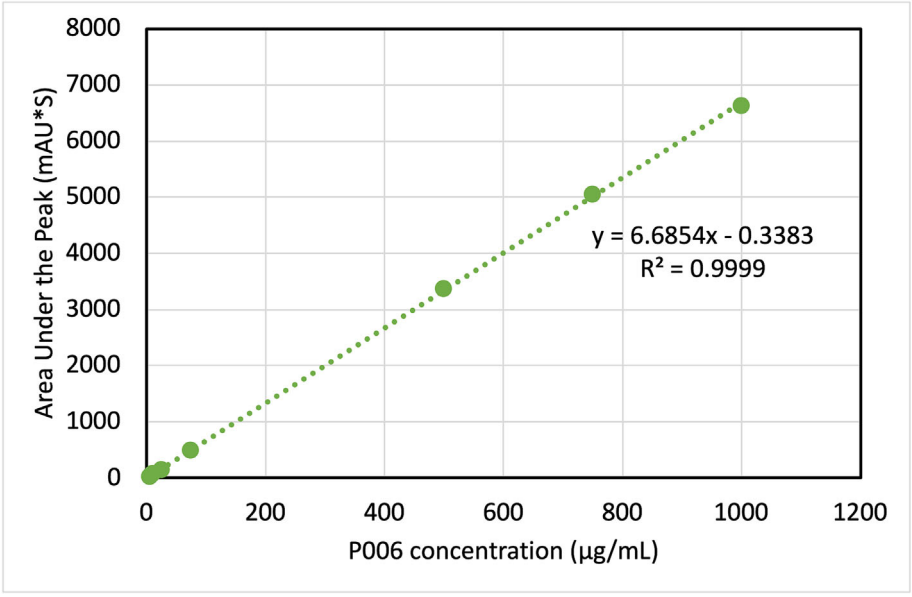

FIGURE 6 P006 calibration in deionised H_2O; concentration range from 5 to 1000 μg/mL.

TABLE 5 Intraday and interday variation and accuracy tested using three different concentrations of peptide within the calibration range. Interday precision was tested at day 0, day 1, day 2, and day 3, with three injections each day. Concentration values are reported as means ± SD.

	Concentration of P006 (μg/mL)	Experimental concentration of P006 ± SD	Accuracy (%)	Confidence interval (%)[a]
Intraday	50	48.84 ± 0.42	102.25 ± 0.43	[102, 103]
	100	100.31 ± 0.26	99.70 ± 0.36	[99.4, 99.9]
	250	252.49 ± 2.25	102.38 ± 1.0	[98.5, 99.5]
Interday	50	48.92 ± 1.00	99.02 ± 0.83	[101, 103]
	100	99.53 ± 1.18	100.48 ± 1.14	[99.8, 101]
	250	253.51 ± 1.13	98.62 ± 1.98	[98.4, 98.8]

[a]Confidence Interval given at 95% confidence level.

TABLE 6 Injection repeatability at three different concentrations (50, 100, and 250 µg/mL). Values are represented as mean ± SD.

Concentration of P006 (µg/mL)	Number of replicates	Peak intensity ± SD (height of peak)	RSD (%)	Assay concentration ± SD (µg/mL)	RSD (%)
50	5	71.36 ± 0.79	1.11	328.96 ± 3.31	1.01
100	5	146.44 ± 1.98	1.35	672.60 ± 2.57	0.38
250	5	356.18 ± 4.20	1.18	1668.48 ± 13	0.78

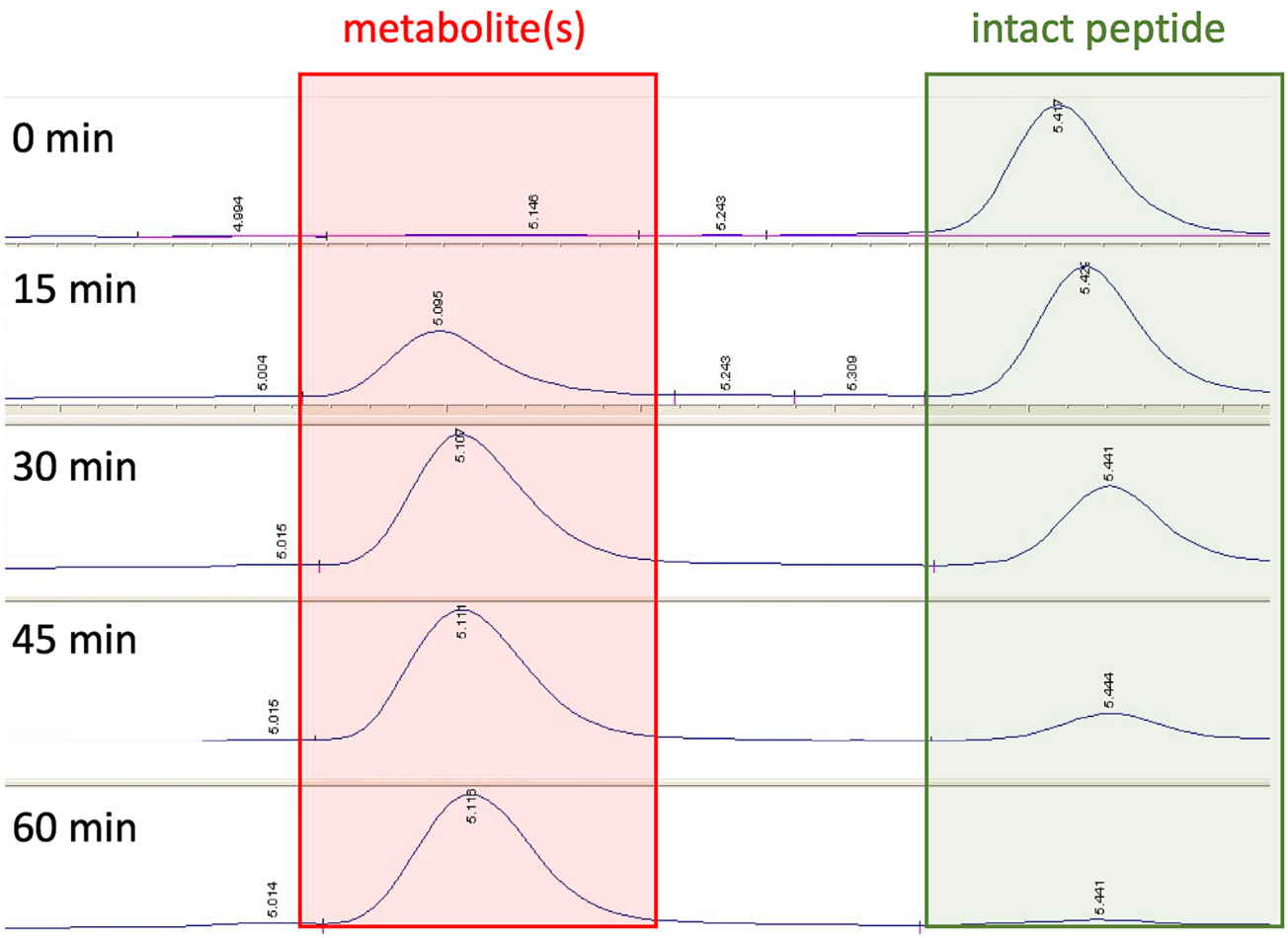

FIGURE 7 Stacked HPLC chromatograms showing the degradation profile of P006 (green) to its metabolite(s) (red) in diluted aq. human serum over 60 min.

with 5% DMSO, deionised H_2O with 2.5% TCA, and blank serum supernatant). This test was designed to prove that the analytical procedure was specific for P006, leading to complete discrimination from other impurities that may be expected to be present in the sample (e.g., human serum supernatant components).

Robustness was part of the parameters studied during the validation of the analytical technique and was tested by changing flow rate, wavelength, and column compartment temperature during analysis of a solution of peptide 250 µg/mL (Table S3). This test is an indication of the analytical procedure capacity to remain unaffected by small, but deliberate variations in method parameters and proves its reliability during routine use. Finally, storage stability was demonstrated at 25°C ± 2°C up to 72 h (Figure S8). This test was essential to confirm that samples could be stored at room temperature for days before analysis without any major change occurring. The validation of the analytical procedure showed that the method was adequate to accurately quantify the peptide P006.

3.5 | Blood serum stability of P006 and in vitro half-life estimation

Analytical method optimisation and validation allowed a more accurate estimation of in vitro half-life of P006. By incubation of P006 in diluted serum, it was possible to derive kinetic information (i.e., degradation profile and in vitro half-life) and to define the main

degradation products. Comparison of HPLC chromatograms derived from analysis of P006 samples after incubation in diluted serum for 0, 15, 30, 45, and 60 min (Table S2) showed a gradual decrease in parent peptide (RT 5.4 min) and an increase in other species forming over the time (RT 5.1 min) (Figure 7).

By measuring the area under the peak at the retention time corresponding to the elution of the parent peptide (i.e., 5.4 min for P006) and comparing with the calibration curve (Figure S7), it was possible to quantify the amount of peptide degraded over 60 min. The kinetic profile for P006 is shown in Figure 8, where the percentage of intact peptide was plotted against time, normalised to the concentration of intact peptide at time zero (taken as 100%). Clearly, P006 underwent fast degradation in diluted serum, being almost completely digested after 60 min (remaining % of intact peptide 4.1 ± 0.1) and this was easily detectable after method optimisation.

The degradation data were used to estimate in vitro half-life in case of intravenous administration of P006. To prove that the degradation profile of P006 could be approximated to a first-order kinetic reaction, ln[P006] was plotted against the time (Figure 9), resulting in a straight line ($R^2 = 0.96$).

To determine P006 in vitro half-life, the constant k was derived from the equation in Figure 9 ($k = 0.052$). Hence, $t_{½}$ for P006 was obtained by $t_{½} = 0.693/0.052 = 13.33$ min. In fact, the degradation profile for P006 was approximated to a first-order reaction with a degradation profile comparable to an exponential decay graph, and the relative half-life was calculated from the slope of the ln[A] versus time graph, using Equation (8).

3.6 | Mass spectrometry analysis and identification of metabolites of P006

Metabolite identification (met ID) is commonly achieved through mass spectrometry.[43] Once quantitative measurements were obtained via RP-HPLC, LC-MS analysis was performed at each time point of the stability study in human serum, to gain a better insight of both the

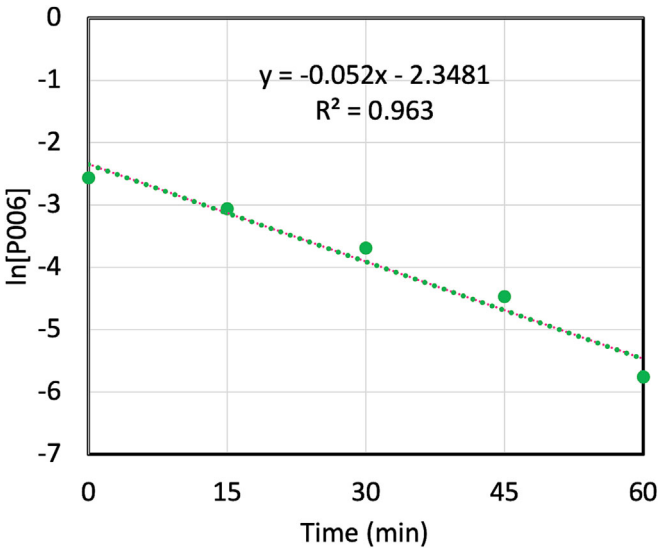

FIGURE 9 First-order reaction plot ln[P006] versus time (min) to determine the rate constant.

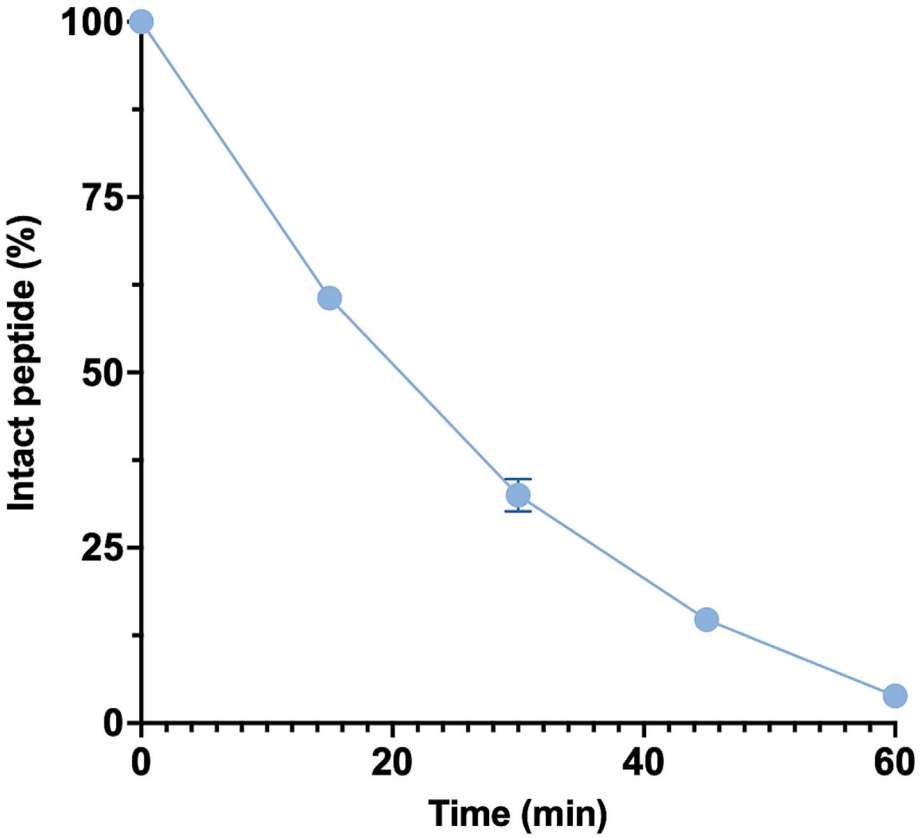

FIGURE 8 Degradation profile of P006 when incubated in diluted serum (25% *v/v*) over 60 min. Concentration of P006 at time zero was taken as 100%. Error bars not shown when smaller than the marker.

Entry	Parent sequence	*m/z* metabolites (time 60 min)	Putative metabolites sequences
P006	VPTDVGPFAF-NH_2	852.5534	TDVGPFAF-NH_2
		751.5021	DVGPFAF-NH_2

TABLE 7 P006 sequence, *m/z* of metabolites forming at 60 min, and putative metabolite(s) sequence.

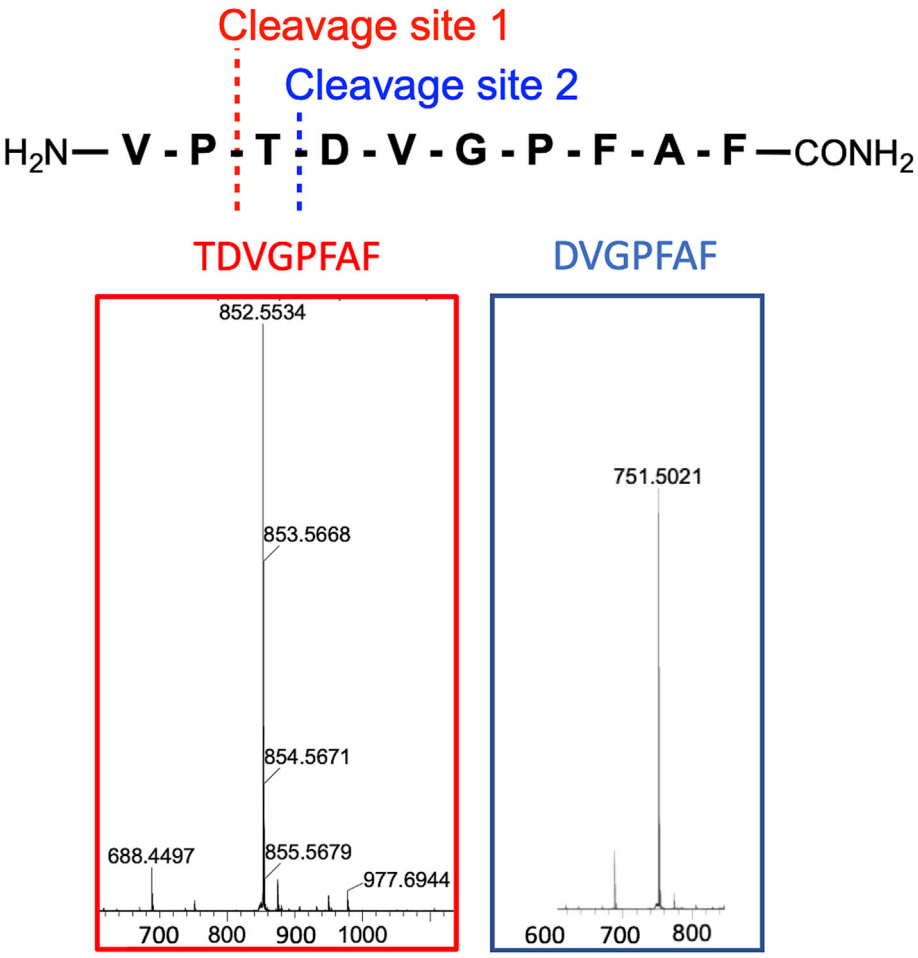

FIGURE 10 Main cleavage sites for P006 and related MS spectra. Note: full spectra are available in Supporting Information.

kinetics of the metabolic reactions occurring, and the metabolites originating from the compound under evaluation. This resulted in the identification of the sites more susceptible to undergoing proteolytic cleavage. Metabolites derived from incubation of P006 in serum and the corresponding putative sequences are shown in Table 7. LC-MS chromatograms for parent peptide and metabolites identified after 60 min are included in the Supporting Information (Figures S9–S11).

The main cleavage site was identified to be at the N-terminal site between Pro29 and Thr30. However, a second metabolite was also observed only using the optimised HPLC method, in which cleavage occurred one amino acid further along the sequence (between Thr30 and Asp31). However, this cleavage occurred considerably more slowly and the metabolite DVGPFAF-NH_2 (*m/z* 751.5021) was only detected after 45 min of incubation in serum, and it was not fully resolved from the main metabolite. These reactions could be ascribed to the action of dipeptidyl-peptidases and tripeptidyl-peptidases, respectively. Figure 10 shows the cleavage sites of P006 and relevant MS.

Overall, there are a number of benefits in the use of this screening tool at early stages of the drug discovery, such as reducing in vivo pharmacokinetic testing to fewer compounds and decreasing the number of animals used as well as the costs associated with these studies.

4 | CONCLUSIONS

The optimisation and validation of an easy-to-use and inexpensive method for the accurate evaluation of in vitro biological stability of small peptides is described in this work. The use of commercially available pooled human serum, instead of serum derived from volunteers, was key for the repeatability of the experiments, while the optimisation of the HPLC method was required to obtain full separation between the intact peptide (P006) and its metabolites. The validation of the analytical method following the ICH guidelines demonstrated its suitability for this purpose. This study neatly highlighted the

significant peptidase sensitivity of P006 and pointed to possible structural modifications that may be made in ongoing lead-optimisation work to increase its serum stability. This may include changes at the major peptidase cleavage site or the preparation of macrocyclic analogues.

Overall, the designs used to optimise the HPLC-analysis of a peptide serum stability assay should serve as a reliable and robust strategy to screen peptide libraries at early stages, thus avoiding or reducing the use of animals to test in vivo half-life or, alternatively, facing in vivo inefficacy, which may be due to lack of biological stability.

CONFLICT OF INTEREST STATEMENT

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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