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Article

Toward Oral Delivery of Biopharmaceuticals: An Assessment of the Gastrointestinal Stability of 17 Peptide Drugs

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ABSTRACT

A major barrier to successful oral delivery of peptide and protein molecules is their inherent instability in the lumen of the gastrointestinal tract. The aim of this study was to determine the stability of 17 disparate peptide drugs (insulin, calcitonin, glucagon, secretin, somatostatin, desmopressin, oxytocin, [Arg⁸]- vasopressin, octreotide, ciclosporin, leuprolide, nafarelin, buserelin, histrelin, [D-Ser]⁴- gonadorelin, deslorelin, goserelin) in gastric and small intestinal fluids from both humans and pigs, and in simulated gastric and intestinal fluids. In human gastric fluid, the larger peptides including somatostatin, calcitonin, secretin, glucagon and insulin were metabolized rapidly, whereas the smaller peptides showed good stability. In human small intestinal fluid, however, both small and large peptides degraded rapidly with the exception of the cyclic peptide ciclosporin and the disulfide-bridge containing peptides octreotide and desmopressin, which showed good stability. From the investigations in simulated gastric and intestinal fluids without their respective enzymes pepsin and pancreatin, it was established that the pH was not responsible for peptide degradation. The stability of peptides in both simulated gastric fluid and pig gastric fluid correlated well with stability in human gastric fluid. However it was not possible to establish such a correlation with the small intestinal fluids because of the rapid rate of peptide degradation. This work has identified the molecular features in the structure of a wide range of peptides that influence their stability in the environment of the gastrointestinal tract, which in turn will allow for better selection of peptide candidates for oral delivery.

KEYWORDS

Biopharmaceuticals, peptides and proteins; metabolism; simulated gastrointestinal fluids; pig gastrointestinal fluids; human gastrointestinal fluids.

INTRODUCTION

The increasingly widespread use of peptides and proteins as therapeutic drugs is rapidly expanding in diverse fields such as neurology, oncology, endocrinology, haematology, as well as in the treatment of inflammatory diseases. Coupled with recent advances in molecular biology and biotechnology, this has led to the synthesis of several peptide and protein molecules which offer the advantages of high activity, high specificity, low toxicity and minimal non-specific or drug-drug interactions. Somewhat inevitably, however, these drugs are also not without their shortcomings: The vast majority of currently marketed peptide drugs are delivered parenterally on account of stability issues; a route often unacceptable to the patient due to issues of pain and discomfort with administration, and which frequently leads to low patient compliance. Equally, there is also the associated issue of high systemic bioavailability and the risk of potentially severe side-effects with the intravenous route, including hypersensitivity responses. Various non-parenteral routes for the systemic delivery of peptide drugs have been so far investigated, including transdermal, nasal, buccal and pulmonary delivery, which have been met with some success in achieving therapeutic goals.¹⁻⁴ The oral route is another increasingly-investigated alternative, given that it offers advantages in terms of ease of administration, lower manufacturing costs and increased patient compliance. To date, however, there are currently only five marketed orally delivered peptide and protein drugs available; desmopressin, ciclosporin, vancomycin, linaclotide and pancreatin. These molecules are orally

available due to either the low systemic dose required (desmopressin),⁵ their high lipophilicity (ciclosporin),⁶ or because they are targeted for local, intestinal delivery (vancomycin, linaclotide and pancreatin).^{7, 8}

The two major barriers to effective oral peptide drug delivery include the instability of peptides in the gastrointestinal (GI) tract, and erratic absorption across the intestinal membrane into the systemic circulation. In terms of luminal stability, the highly acidic condition of the stomach is believed to be responsible for degradation of peptide drugs through destabilization of the three-dimensional structural folding by interference with ionic and hydrogen bonding.⁹ Moreover the gastric-secreted enzyme pepsin and the intestinally-secreted enzymes trypsin, chymotrypsin, elastase and brush-border membrane-bound enzymes carboxypeptidases A and B cause hydrolysis of specific bonds, leading to peptide degradation and loss of structure and activity.^{9, 10}

The stability of peptides with GI enzymes and fluids have been assessed previously, but these studies have typically involved investigating only 1 or 2 peptide drugs, making it difficult to compare across studies due to variations in experimental conditions, biological fluids and analytical techniques. For instance, studies have included desmopressin stability in the presence of gastrointestinal enzymes,¹¹ oxytocin and Arg-vasopressin in human stomach and small intestinal fluids,¹² and insulin in pig gastric and intestinal luminal fluids.¹³ It would be of use, therefore, to assess the intestinal stability of a wider range of peptide drugs using the same biological fluids and analytical techniques within the same study in order to allow accurate comparisons to be made. This would enable the identification of those structural features that influence gastric and intestinal stability, allow for better selection of oral delivery candidates, and determine suitable formulation strategies.

The aim of this study was to evaluate the stability of 17 disparate peptide drugs in human gastric (HGF) and small intestinal fluids (HIF), pig gastric (PGF) and small intestinal fluids (PIF) and simulated gastric (SGF) and intestinal fluids (SIF). The drugs display different physical and chemical properties and were selected to understand structural influences on gastric and intestinal stability. A schematic representation of the peptides along with their physicochemical properties is shown in Table 1. The molecules can be separated into three groups; a) small peptides with fewer than 12 amino acids and a partial or complete cyclic structure (desmopressin, oxytocin, Arg-vasopressin, octreotide and ciclosporin), b) small and linear peptides with fewer than 12 amino acids (nafarelin, buserelin, goserelin, histrelin, leuprolide, [D-Ser]⁴-Gonadorelin and deslorelin) and c) large peptides with more than 12 amino acids (somatostatin, calcitonin, glucagon, secretin and insulin). This is the first study to comprehensively evaluate the gastrointestinal stability of a large number of peptides, and the data from the study will be useful in **the selection of peptide candidate for oral peptide delivery.**

Table 1 Physical properties of peptide molecules calculated by ChemSpider using the ACD/PhysChem Suite and software ChemAxon or unless referenced otherwise

Peptides	Molecular weight	No. of amino acids	Isoelectric point	Log P	No. of H bond acceptors	No. of H bond donors	No. of Freely rotating bonds	Polar Surface area
Small Cyclic Peptides								
Ciclosporin	1202.6	11	4.70	3.64	23	5	16	278.80
Oxytocin	1007	9	8.57	-5.00	24	16	19	399.53
Vasopressin	1060.2	9	9.75	-6.77	25	18	22	425.55
Desmopressin	1128.3	9	10.22	-6.13	26	18	20	435.41
Octreotide	1019.2	8	10.97	-1.69	20	15	22	329.32
Small Linear Peptides								
[D-Ser] ⁴ Gonadorelin	1182.2	10	10.19	-6.26	30	19	33	471.73
Nafarelin	1322.4	10	10.15	-2.97	33	22	35	469.23
Histrelin	1323.5	9	9.95	-2.41	30	17	36	443.96
Buserelin	1299.4	9	10.17	-3.35	29	17	35	437.87
Leuprolide	1209.4	9	10.07	-2.75	28	17	34	428.64
Goserelin	1269.4	10	9.72	-5.11	32	20	35	492.99
Deslorelin	1282.4	9	9.96	-2.44	29	18	34	439.03
Large peptides								
Somatostatin	1638.1	14	10.20	-8.50	37	26	32	610.33
Calcitonin	3432	32	10.14	-28.49	54	52	98	1508.91

Glucagon	3485	29	8.20	-26.70	92	63	124	1580.71
Secretin	3039	36	10.68	-24.89	47	52	109	1401.22
Insulin	5809	51	5.40 ¹⁴	-	88	89	166	2450

MATERIALS AND METHODS

Materials

Oxytocin acetate, buserelin acetate, goserelin acetate, nafarelin acetate, desmopressin acetate, leuprolide acetate, histrelin, [D-Ser]⁴-Gonadorelin, human recombinant insulin and [Arg⁸]-vasopressin acetate were obtained from Sigma-Aldrich, UK. Octreotide, deslorelin, somatostatin, glucagon, secretin and salmon calcitonin were purchased from Oxford Expression Technologies, UK. Ciclosporin was sourced from Abcam plc, UK. Trifluoroacetic acid, tween 80, pepsin from porcine gastric mucosa, 469 units/mg solid, pancreatin from porcine pancreas, activity at least 3 times more than USP specifications were obtained from Sigma-Aldrich, UK. All other chemicals and solvents were of analytical reagent grade and used without further purification. All the salts were obtained from standard commercial sources.

Sourcing and preparation of gastrointestinal fluids

Human gastric fluid (HGF) and human intestinal fluid (HIF)

HGF and HIF were provided by AstraZeneca, Sweden, through the University of Uppsala, Sweden. The sampling of fluids from the stomach and upper jejunum was achieved using an intestinal intubation device via the Loc-I-Gut[®] procedure.^{15, 16} The pH of the HGF and HIF was 1.8 and 7.6 respectively.

Pig gastric fluid (PGF) and pig intestinal fluid (PIF)

Pig gastrointestinal tracts were obtained from three cross-breed of large white and landrace pigs (95-110 kg, 6 months old) at a meat factory (Cheale Meats, Essex, UK). The gastrointestinal tracts from pigs were then dissected to collect the fluid from the stomach and upper small intestine (duodenum and jejunum). The fluid was collected within 5 hours at 4^oC after sacrifice of the pigs and immediately stored at -80^oC. The fluid was centrifuged at 10,000 rpm for 10 minutes, and the supernatants were used in the stability studies. The pH of the gastric and small intestinal supernatants was 3.4 ± 0.4 and 6.6 ± 0.2 respectively.

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

SGF was prepared according to USP specifications (Test Solutions, United States Pharmacopeia 35, NF 30, 2012). 0.2 g of sodium chloride was added to a 100 ml flask and dissolved in 50 ml water. 0.7 ml of 10 M HCl was added to adjust the pH of the solution to 1.2. To this, 0.32 g of pepsin was added and dissolved with gentle shaking, and the volume made up to 100 ml with water. Pepsin was added only after the pH was adjusted to 1.2. SIF was prepared according to USP specifications (Test Solutions, United States Pharmacopeia 35, NF 30, 2012). 0.68 g of monobasic potassium phosphate was dissolved in 25 ml water, then 7.7 ml of 0.2 N NaOH was added to adjust the pH to 6.8. To this, 1g of pancreatin was added and shaken gently until dissolved, and the volume adjusted to 100 ml with water. Pancreatin was added after adjusting the pH of the solution to 6.8 to avoid precipitation of the enzyme.

Determination of protease activity of the small intestinal fluids

A protease activity kit (Sigma Aldrich, USA) was used to investigate the protease activity of HIF, PIF and SIF. The protease activity kit contained casein labelled with fluorescein isothiocyanate (FITC) as the substrate, with an excitation/emission maxima of 485/520 nm. 20 µl of incubation buffer, 20 µl of FITC-casein substrate and 10 µl of test sample (SIF with pancreatin/PIF/HIF) were added to an eppendorf tube and incubated at 37°C for 2 hours. After incubation, 150 µl of trichloroacetic acid (TCA) was added to the solution. This was centrifuged, and the fluorescence of the supernatant was measured using a fluorometer (BMG LABTECH PheraStar, Germany). The protease activity was calculated using MARS data analysis software. One unit of activity was defined as the amount of protease needed to hydrolyze FITC casein to produce a fluorescence intensity equivalent to 1ng of trypsin hydrolyzed FITC-Casein per hour at 37 °C.

Peptide stability studies

A stock solution of glucagon was prepared in 0.01N HCl, while secretin and calcitonin were prepared in a 0.9% NaCl solution to obtain a concentration of 6.7×10^{-4} mM. Insulin stock solution was prepared in 0.001N HCl at a concentration of 3.4×10^{-4} mM. All other peptide drugs were prepared by dissolving the drug in 0.9% NaCl solution to obtain a concentration of 6.7×10^{-4} mM.

The peptide incubation studies were performed in a Gallenkamp® shaking incubator at 37°C and 100 rpm for 120 minutes. Peptide drug stock solution (6.7×10^{-4} mM) was added to the GI fluids and the final concentration of each peptide drug, with the exception of insulin, was 3.4×10^{-5} mM. For insulin, the stock solution (3.4×10^{-4} mM) was added to GI fluids to obtain a final concentration of 6.9×10^{-5} mM during incubation. The higher concentration of insulin was required due to the low limit of detection of insulin by high performance liquid chromatography (HPLC). All incubations were done in triplicates except for the study in human fluids, which were done in duplicates.

Samples (150µl) were withdrawn at frequent time intervals and added to ice-cold stop reagent (450µl), i.e. methanol for gastric fluids and 0.1N HCl for intestinal fluids, to inactivate the enzymes and allow quantitative determination of intact peptide remaining. For insulin, the sample was added to 0.002M NaOH and 0.01N HCl to inactivate the gastric and intestinal enzymes respectively. Initial experiments had confirmed that the stop reagent had no degradative effect on peptide drug stability. All the samples were centrifuged at 10,000 rpm (9600 g) for 10 minutes, and the supernatant was analysed quantitatively by HPLC.

Chromatographic analysis was performed with a HPLC system (Agilent Technologies, 1260 Infinity) equipped with pump (model G1311C), autosampler (model G1329B) and a diode-array UV detector (model G1314B). The different chromatographic parameters for the peptides are shown in Table 2. The calibration curves were linear in the range 1.7×10^{-6} mM to 3.4×10^{-5} mM for all peptides. The peptide peak could be separated from the peaks from degradation product and protein peaks from gastric and intestinal fluids.

Table 2 HPLC parameters for quantitative analysis of peptide drugs

	Somatostatin	Glucagon	Calcitonin	Secretin	Oxytocin	Buserelin	Desmopressin	Goserelin	Nafarelin	
Column	Luna 5 μ C18 150x4.6 mm	Luna 5 μ C18 150x4.6 mm	Luna 5 μ C18 150x4.6 mm	Luna 5 μ C18 150x4.6 mm	Luna 5 μ C18 150x4.6 mm	Luna 5 μ C18 150x4.6 mm	Luna 5 μ C18 150x4.6 mm	Luna 5 μ C18 150x4.6 mm	Luna 5 μ C18 150x4.6 mm	
	Phenomenex [®]	Phenomenex [®]	Phenomenex [®]	Phenomenex [®]	Phenomenex [®]	Phenomenex [®]	Phenomenex [®]	Phenomenex [®]	Phenomenex [®]	
Temperature	40°C	25°C	25°C	25°C	25°C	33°C	25°C	33°C	25°C	
Injection Volume	50 μ l	50 μ l	50 μ l	50 μ l	50 μ l	50 μ l	50 μ l	50 μ l	50 μ l	
Flow rate	1 ml/min	1 ml/min	1 ml/min	1 ml/min	1 ml/min	1 ml/min	0.7 ml/min	1 ml/min	1 ml/min	
UV absorbance (nm)	214 nm	220 nm	220 nm	210 nm	220 nm	214 nm	220 nm	214 nm	224 nm	
Mobile Phase	Acetonitrile (ACN) : Water : Phosphoric acid (22:78:0.5)	Time ACN Water (min) (0.1%TFA)	Time ACN Water (min) (0.1%TFA)	Time ACN Water (min) (0.1%TFA)	Acetonitrile : Water : 70% perchloric acid (37:63:0.5)	Water (0.1%TFA) : Acetonitrile (83:17)	Water (0.1%TFA) : Acetonitrile (77:23)	Water (0.1%TFA) : Acetonitrile (83:17)	Water (0.1%TFA) : Acetonitrile (78:22)	Water (0.1%TFA) : Acetonitrile (73:27)
		0 20% 80%	0 10% 90%	6 37% 63%						
		6 39% 61%	6 37% 63%	12 37% 63%						
		12 39% 61%	12 37% 63%	14 10% 90%						
		14 20% 80%	14 10% 90%	18 10% 90%						
		18 20% 80%	18 10% 90%							

	Histrelin	Leuprolide	[D-Ser] ⁴ - Gonadorelin	[Arg ⁸]- vasopressin	Octreotide	Deslorelin	Ciclosporin	Insulin
Column	Luna 5µ C18 150x4.6 mm Phenomenex [®]	Discovery 300°A C18 150 x 4.6mm						
Temperature	25°C	25°C	25°C	25°C	25°C	25°C	70°C	40°C
Injection Volume	50 µl	20µl						
Flow rate	1 ml/min	0.6 ml/min	1 ml/min	1 ml/min	1 ml/min	1 ml/min	0.7 ml/min	1ml/min
UV absorbance (nm)	210 nm	210 nm	214 nm	214 nm	220 nm	210 nm	210 nm	210nm
Mobile Phase	Water (0.1%TFA) : Acetonitrile (77:23)	Water (0.1%TFA) : Acetonitrile (70:30)	Water (0.1%TFA) : Acetonitrile (84:16)	Water (0.1%TFA) : Acetonitrile (87:13)	Water (0.1%TFA) : Acetonitrile (82:18)	Water (0.1%TFA) : Acetonitrile (75:25)	Water (0.1%TFA) : Acetonitrile (80:20)	Water (0.1% TFA): Acetonitrile (0.1% TFA) (30-60%)

Data analysis

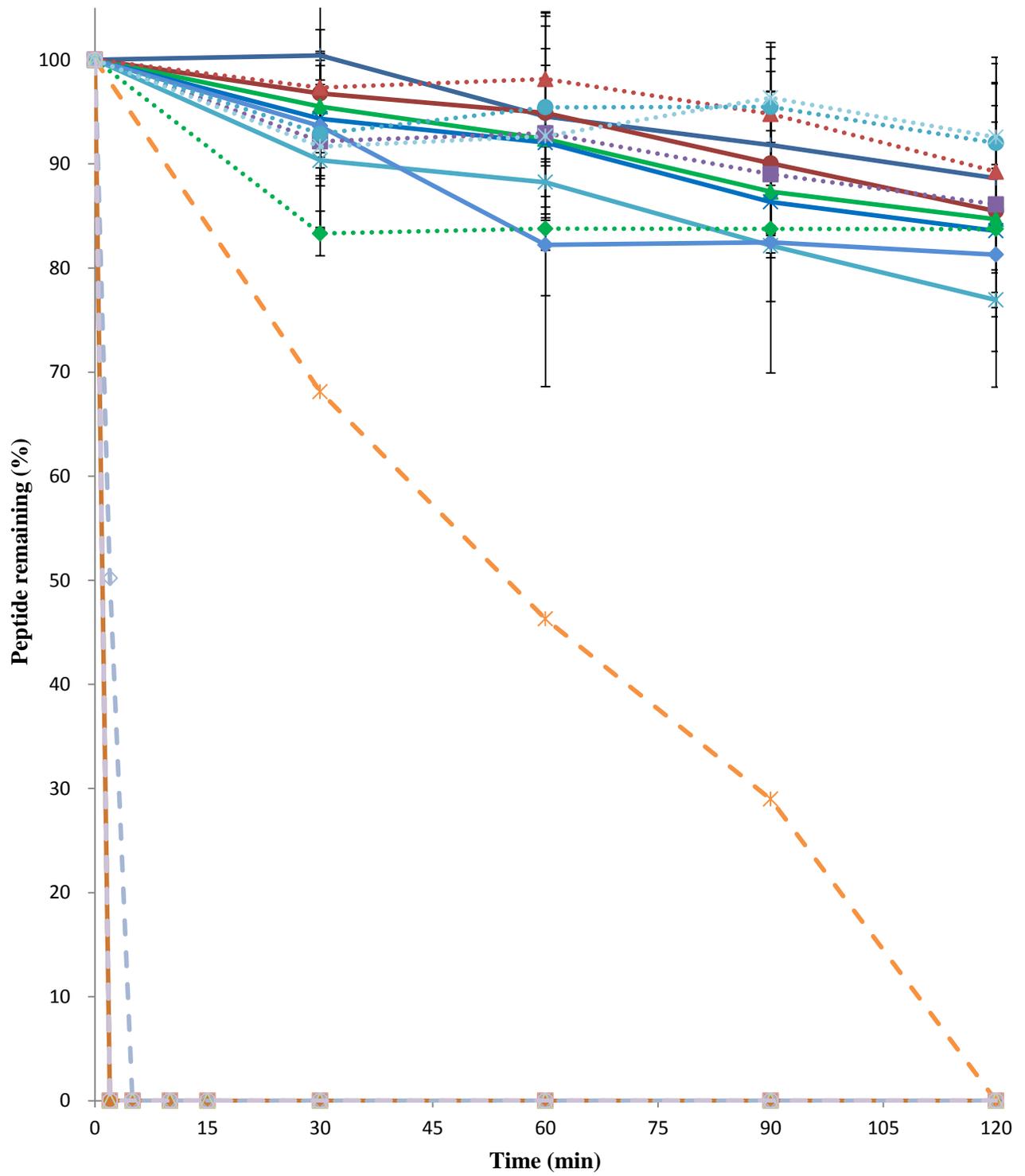
All experiments were performed in the same batch of GI fluids to avoid inter-day variability, and the results are presented as mean \pm standard deviation (SD). The half-life ($t_{1/2}$) of peptides in GI fluids was calculated from their degradation profiles, by linear regression analysis assuming zero-order or first order degradation kinetics, using Origin 9 (OriginLab Corporation, MA, USA).

RESULTS

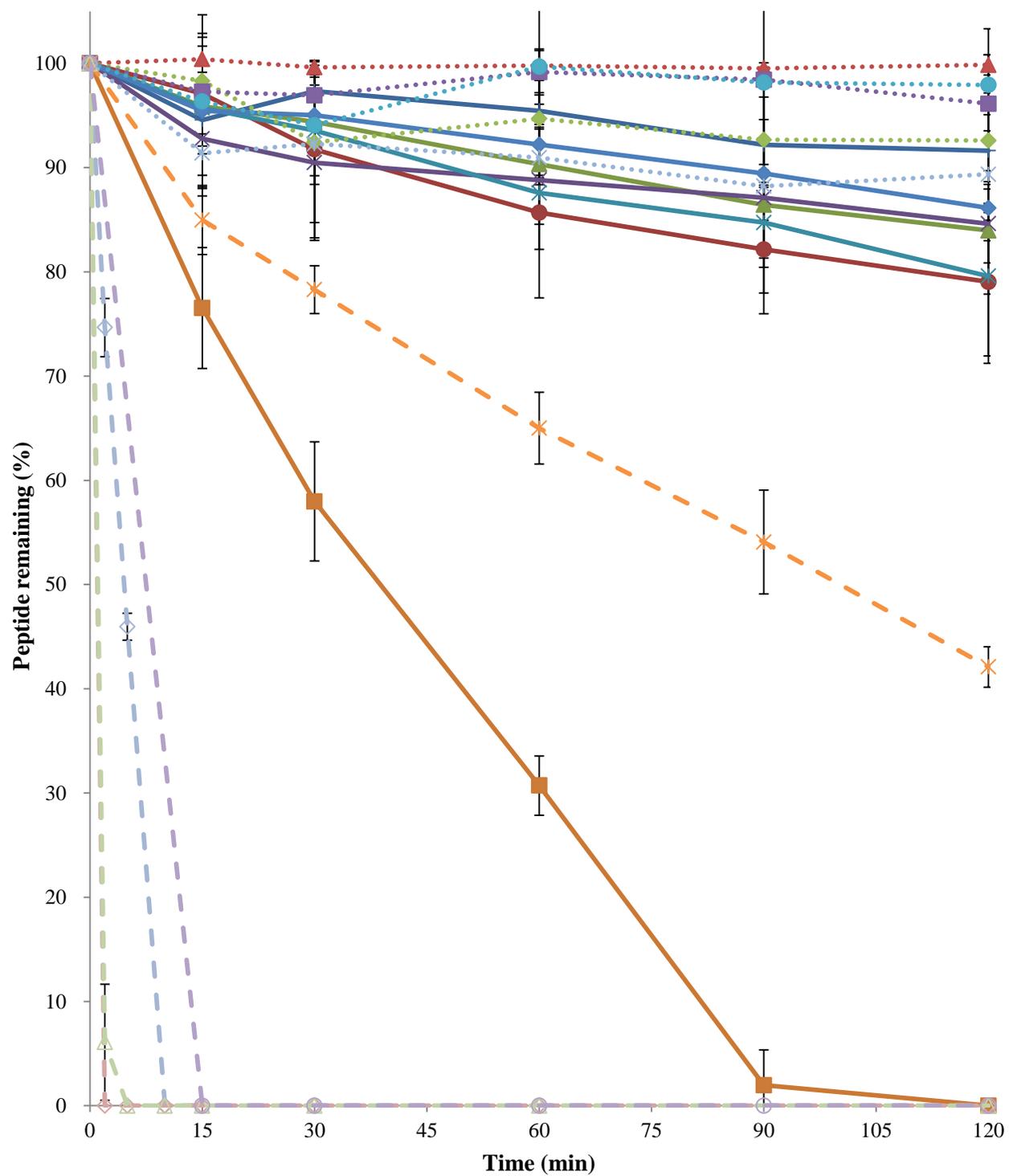
The stability of the peptides in human gastric fluid (HGF) is shown in Figure 1a. The stability of the peptides in pig gastric fluid (PGF) and simulated gastric fluid (SGF) with pepsin is shown in Figures 1b and 1c respectively. With the exception of [D-Ser]⁴-Gonadorelin, the small peptides were stable in all three gastric fluids. In contrast, the large peptides were rapidly degraded within 10 minutes. Somatostatin was the exception as it showed relatively high stability in all gastric fluids.

The stability of the peptide drugs in human intestinal fluid (HIF) is shown in Figure 2a. The stability of the peptide drugs in PIF and SIF is shown in Figures 2b and 2c respectively. The large peptides as well as the small peptides busserelin, goserelin, deslorelin, histrelin and leuprolide degraded rapidly within 2-10 minutes in all three small intestinal fluids. The small peptides which did not follow this trend were nafarelin and [D-Ser]⁴-Gonadorelin which showed higher stability in PIF but rapid degradation HIF and SIF. Oxytocin showed uniform stability in all three small intestinal fluids, with complete degradation achieved after 30 minutes, while Arg-vasopressin was more stable in SIF than HIF and PIF. The peptides octreotide and desmopressin also showed relatively higher stability, but stability was variable between the fluids with fastest degradation observed in HIF followed by PIF and SIF. Ciclosporin was stable in all three small intestinal fluids.

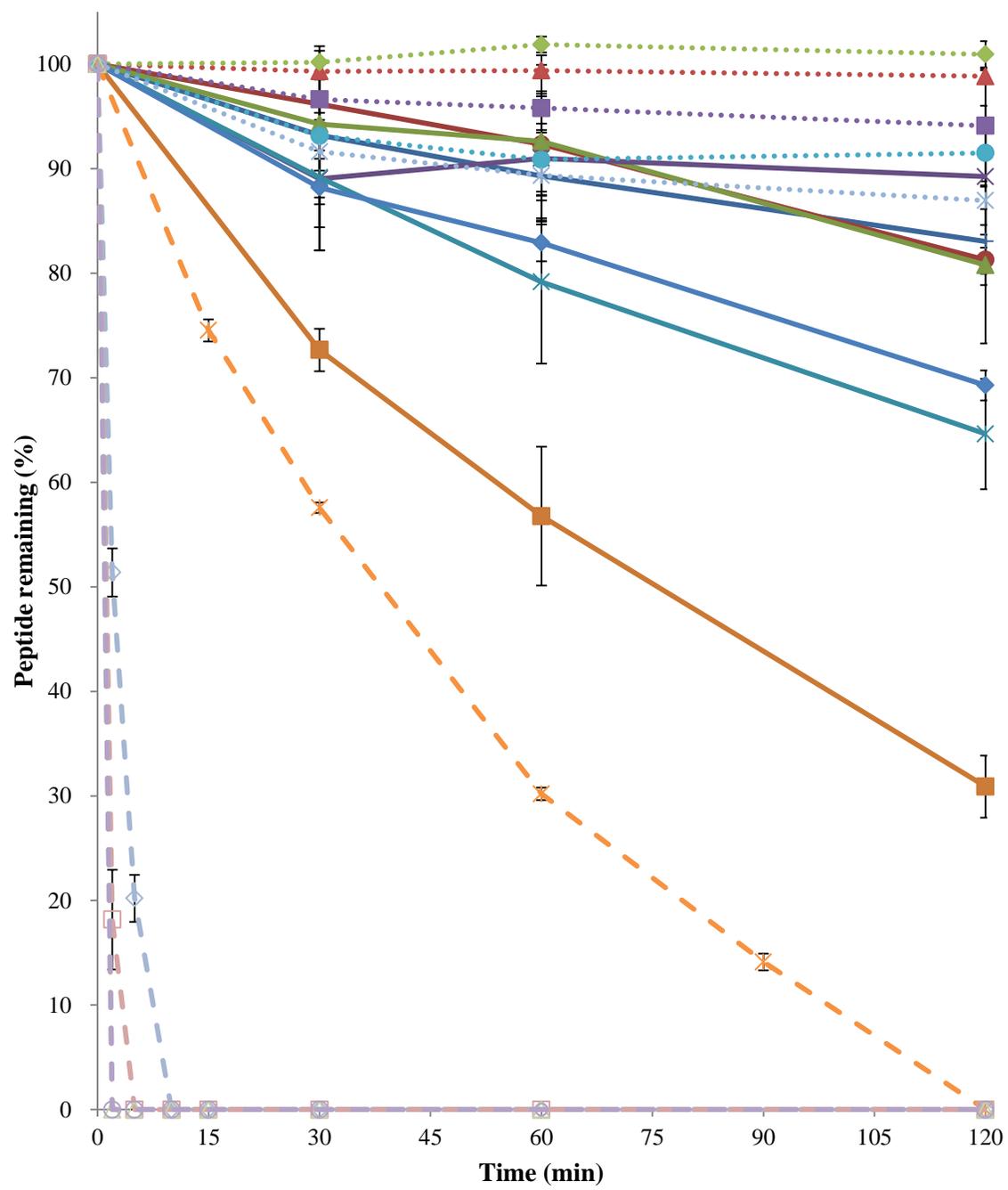
Figure 1. Stability of peptide drugs in human gastric fluid (a), pig gastric fluid (b) and simulated gastric fluid with pepsin (c). Nafarelin (—+—), Buserelin (—●—), Goserelin (—▲—), Histrelin (—×—), Leuprolide (—*—), Gonadorelin (—■—), Deslorelin (—◆—), Desmopressin (··▲··), Oxytocin (··◆··), Arg-Vasopressin (··■··), Octreotide (··●··), Somatostatin (—✱—), Calcitonin (—◇—), Glucagon (—□—), Secretin (—△—), Insulin (—⊕—), Ciclosporin (··✱··).



a)

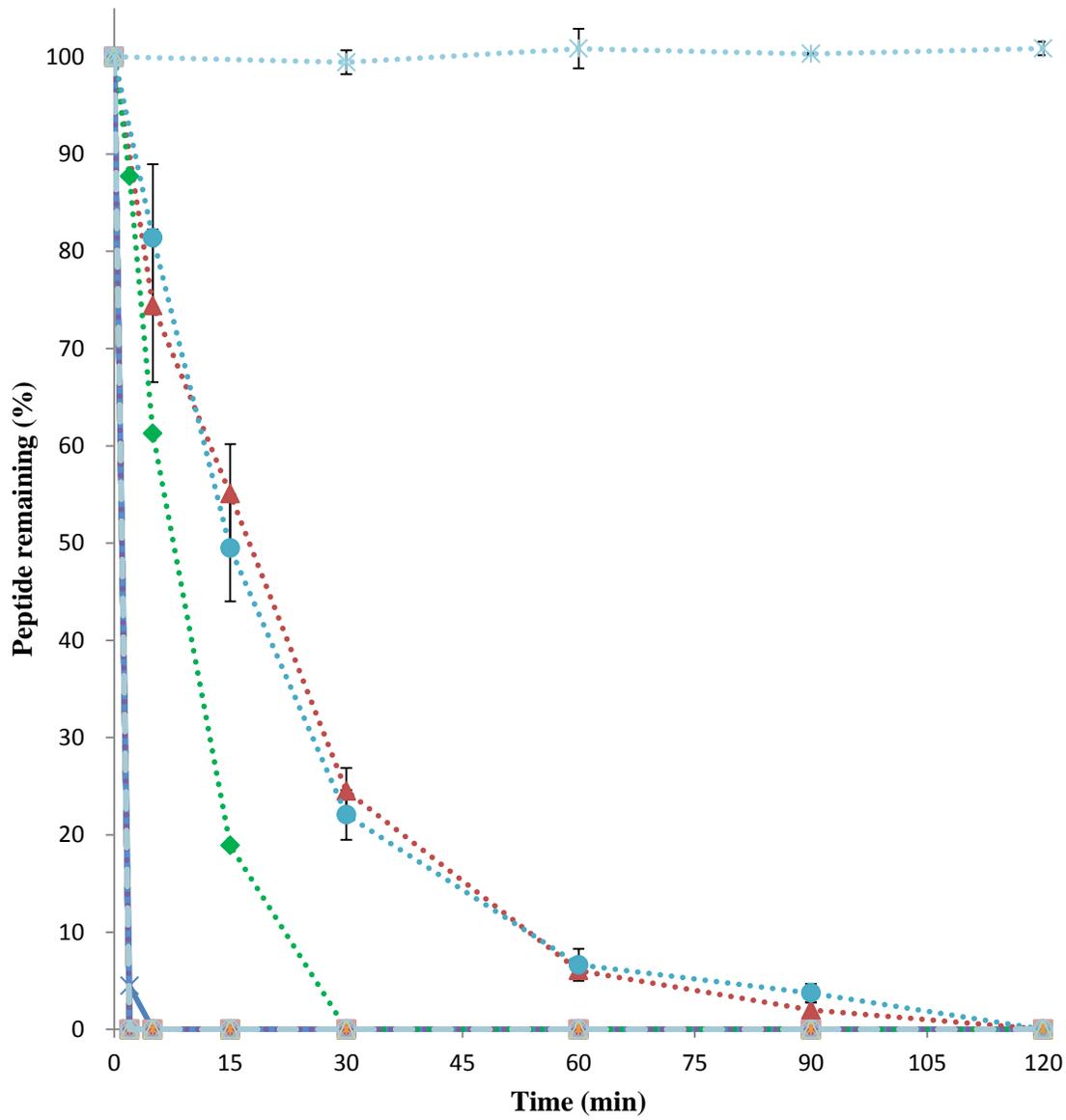


b)

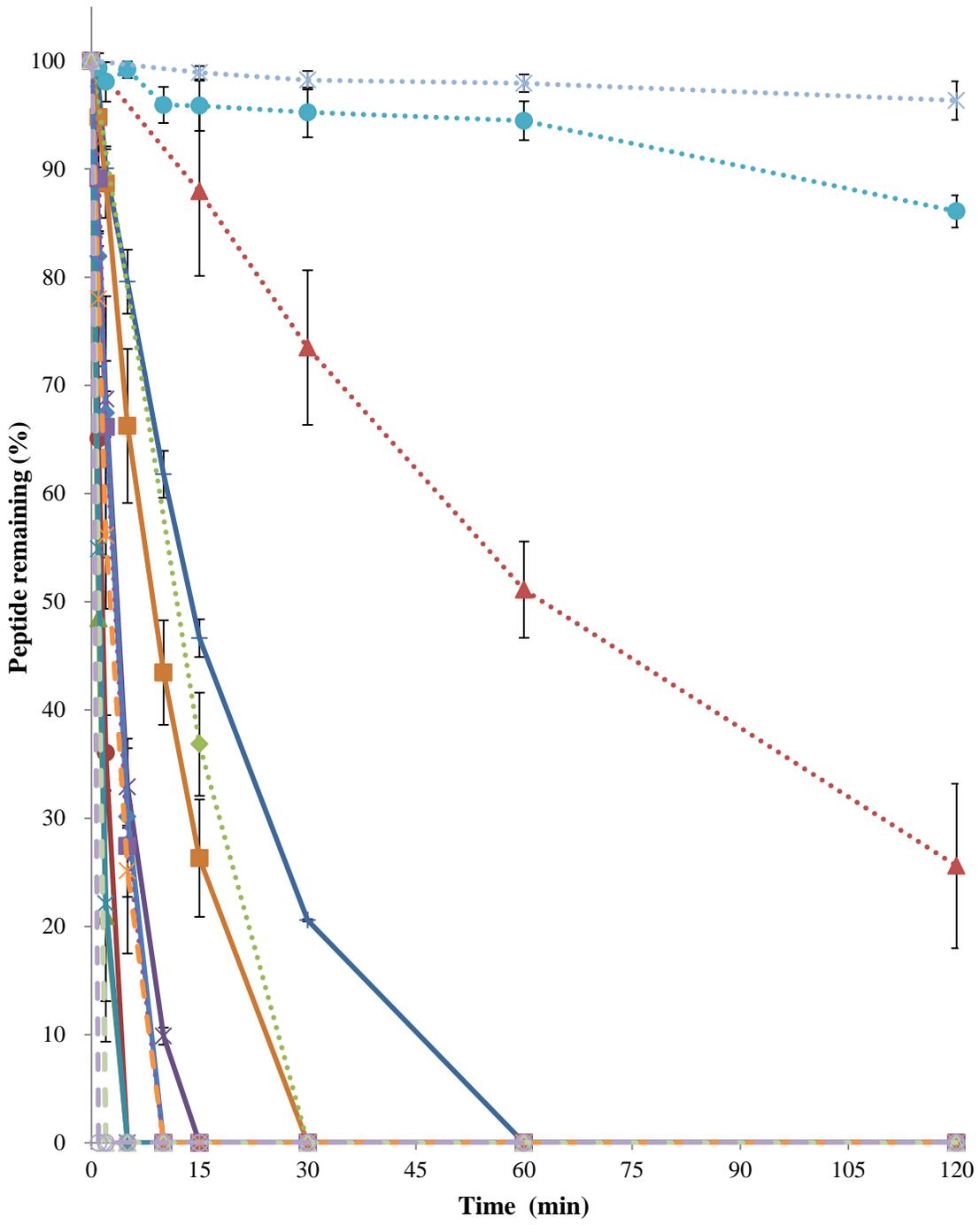


c)

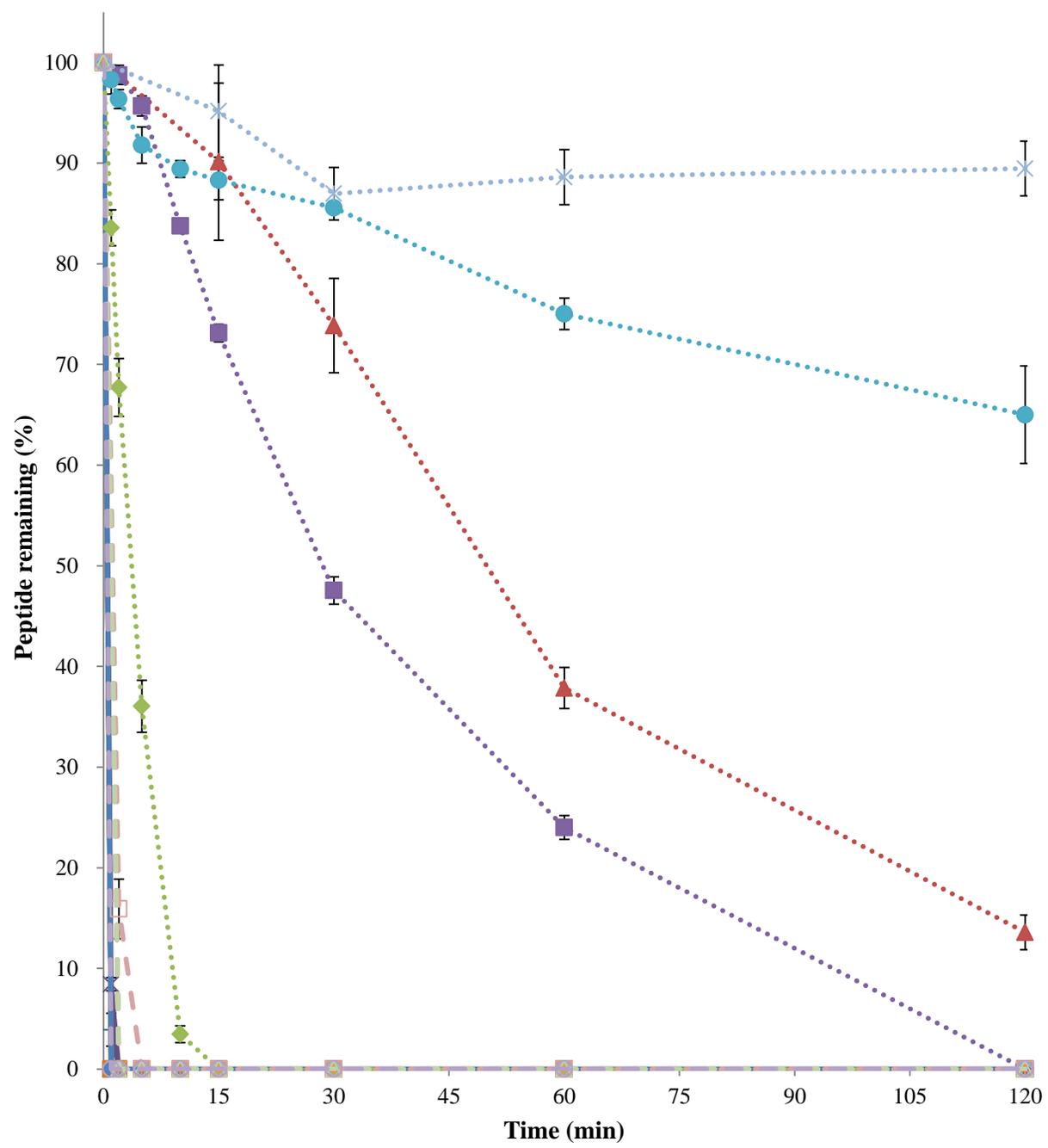
Figure 2. Stability of peptide drugs in human small intestinal fluid (a), pig intestinal fluid (b) and simulated intestinal fluid with pancreatin (c). Nafarelin (—+—), Buserelin (—●—), Goserelin (—▲—), Histrelin (—×—), Leuprolide (—*—), Gonadorelin (—■—), Deslorelin (—◆—), Desmopressin (··▲··), Oxytocin (··◆··), Arg-Vasopressin (··■··), Octreotide (··●··), Somatostatin (—*—), Calcitonin (—◇—), Glucagon (—□—), Secretin (—△—), Insulin (—⊕—), Ciclosporin (··*··).



a)



b)



c)

The stability of the peptides in SGF and SIF without their respective enzymes pepsin and pancreatin is shown in Figures 3 and 4 respectively. In both fluids, all peptides were stable with more than 80% drug recovery after 2 hours (with the exception of calcitonin in SIF without pancreatin), highlighting the dominant role of GI enzymes in the breakdown and cleavage of the peptide structure.

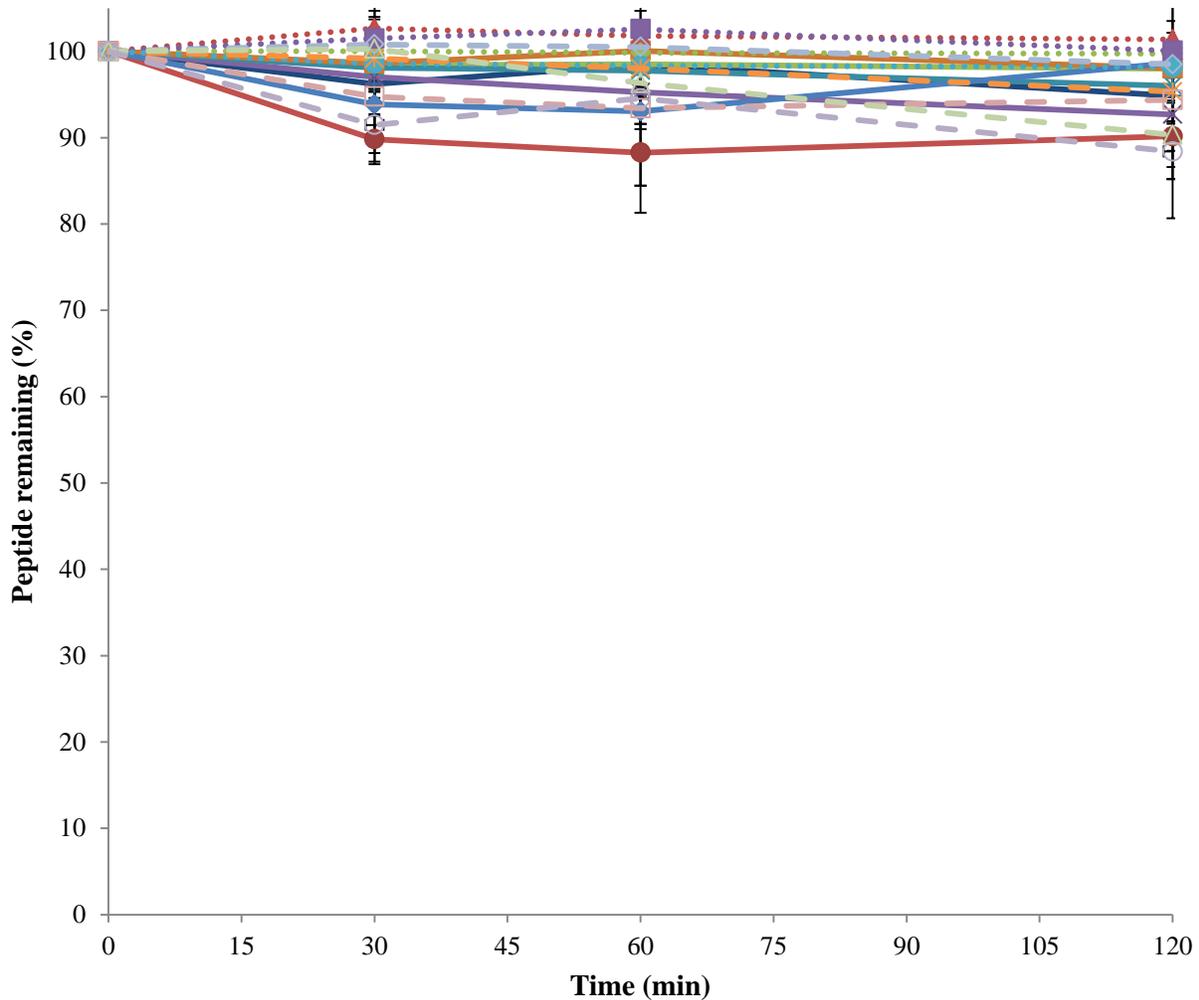


Figure 3. Stability of peptide drugs in intestinal fluid with pancreatin. Each value represents mean \pm S.D (n=3). Nafarelin (—+—), Buserelin (—●—), Goserelin (—▲—), Histreltin (—×—), Leuprolide (—✕—), Gonadorelin (—■—), Deslorelin (—◆—), Desmopressin (...▲...), Oxytocin (...◆...), Arg-Vasopressin (...■...), Octreotide (...●...), Somatostatin (—✱—), Calcitonin (—◇—), Glucagon (—□—), Secretin (—△—), Insulin (—○—), Ciclosporin (...✱...).

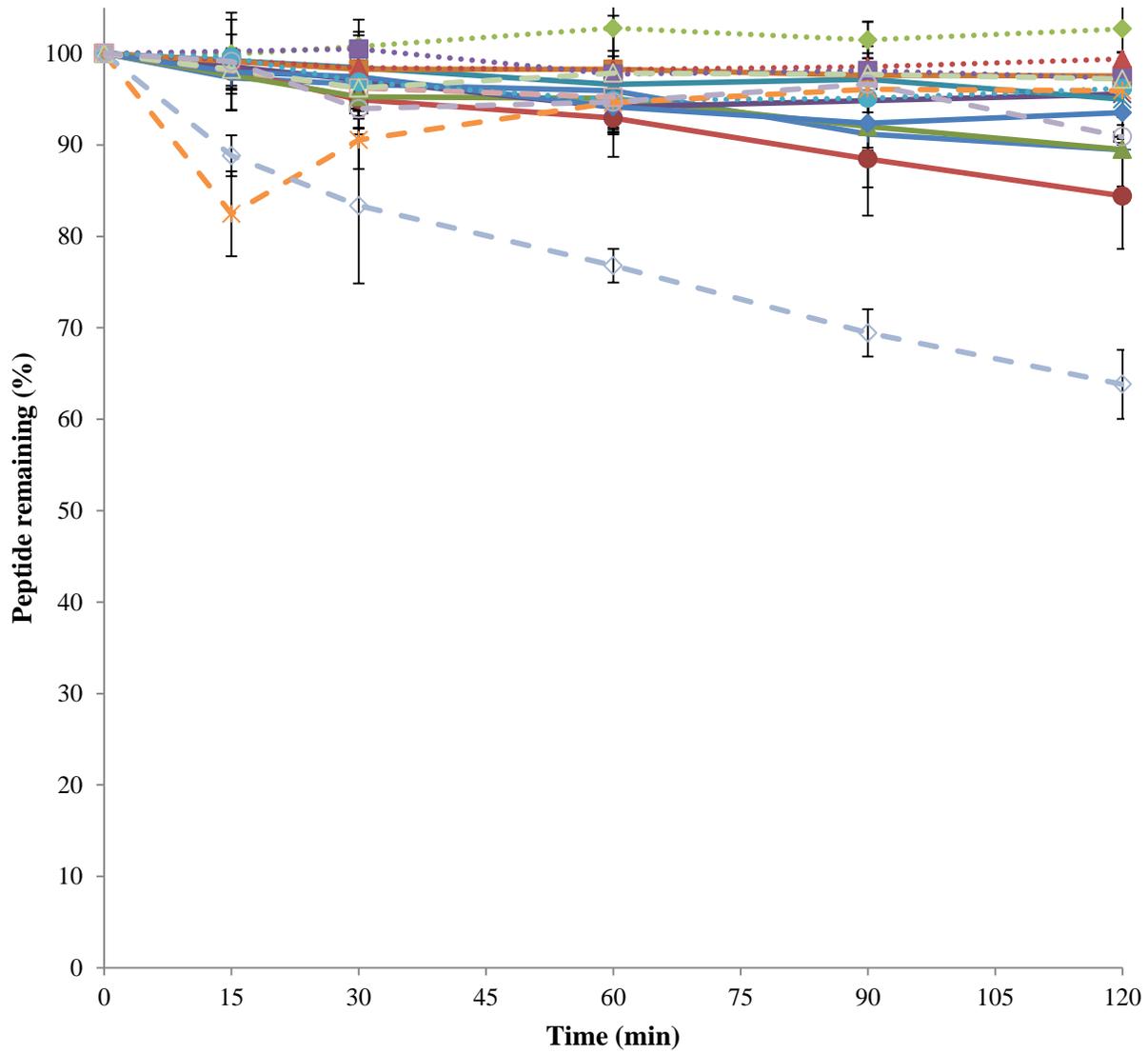


Figure 4. Stability of peptide drugs in simulated gastric fluid without pepsin enzyme. Each value represents mean \pm S.D (n=3). Nafarelin (—+—), Buserelin (—●—), Goserelin (—▲—), Histrelin (—×—), Leuprolide (—*—), Gonadorelin (—■—), Deslorelin (—◆—), Desmopressin (—▲—), Oxytocin (—◆—), Arg-Vasopressin (—■—), Octreotide (—●—), Somatostatin (—*—), Calcitonin (—◇—), Glucagon (—□—), Secretin (—△—), Insulin (—⊖—).

In order to further understand the role of protease enzymes in the degradation of peptides in gastric and small intestinal fluids, the protease activity of all these fluids was measured. The protease activity in SGF with pepsin, PGF and HGF was 14237 ± 3099 , 6749 ± 2464 and 10072 ± 3157 units/ml respectively while the protease activity in SIF with pancreatin, PIF and HIF was 49649 ± 2610 , 30730 ± 5575 , 50608 ± 3880 units/ml respectively. The half-lives of the peptide drugs in all three gastric and small intestinal fluids are shown in Table 2 and 3 respectively; the half-lives of large peptides could not be obtained due to rapid degradation within 2 minutes. Good correlations were observed for the stability of the peptides in SGF and HGF ($R^2 = 0.917$), PGF and HGF ($R^2 = 0.932$) and SGF and PGF ($R^2 = 0.917$). This suggests the ability of SGF and PGF to be used as an *in-vitro* model to predict the stability of peptides in human stomach, as well as the usefulness of SGF to simulate pig gastric conditions.

Table 2

The half lives and % peptide remaining after 2 hours in HGF, PGF and SGF with pepsin.

Peptide	HGF		PGF		SGF	
	Half life (mins)	% Drug remaining after 2 hours	Half life (mins)	% Drug remaining after 2 hours	Half life (mins)	% Drug remaining after 2 hours
Nafarelin	491	89	822	92	357	83
Buserelin	424	85	274	79	324	81
Goserelin	385	85	376	84	322	81
Histrelin	363	84	440	85	661	89
Leuprolide	267	77	300	80	166	65
[D-Ser] ⁴ -Gonadorelin	*	0	46	0	80	31
Deslorelin	294	81	468	86	195	69
Desmopressin	633	89	~	100	~	99
Oxytocin	405	84	884	93	~	100
[Arg ⁸]-vasopressin	468	86	~	96	1093	94
Octreotide	1071	92	~	98	761	92
Ciclosporin	1374	93	667	89	480	87
Somatostatin	58	0	98	42	49	0
Calcitonin	2.0	0	4.6	0	2.1	0
Glucagon	*	0	*	0	1.2	0
Secretin	*	0	1.1	0	*	0
Insulin	*	0	*	0	*	0

~ Stable, >95% peptide recovery after 2 hours

* Unable to calculate $t_{1/2}$ due to very fast degradation within 2 minutes

Table 3

The half lives and % peptide remaining after 30 minutes in HIF, PIF and SIF with pancreatin.

Peptide	HIF		PIF		SIF	
	Half life (mins)	% Drug remaining after 30 mins	Half life (mins)	% Drug remaining after 30 mins	Half life (mins)	% Drug remaining after 30 mins
Nafarelin	*	0	14	21	0.5	0
Buserelin	*	0	1.4	0	*	0
Goserelin	*	0	0.9	0	*	0
Histrelin	1.1	0	3.2	0	*	0
Leuprolide	*	0	1.0	0	*	0
[D-Ser] ⁴ -Gonadorelin	*	0	8.0	0	*	0
Deslorelin	*	0	2.9	0	*	0
Desmopressin	15	25	63	73	50	74
Oxytocin	6.8	0	8.9	0	3.2	0
[Arg ⁸]-vasopressin	*	0	2.9	0	30	48
Octreotide	15	22	554	95	163	86
Ciclosporin	~	99	~	98	499	87
Somatostatin	*	0	2.4	0	*	0
Calcitonin	*	0	*	0	*	0
Glucagon	*	0	*	0	*	0
Secretin	*	0	*	0	*	0
Insulin	*	0	*	0	*	0

~ Stable, >95% peptide recovery after 2 hours

* Unable to calculate $t_{1/2}$ due to very fast degradation within 2 minutes

DISCUSSION

The high stability of peptides in SGF without pepsin and the degradation of peptides in SGF with pepsin established the negligible effect of pH and the importance of the enzyme in the degradation of peptides in gastric conditions. Pepsin specifically cleaves amino acids at L-Phe-, L-Met-, L-Leu-, and L-Trp, which are adjacent to a hydrophobic amino acid.^{17, 18} Therefore the high gastric stability of the small peptides oxytocin, Arg-vasopressin, desmopressin, goserelin, buserelin, nafarelin and histrelin can be linked to the absence of pepsin-susceptible peptide bonds. Additionally, the peptides oxytocin, vasopressin and desmopressin feature disulfide bridges between their Cys amino acids which partially cyclizes their structure and results in high resistance to pepsin cleavage and their subsequent stability in gastric fluids. These results are in line with previous studies showing the high stability of Arg-vasopressin and desmopressin in gastric fluid.^{11, 12} Interestingly, ciclosporin, octreotide, leuprolide and deslorelin which all contain pepsin-vulnerable peptide bonds were found to be stable in SGF with pepsin. However, the pepsin vulnerable amino acids at position 6 in leuprolide and deslorelin, and position 4 in octreotide, are in the D-confirmation which may render them more resistant to pepsin cleavage due to the enzyme's specificity towards L-amino acids.¹⁹ [D-Ser]⁴-Gonadorelin was degraded in gastric fluids even though it does not possess pepsin vulnerable bonds. This might be due to the L-confirmation of [D-Ser]⁴-Gonadorelin amino acid at position 6 compared to the D-confirmation in other LHRH analogues. It was reported that the D-amino acid at position 6 could stabilize the U-shaped conformation of LHRH analogues, which could improve the binding affinity.²⁰ Previous study has showed an analogue of gonadorelin with D-Lys amino acid substitution at position 6 to be stable in simulated gastric fluids with pepsin with 80% intact drug remaining after 2 hours.²¹ This may indicate the importance of D-confirmation of amino acid at position 6 for the stability of LHRH analogues in gastric fluids. The faster degradation of large peptides in gastric fluids may be due to multiple factors such as the presence of high number of pepsin susceptible peptide bonds, high structural flexibility and a greater number of H bond acceptors/donors, producing a higher polar surface area enabling increased interaction with pepsin **8**.

Pancreatin contains the proteases trypsin, chymotrypsin and elastase which are largely responsible for intestinal breakdown of peptide molecules.⁹ The peptide molecules were found to be degraded at different rates in small intestinal fluids, even though they all contain protease susceptible peptide bonds. This might be attributed to the differences in their physicochemical properties, as well as the different protease activity of the small intestinal fluids used in the current study. Indeed, the measured protease activity of SIF and HIF was almost 2-fold higher than for PIF, which may explain the higher stability of peptides such as octreotide, desmopressin and leuprolide in PIF. The greater stability of oxytocin, desmopressin, octreotide and ciclosporin as compared to the linear peptides in SIF could be attributed to their partial or complete cyclic structure and resultant reduced flexibility, fewer H bond acceptors/donors and fewer freely rotating, thereby restricting protease digestion.^{22, 23} The superior stability of desmopressin over Arg-vasopressin may be due to the D-arginine substitution at residue 8 which may have increased its resistance towards trypsin.²⁴ The faster degradation of large peptides in small intestinal conditions could be attributed to similar reasons, as observed in gastric fluids. Similar findings have previously been reported with salmon calcitonin and insulin, which were shown to be vulnerable to digestion in SIF,²⁵⁻²⁷ rat GI fluids,²⁸⁻³⁰ and PIF.²⁷ A much slower degradation of insulin was observed in another study in the presence of proteases, with complete drug loss achieved only after 1 hour.³¹ This highlights the significance of studying the

stability of peptides under identical experimental conditions in biological fluids obtained from the same source, and using the same analytical techniques/instruments for analysis, to minimize the variability observed in the results obtained from different studies.

Owing to the ethical limitations on access to human GI fluids, the need to develop a suitable *in-vitro* system that can be used for prediction of biopharmaceutical drug stability in early-stage drug development would be beneficial. The current study has highlighted the use of simulated and pig GI fluids as an effective *in-vitro* tool to predict the stability of biopharmaceuticals in the human GI tract by mimicking the physiological conditions. Considering the correlation observed between the half-life of peptides in SGF and PGF compared to HGF, both models appear to effectively mimic the *in-vitro* stability in human gastric conditions. However, the half-life of some peptides in small intestinal fluids could not be determined due to the extremely rapid rate of degradation; hence the same correlation could not be achieved between the stability of peptides in small intestinal fluids. However, the degradation of peptides in PIF and SIF showed similar trends as that observed in HIF, highlighting their ability to be implemented as good surrogates to HIF.

CONCLUSIONS

The stability of proteins and peptides in gastrointestinal fluids is an inherent problem associated with the oral delivery of these biopharmaceuticals. In order to ascertain whether differences existed in terms of peptide stability relating to factors such as molecular weight and size as well as regional exposure to gut fluids, we investigated the stability of 17 peptide drugs in human, pig and simulated fluids. Peptide stability was shown to vary widely depending on the amino acid sequence and higher structures. Cyclisation due to disulfide bond linkages resulting in high rigidity and low flexibility appeared to provide resistance against enzymatic cleavage of susceptible peptide bonds. Larger peptides, however, were more vulnerable to enzymatic cleavage due to the higher number of enzyme susceptible peptide bonds, and high structural flexibility. SGF and PGF appeared to be reasonably good models for predicting peptide stability in the human stomach, as they both demonstrated good correlation in terms of peptide stability to HGF. Despite similarities in the trend in peptide degradation in both PIF and SIF compared to HIF, a correlation of the half-lives could not be obtained due to the rapid rate of degradation in this segment of the gut. The overall outcome of the work has made it possible to identify those features in the structure of the molecules that influence gastric and intestinal stability, allow for better selection of peptide candidates for oral delivery, and determine suitable formulation strategies for those molecules with inherent stability issues.

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