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Original Research Article

Development of a novel stability indicating RP-HPLC method for quantification of Connexin43 mimetic peptide and determination of its degradation kinetics in biological fluids

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ABSTRACT

Connexin43 mimetic peptide (Cx43MP) has been intensively investigated for its therapeutic effect in the management of inflammatory eye conditions, spinal cord injury, wound healing and ischemia-induced brain damage. Here, we report on a validated stability–indicating reversed-phase high performance liquid chromatography (RP-HPLC) method for the quantification of Cx43MP under stress conditions. These included exposure to acid/base, light, oxidation and high temperature. In addition, the degradation kinetics of the peptide were evaluated in bovine vitreous and drug-free human plasma at 37 °C. Detection of Cx43MP was carried out at 214 nm with a retention time of 7.5 min. The method showed excellent linearity over the concentration range of 0.9–250 μg/mL (R^2≥0.998), and the limits of detection (LOD) and quantification (LOQ) were found to be 0.90 and 2.98 μg/mL, respectively. The accuracy of the method determined by the mean percentage recovery at 7.8, 62.5 and 250 μg/mL was 96.79%, 98.25% and 99.06% with a RSD of < 2.2%. Accelerated stability studies revealed that Cx43MP was more sensitive to basic conditions and completely degraded within 24 h at 37 °C (0% recovery) and within 12 h at 80 °C (0.34% recovery). Cx43MP was found to be more stable in bovine vitreous ($t_{1/2slow}$= 171.8 min) compared to human plasma ($t_{1/2slow}$ = 39.3 min) at 37 °C according to the two phase degradation kinetic model. These findings are important for further pre-clinical development of Cx43MP.

1. Introduction

Connexin43 mimetic peptide (Cx43MP) is a twelve amino acid peptide (Mol. wt. 1396 g/mol) which has been investigated for its therapeutic efficacy in the management of inflammatory eye diseases [1,2], spinal cord injury [3,4], wound healing [5,6] and ischemia-induced brain damage [7]. We have recently shown that Cx43MP can be loaded into polymeric nanoparticles for sustained peptide delivery in the treatment of retinal diseases [1,8,9]. However, for successful clinical translation, the ability of Cx43MP to withstand physiological and formulation processing conditions has to be determined.

Stability studies can be classified into long-term, accelerated and stress-stability studies [10–12]. Long-term stability studies are usually carried out to evaluate the drug's shelf-life (time taken for the amount of active ingredient to fall by 10% of the original amount). Compared to long-term studies, accelerated stability studies reduce the time required to predict the shelf-life of a drug-product at elevated environmental conditions. Stress-stability studies use even more extreme conditions to detect and separate active pharmaceutical ingredients from their degradation products. Degradation products generated during such stress-based stability studies may or may not form under normal storage conditions; however, they are useful in the development of a stability indicating HPLC method [13].

To the best of our knowledge, a validated stability-indicating HPLC method for the quantification of Cx43MP has not been reported in

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literature to date. Thus, the present investigation aimed to develop a suitable, simple, precise, accurate, robust, and reproducible RP-HPLC method for the quantification of Cx43MP. The validation of the method was accomplished according to Food and Drug Administration (FDA) guidelines [14] with the stability of Cx43MP evaluated under various stress conditions including acidic/basic hydrolysis as well as oxidative, thermal and photolytic degradation. The stability studies were conducted at two different temperatures (37 and 80 °C) to evaluate the ability of the peptide to withstand extreme conditions and to determine any degradation products which may have not appeared at 37 °C. The degradation kinetics of Cx43MP in biological fluids such as bovine vitreous and drug-free human plasma was also evaluated.

2. Materials and methods

2.1. Chemicals

Cx43MP (VDCFLSRPTEKT, purity > 95%) was purchased from ChinaPeptides Co., Ltd. (Suzhou, China). Sodium hydroxide (NaOH), hydrochloric acid (HCl), hydrogen peroxide (H_2O_2), formic acid (FA) and acetonitrile (ACN) were obtained from Sigma Aldrich and Thermo Fisher Scientific (Auckland, New Zealand). Bovine eyes were obtained from a local abattoir (Auckland Meat Processors Ltd., Auckland, New Zealand). Milli-Q water was obtained from a Millipore water purification system (Massachusetts, USA). All chemicals used were of HPLC or higher grade.

2.2. Instrumentation and chromatographic conditions

HPLC was performed on an Agilent 1100 series HPLC system coupled with a diode array detector (Agilent, USA). Chromatographic separation was achieved using a Gemini® C_{18} reverse phase column (150 mm × 3 mm, 3 µm 110Å, Phenomenex; PN 00F-4439-Y0) protected with a SecurityGuard™ C_{18} (8 mm × 3 mm, 3 µm, Phenomenex; PN AJ0-4287) guard column. Chromatographic separation was performed in gradient mode with a flow rate of 0.4 mL/min consisting of an aqueous (B, 0.01% (v/v) FA in Milli-Q water) and an organic phase (A, 0.01% (v/v) FA in ACN) with the column temperature set to 30 °C. The injection volume was 10 µL and UV detection of Cx43MP was accomplished at 214 nm. The volume of injection loop was 20 µL. The gradients used are 0 min (A-5% and B-95%), 15 min (A-50% and B-50%), 17 min (A-100% and B-0%), 19 min (A-5% and B-95%) and 25 min (A-5% and B-95%). Instrument control and data acquisition was achieved by ChemStation B.04.03-SP2 (Agilent, USA).

2.3. Preparation of stock solutions, working solutions, calibration standards and quality control samples

A standard stock solution of Cx43MP was prepared in Milli-Q water at a concentration of 1 mg/mL. The stock solution was diluted with Milli-Q water to obtain Cx43MP working solutions (7.8, 62.5 and 250 µg/mL); these intermediate solutions were used for preparation of the calibration standards and quality control (QC) samples. All stock and working solutions were stored at −80 °C and protected from light.

2.4. Method validation

The developed HPLC method was validated in terms of linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy and robustness according to FDA guidelines [14]. All experiments were performed in triplicate (n=3).

2.4.1. Linearity

The calibration curve was prepared from the Cx43MP stock solution (1 mg/mL) at concentrations ranging from 0.9 to 250.0 µg/mL. The Cx43MP area under the peak was plotted against its corresponding concentration in Milli-Q water and the linearity was determined by the least square linear regression analysis.

2.4.2. LOD and LOQ

LOD and LOQ were determined from the calibration curve of Cx43MP according to Eqs. (1) and (2), where Sy is the standard error and a is the slope of the corresponding calibration curve.

$$\mathrm{LOD} = 3.3\ \mathrm{Sy}/a \quad (1)$$

$$\mathrm{LOQ} = 10\ \mathrm{Sy}/a \quad (2)$$

2.4.3. Precision and accuracy

The precision of the assay was determined in relation to the repeatability (intra-day) and the intermediate precision (inter-day). In order to evaluate the repeatability of the method, three QC samples of Cx43MP (7.8, 62.5 and 250 µg/mL; n=3 each) were quantified on the same day and on three consecutive days. The precision value was expressed as the percentage relative standard deviation (% RSD) with a value < 15% considered satisfactory. The accuracy was assessed by analysing six replicates (n=6) of the three QC samples (7.8, 62.5, and 250 µg/mL) and was expressed as % RSD. Accuracy was expressed as the percentage recovery which was calculated from the following Eq. (3):

$$\text{Recovery (\%)} = (\text{concentration calculated from the calibration curve /nominal concentration}) \times 100 \quad (3)$$

2.5. Stress degradation studies

The specificity of the method can be demonstrated through forced degradation studies performed under acidic, basic, oxidative, heat and ultraviolet light conditions. A specific method should be able to separate and equivocally identify the test compound from the various degradation products. All experiments were conducted in triplicate (n=3).

2.5.1. Acid and base hydrolysis

Cx43MP (250 µg/mL) was prepared in HCl (1 M) and NaOH (1 M), respectively. Aliquots were kept at 37 °C for 24 h (Thermo Scientific Heraeus® microbiological incubator, USA) and 80 °C (AccuBlock™ Digital Dry Baths, Labnet international, USA) for 12 h.

2.5.2. Oxidative degradation

Cx43MP (250 µg/mL) was prepared in 30% (v/v) hydrogen peroxide (H_2O_2). Aliquots were incubated at 37 °C for 24 h and 80 °C for 12 h.

2.5.3. Thermal degradation

Cx43MP (250 µg/mL) was prepared in Milli-Q water and aliquots were kept at room temperature (22–25 °C), 37 °C, and −80 °C for 24 h, and at 80 °C for 12 h. Cx43MP solutions were also subjected to two freeze-thaw cycles with the sample first frozen at −80 °C for 24 h after which it was defrosted under running tap water at room temperature for 2 h. In the second cycle, samples were frozen at −20 °C and then defrosted under running tap water at room temperature for 2 h.

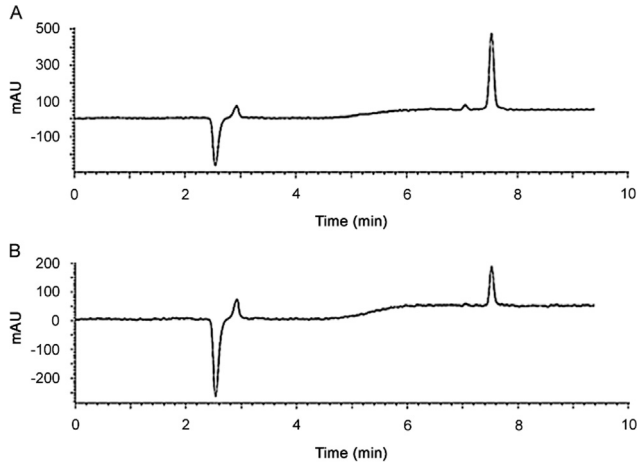

Fig. 1. Typical chromatograms of Cx43MP (A) 250 µg/mL and (B) 62.5 µg/mL.

Table 1
Linearity for Cx43MP.

Parameters	Slope	Y-intercept	R^2
Linearity-1	10.392	21.48	0.9985
Linearity-2	10.306	28.33	0.9982
Linearity-3	10.159	25.49	0.9984
Avg	10.285	25.10	0.9983
SD		3.07	

2.5.4. UV degradation

Cx43MP (250 µg/mL) was prepared in Milli-Q water and aliquots were kept in clear plastic vials to avoid unwanted UV absorption which may occur with glass vials. Samples were then exposed to UV light (365 nm, Spectroline® E-Series UV lamp, Spectronics Corp., USA) for a duration of 7 h.

2.6. Stability in bovine vitreous and human plasma

Cx43MP (250 µg/mL) was added to bovine vitreous and human plasma, respectively. Solutions were kept at 37 ± 1 °C and at predetermined time intervals (0, 5, 10, 15, 30, 60, 120, 180 and 240 min), 100 µL of sample was withdrawn, mixed with ice cold ACN (1:3, v/v) and centrifuged at 13,000 rpm for 10 min to precipitate any proteins. The clear supernatant (100 µL) was diluted with Milli-Q water (1:1, v/v) and analyzed by HPLC. The percentage remaining versus time data in bovine vitreous and human plasma was fitted to a non-linear two-phase decay kinetic model using Prism 7 (GraphPad, La Jolla USA). Important kinetic parameters, such as half-life ($t_{1/2}$), degradation rate constant (k), area under the curve (AUC) and correlation coefficient (R^2) were determined.

2.7. Peak purity

Often degradation products co-elute with the test compound; and therefore, it is important to establish the spectral purity of the analyte in the chromatogram. The peak spectral purity assessment was checked by comparison of the UV spectrum of the analyte (Cx43MP at 7.5 min) with those of the authentic Cx43MP standard using the peak purity function in the Agilent ChemStation software (ver B.04.03-SP2; Agilent, USA)

3. Results and discussion

3.1. Method development and validation

An HPLC method was developed, which allowed for quantification of Cx43MP. The detection wavelength was decided on the basis of its UV–visible spectrum, which showed a peak at 214 nm. The mobile phase was optimized after several trials with ACN and water in various proportions to obtain a sharp peak. The best results (peak width and retention time, ~7.5 min) were obtained when the mobile phase consisted of a changing volume ratio of aqueous (Milli-Q water + 0.01% (v/v) FA) and organic phase (ACN + 0.01% (v/v) FA) over time. Typical chromatograms of Cx43MP at the optimized conditions are shown in Fig. 1. At the set chromatographic conditions, Cx43MP was found to elute at 7.5 min with a total run time of 25 min. The method showed good linearity with $R^2 > 0.998$ (Table 1). LOD and LOQ were found to be 0.90 and 2.98 µg/mL, respectively. The % RSD of Cx43MP in Milli-Q water during the intra-day precision ranged from 0.98 to 2.07 and the inter-day precision from 1.13 to 2.0 (Table 2). Both RSD values were < 15%, which is within the acceptable limits according to the FDA guidelines [15]. The accuracy determined by the mean percentage recovery of various peptide concentrations (7.8, 62.5 and 250 µg/mL) was 96.79, 98.25 and 99.06, respectively with a % RSD of < 2.2% The method therefore showed good accuracy with over 95% recovery of Cx43MP (Table 2).

3.2. Stress degradation

Cx43MP (250 µg/mL) solutions prepared with Milli-Q water and stored at 37 °C for 24 h were found stable with a recovery of 99.04% (Table 3, Fig. 2A). Cx43MP was found to moderately tolerate acidic conditions (1 M HCl) with a recovery of approximately 72% at 37 °C, whereas approximately 6% was remaining at 80 °C (Table 3, Figs. 2B and 3B). The degradation of Cx43MP in acidic conditions at 37 °C could be due to the acid catalyzed formation of a cyclic imide intermediate which can result in cleavage of the peptide chain [16]. This acid-based degradation was further accelerated at a higher temperature (80 °C). Under basic conditions (1 M NaOH) at 37 and

Table 2
Intra- and inter-day precision and accuracy.

Conc. injected (µg/mL)	Intra-day (n=3)			Inter-day (n=3)			Accuracy (n=6)		
	Conc. found (Mean ± SD, µg/mL)	Recovery (%)	RSD (%)	Conc. found (Mean ± SD, µg/mL)	Recovery (%)	RSD (%)	Conc. found (Mean ± SD, µg/mL)	Recovey (%)	RSD (%)
7.8	7.58 ± 0.13	97.17	2.02	7.42 ± 0.13	95.12	2.00	7.55 ± 0.15	96.79	2.19
62.5	61.43 ± 0.55	98.28	0.98	61.41 ± 0.63	98.25	1.13	61.41 ± 0.92	98.25	1.64
250	246.98 ± 4.66	98.78	2.07	249.81 ± 3.39	99.92	1.49	247.67 ± 2.70	99.06	1.20

Table 3
Stability of Cx43MP under various conditions (Mean ± SD; n=3).

Condition	Conc. injected (μg/mL)	Conc. found (Mean ± SD, μg/mL)	Recovery (%)	RSD (%)
Acidic degradation (1 M HCl)				
37 °C (24 h)	250	182.13 ± 2.13	72.85	0.40
80 °C (12 h)	250	14.73 ± 2.54	5.89	3.90
Basic degradation (1 M NaOH)				
37 °C (24 h)	250	0.00 ± 0.00	0.00	0.00
80 °C (12 h)	250	0.00 ± 0.00	0.00	0.00
Oxidative degradation (30% H_2O_2)				
37 °C (24 h)	250	199.76 ± 3.21	79.90	1.08
80 °C (12 h)	250	24.13 ± 2.32	9.65	1.87
Thermal degradation				
Room temperature (24 h)	250	248.36 ± 2.11	99.34	0.40
37 °C (24 h)	250	247.60 ± 3.22	99.04	0.76
80 °C (12 h)	250	103.10 ± 3.11	41.24	10.81
−80 °C (24 h)	250	248.92 ± 2.67	99.56	1.17
Two freeze-thaw cycles (24 h (−80 °C)–2 h (22 °C)–2 h (−20 °C))	250	247.54 ± 3.55	89.03	8.39
Photolytic degradation (365 nm UV light, 7 h)	250	242.75 ± 2.45	97.10	2.44

80 °C, Cx43MP completely degraded with no recovery. The chromatograms showed complete loss of the characteristic Cx43MP peak under basic conditions with the appearance of a small chromatographic peak representing a degradation product (Table 3, Figs. 2C and 3C). The instability under basic condition (pH > 8) could be due to the reverse oxidation of cysteine and the formation of intra-chain or inter-chain disulfide bonds [17]. Under oxidising conditions (30% (v/v) H_2O_2) about 79.90% and 9.65% were recovered at 37 and 80 °C, respectively (Table 3, Figs. 2D and 3D). The cysteine residue within the peptide is prone to reversible oxidation with the thiol group of cysteine easily deprotonated and readily forming intra-chain or inter-chain disulfide bonds [17]. Therefore, a slight instability of peptide was observed under oxidising conditions which appeared to be temperature dependent with higher degradation at 80 °C.

Recoveries of Cx43MP subjected tovarious temperatures are shown in Table 3. The peptide was found to be stable at room temperature and 37 °C with a recovery of over 99%; however, at a higher temperature (80 °C) only 41.24% was found after 12 h. At −80 °C, Cx43MP was found stable with a recovery of over 99%. After two freeze-thaw cycles, 89.03% of the peptide was still recovered. It is known that repeated freezing and thawing can cause some degree of peptide degradation [18,19]. If peptide samples need to be frequently or periodically taken from the stock, it is therefore recommended to make a series of working aliquots from the stock which are stored at −20 °C or even lower temperature. Upon UV light exposure (365 nm) for 7 h, 97.10% of Cx43MP was recovered (Table 3), suggesting that Cx43MP is stable under UV light, which is important from a formulation and packaging point of view. Nevertheless, peptide containing formulations should generally be protected from light by keeping them in light resistant containers [20].

3.3. Stability in bovine vitreous and human plasma

Peptides are known to undergo enzymatic degradation in biological fluids. As our ongoing study intended to use Cx43MP for the treatment of inflammatory retinal diseases, it was imperative to test its stability in bovine vitreous and human plasma. Figs. 4 and 5 show Cx43MP chromatograms in bovine vitreous and human plasma, with Cx43MP recoveries shown in Fig. 6. Immediately after addition of Cx43MP to bovine vitreous (initial time point, 0 min), 67% was recovered and by the end of incubation period (240 min) only 17% remained (Fig. 6). This finding was similar to what was previously reported with regard to Cx43MP stability in bovine vitreous [21]. The chromatogram of Cx43MP in bovine vitreous (Fig. 4) showed three new distinctive peaks related to the degradation products which appeared to increase in area over the incubation period. Enzymes, such as chondroitinase, hyaluronidase, dispase and plasmin/microplasmin, are present in the vitreous, which may contribute to the enzymatic degradation of the peptide [22–25]. Cx43MP was found to be rather unstable in human plasma with recovery of only about 40% at the initial time point (sampled immediately after the addition, 0 min) and only 0.4% remaining at the end of incubation period (240 min). Peptides undergo rapid degradation in human plasma due to the presence of many proteases and peptidases. In this study heparinised human plasma was used for Cx43MP incubation without any enzyme inhibitor to mimic physiological condition.

A previous study has shown that the stability of Cx43MP in the vitreous can be increased almost two-fold by addition of two C-12 lipoamino acid groups at the N-terminus of the peptide [21]. Another approach to increasing the stability and half-life in the vitreous could be to incorporate the peptide into polymeric particles and hydrogels indented for intravitreal injection [26–29].

The chromatogram of Cx43MP immediately after the addition to human plasma (Fig. 5) showed a distinctive peak related to a degradation product which appeared to increase rapidly in area over the following 5 min of incubation, while the main peptide peak started disappearing after 15 min. Cx43MP was found to be relatively unstable in human plasma with recovery of only about 40% at the initial time point (sampled immediately after the addition, 0 min) and only 0.4% remaining at the end of incubation period (240 min) (Fig. 6).

Werle et al. [30] have previously discussed protein and peptide stability in human plasma as well as strategies to improve it in detail. Studies have shown that somatostatin, a 14 amino acid sequence peptide, has a very short plasma half-life ($t_{1/2}$= 2–3 min) which limits its effective clinical application in its native from. To overcome this drawback, octreotide (Sandostatin®) was developed by shortening the overall amino acid sequence of somatostatin from 14 to 8 amino acids with the L-amino acids also replaced by D-amino acids. These modifications led to an improvement in plasma half-life from a few minutes to about 1.5 h [31]. Unmodified peptides usually do not circulate in the blood for more than a few minutes due to enzymatic degradation, and have generally poor bioavailability in tissues and organs. Once in the tissue or cellular compartments, the inactivation of the peptide involves specific endopeptidases, while in the systemic

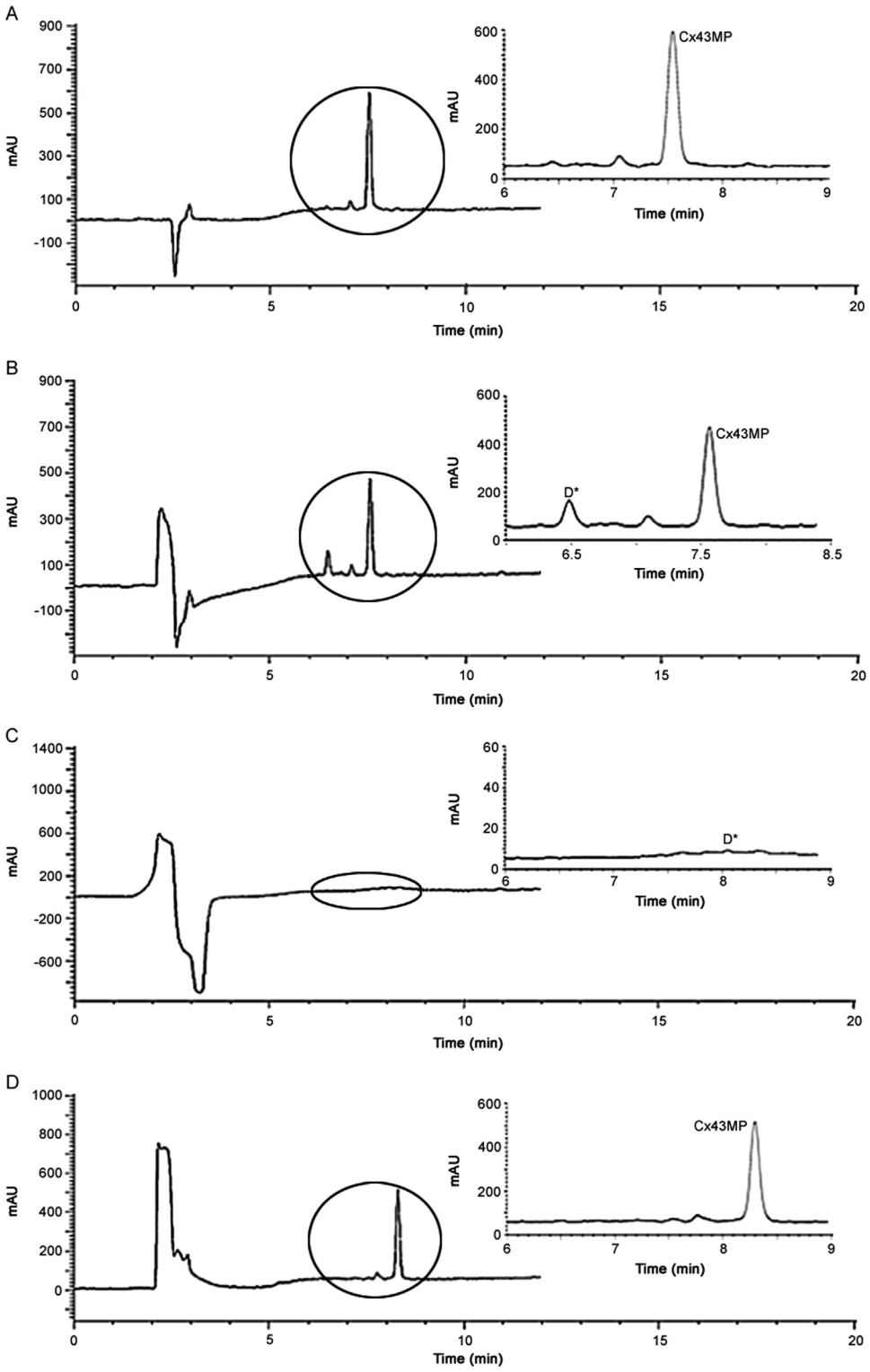

Fig. 2. Stability of Cx43MP at 37 °C in (A) Milli-Q water; (B) 1 M HCl; (C) 1 M NaOH; and (D) H_2O_2 (30% (v/v)). The degradation products are indicated by D*.

circulation and peripheral organs less specific exopeptidases (amino- and carboxy-peptidases) are responsible [32]. Overall, chemical modifications of peptides at the potential enzymatic cleavage sites may significantly increase their in-vivo stability.

3.4. Degradation kinetics

The non-linear two-phase decay kinetic model described the best fit and was used to determine the degradation kinetics of Cx43MP in bovine vitreous and human plasma. The degradation followed two-phase decay, at first a shorter but rapid followed by a longer but slower phase (Fig. 6). As outlined above, Cx43MP had better stability in bovine vitreous compared to human plasma, which is due to the larger number and quantity of protein and peptide degrading enzymes present in the plasma compared to the vitreous [33–35]. The kinetic parameters obtained for Cx43MP in bovine vitreous were

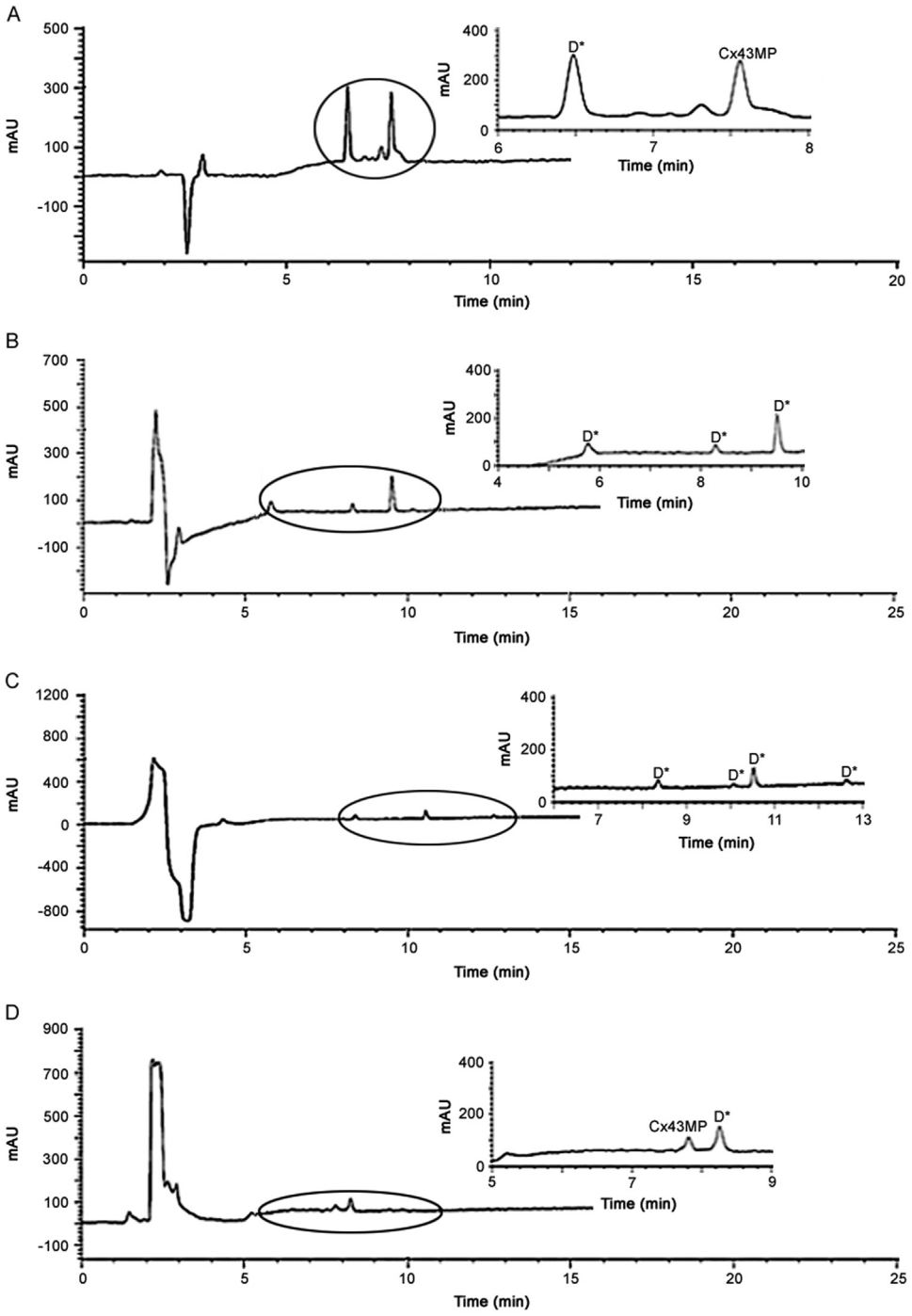

Fig. 3. Stability of Cx43MP at 80 °C in (A) Milli-Q water; (B) 1 M HCl; (C) 1 M NaOH; and (D) H_2O_2 (30% (v/v)). The degradation products are indicated by D*.

half-life ($t_{1/2fast}$=4.51 and $t_{1/2slow}$=171.8 min) and rate constant (k_{fast}=0.01574 and k_{slow} = 0.001033 min^{-1}) with a high correlation coefficient (R^2 = 0.999) and an area under the curve (AUC) of 6643 mg·h/L. The kinetic parameters for Cx43MP in human plasma were half-life ($t_{1/2fast}$=5.06 and $t_{1/2slow}$=39.27 min) and rate constant (k_{fast}=0.004817 and k_{slow}=0.008802 min^{-1}), with a correlation coefficient of R^2 = 0.999 and an AUC of 818.2 mg·h/L. Apparently, $t_{1/2fast}$ (4.51 vs 5.06 min) was found to be similar in bovine vitreous and human plasma; however, the slower phase was quite distinctive and indicated that the Cx43MP was more stable in bovine vitreous than human plasma. This may be explained by the fast enzymatic action as the peptide is mixed with the biological fluids. However, over time the enzymes present become saturated thus resulting in a slower degradation rate during the second phase. However, further investigations are warranted to explain the underlying mechanisms.

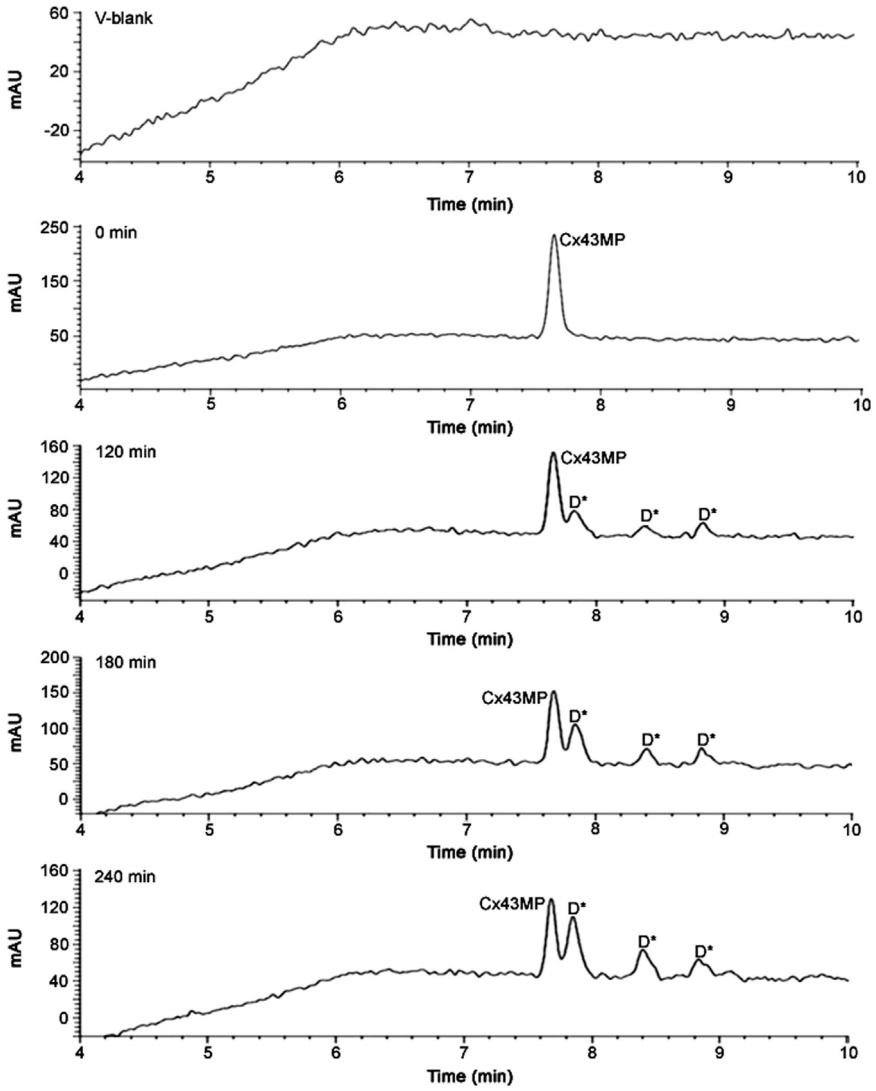

Fig. 4. Chromatograms of Cx43MP incubated within bovine vitreous (V) at different time points (0, 120, 180 and 240 min). The degradation products are indicated by D*.

3.5. Peak purity

The peak spectral purity assessment of the analyte at 7.5 min in bovine vitreous was checked by comparison of the UV spectrum with that of the authentic Cx43MP standard and was found to be within the threshold limit (threshold 778.24; purity factor 871.94), indicating that there was no co-eluting peak at the retention time of Cx43MP.

4. Conclusion

The reported HPLC method was found simple, reliable, sensitive, and precise for the analysis of Cx43MP and can therefore be used to determine the peptide stability in various formulations intended for pre-clinical formulation and pharmacokinetic studies. Cx43MP was found to moderately tolerate the majority of the stress conditions excluding basic conditions. It should be noted that the described stress conditions are extreme and are not generally encountered in peptide drug development and formulation. With regard to clinical applicability, Cx43MP was found to be more stable in bovine vitreous than in human plasma. The information presented here will be useful in designing a sustained release drug delivery system for clinical application of Cx43MP in treatment of inflammatory retinal diseases.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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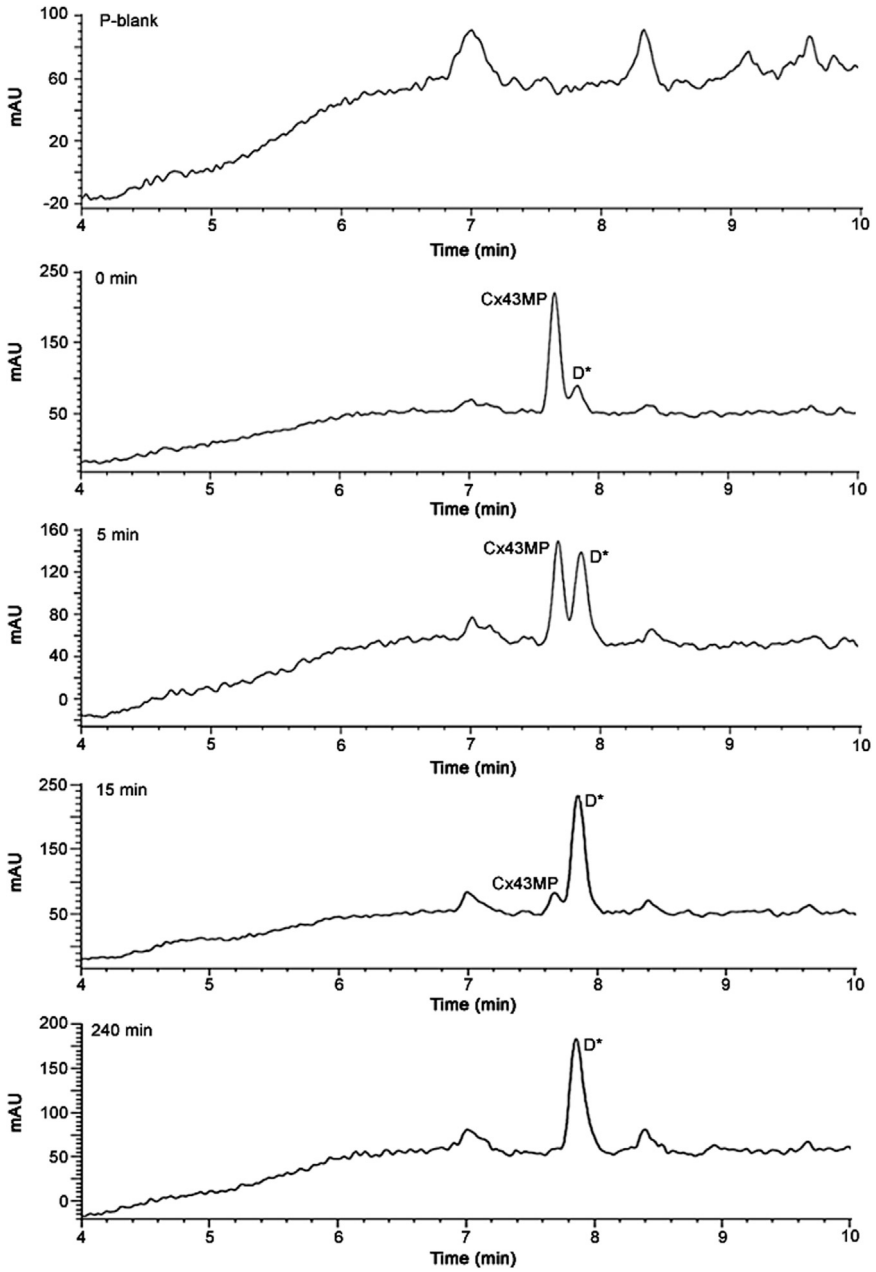

Fig. 5. Chromatograms of Cx43MP incubated with human plasma (P) at different time points (0, 5, 15 and 240 min). The degradation product is indicated by D*.

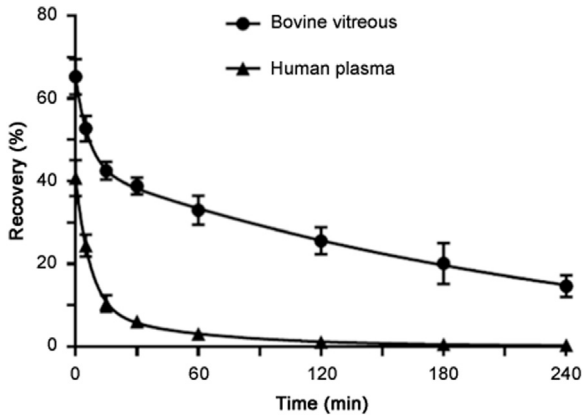

Fig. 6. Non-linear two-phase degradation kinetics of Cx43MP in bovine vitreous and human plasma.

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