Supplementary information to 'Biological characterization of somatropin-derived cryptic peptides'.

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SI 1. Chemical identification and purity quantification of the synthetically prepared SDPs.

1. Introduction

The SDPs were synthesized using solid-phase peptide synthesis (SPPS). Chemical characterization (identification and purity) of these peptides is designated to control if peptide related impurities (deletion, insertion of amino acids, racemization, …) (D'Hondt et al. 2014) or peaks coming from other peptides than the ordered ones are present since they can influence the reliability of the results of initial functionality studies (De Spiegeleer et al. 2008; Verbeke et al. 2015). The peptides were ordered with a requested purity of ≥ 95% which is the recommended target purity to perform ligand binding assays, *in vitro* assays, and *in vivo* studies.

The analytical quality control strategy consists of identification of the SDPs by LC-MS (FA as acidic modifier) and quantification of the purity by LC-UV detection at 210 nm (TFA as acidic modifier), both methods relying on established reversed phase chromatographic separations (De Spiegeleer et al. 2008).

2. Materials and methods

2.1. LC-MS analysis for chemical identification

A Vydac Everest C18 column (4.6 x 250 mm, 5 µm) with suitable guard column (Grace, Lokeren, Belgium) and mobile phases A: 0.1% (m/V) FA in 95/5 (V/V) H_2O/ACN and B: 0.1% (m/V) FA in 5/95 (V/V) H_2O/CAN were prepared for separation of $SDP_{39\text{-}10}$ and $SDP_{165\text{-}177,1\text{-}18,M+1\text{-}18,101\text{-}121}$. For $SDP_{167\text{-}175}$, an adaption to the method was made since there was no retention under the applied conditions. For $SDP_{167\text{-}175}$, the prevail organic acid column (4.6 x 250 mm, 5 µm) (Grace, Lokeren, Belgium) with suitable guard column to obtain longer retention was used. Both columns were thermostated at 40°C and a fixed injection volume of 20 µL and a flow rate of 0.5 mL/min were applied. The linear gradient program started with a 2 min isocratic hold at 100% (V/V) A, followed by a linear gradient to 60% (V/V) A + 40% (V/V) B at 32 min. The method also included a rinsing step at 100% B, followed by returning to the initial conditions and re-equilibration giving a total run of 50 min. UV detection was at 210 nm and the MS parameters were as described in peptide mapping section in reference (Bracke et al. 2014). Prediction of peak identity was performed upon comparison of m/z values with the SEQUEST algorithm of the Thermo BioWorks software (San José, CA, USA).

2.2. HPLC-UV analysis for purity quantification

Each sample was separated on a Vydac Everest C18 column (4.6 x 250 mm, 5 µm) with suitable guard column (Grace, Lokeren, Belgium) using chromatographic parameters as described in 2.1 (of SI 1), using TFA instead of

FA as acidic modifier. $SDP_{167-175}$ had sufficient retention using these conditions. UV detection was performed from 190 to 400 nm, whereas quantification was performed at 210 nm.

3. Results

For all ordered SDPs, the major peak was identified to be the wanted sequence by peak assignment in the MS and MS^2 spectra (***Figure 1, 2***). In ***Table 1***, the purity of each peptide is given. The purity was compliant to the stated purity (≥ 95%) except for SDP_{39-70} which had only a purity of 90.48%.

Table 1: Purity results of the peptides.

Peptide	**Purity results (%)**	**Purity as stated on suppliers' CoA (%)**
SDP_{39-70}	90.48	95.55
$SDP_{167-175}$	100.00	97.91
$SDP_{165-177}$	97.70	96.61
SDP_{1-18}	95.22	99.07
SDP_{M+1-18}	99.78	95.12
$SDP_{101-121}$	100.00	99.32

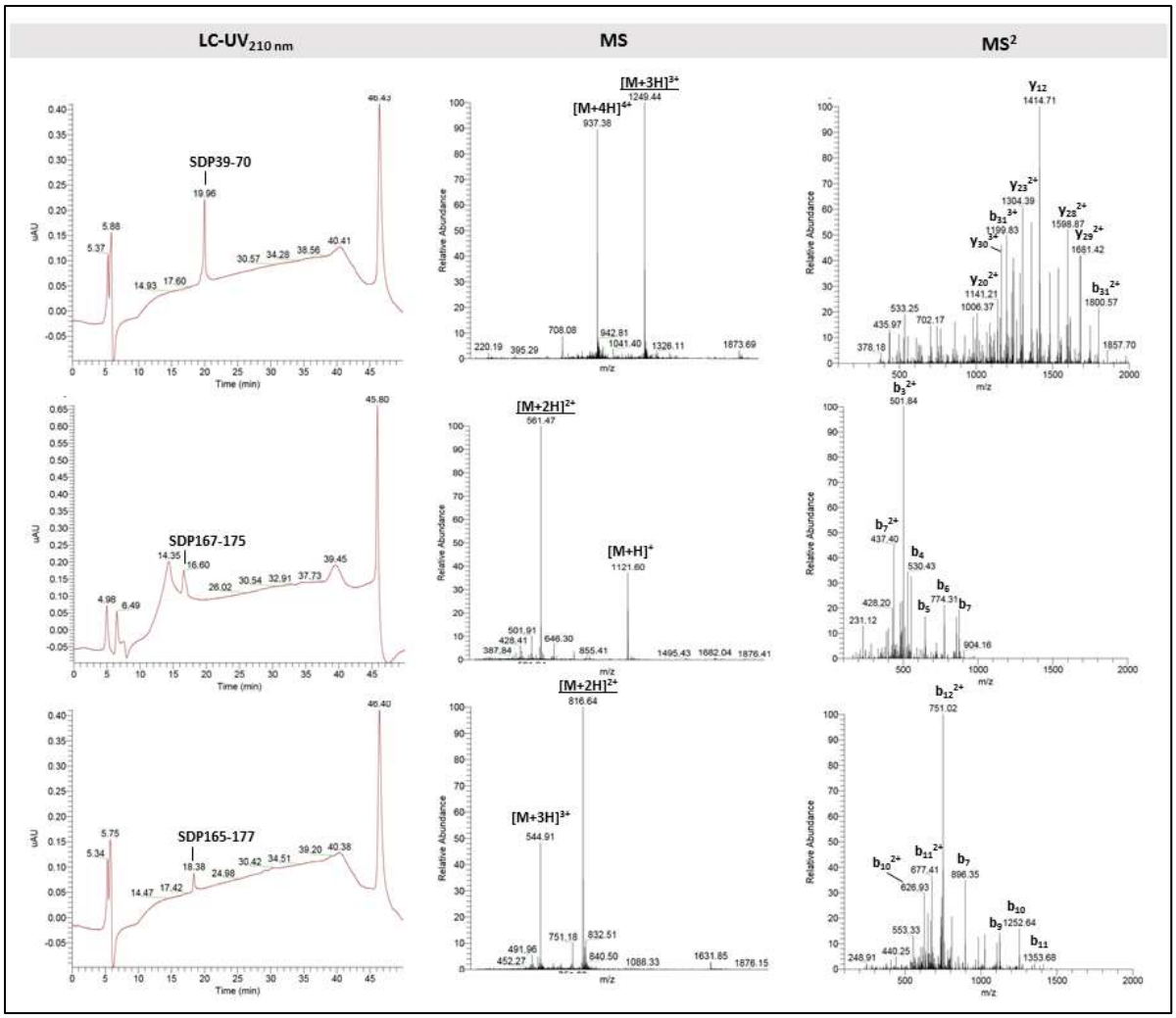


Figure 1: Chemical characterization of the SDPs. Left: LC-UV$_{210\ nm}$ chromatogram, middle: MS spectrum, right: MS² spectrum.

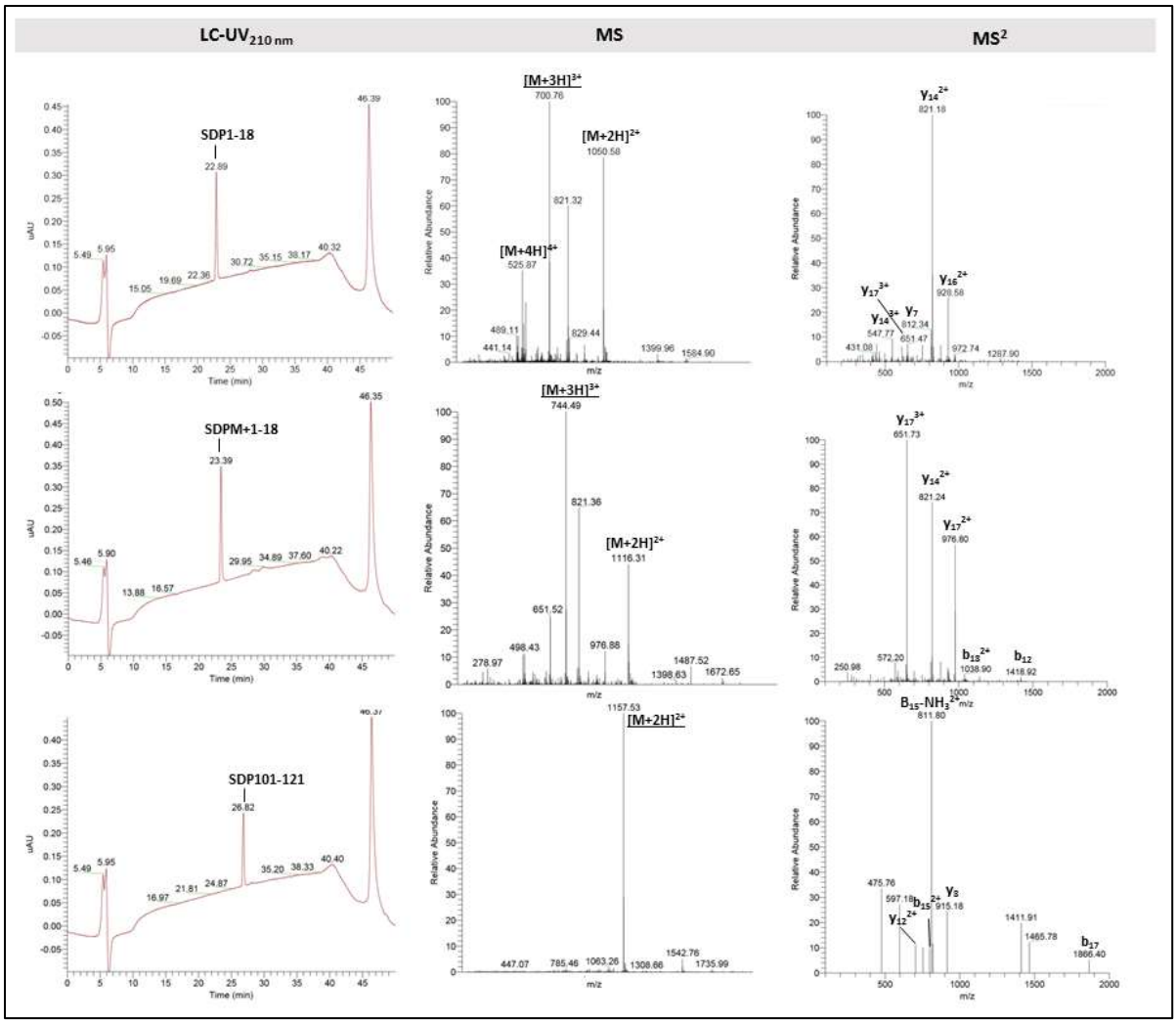


Figure 2: Chemical characterization of the SDPs. Left: LC-UV$_{210\ nm}$ chromatogram, middle: MS spectrum, right: MS² spectrum.

SI 2. Radiolabelling of $SDP_{167-175}$ and $SDP_{101-121}$.

1. Introduction

$SDP_{167-175}$ and $SDP_{101-121}$ were radiolabelled in order to evaluate their tissue distribution in brain, kidney, heart, serum, spleen, lungs, and liver. A description of the materials, methods, pharmacokinetic elimination results and compartmental distribution in the brain is given below.

2. Materials and methods

The radiolabelling of $SDP_{101-121}$ was performed according to the chloramine T (CAT) method: 20 µL of a 0.56 mg/mL NaI solution is added to 50 µL of a 1 mM peptide $SDP_{101-121}$ solution, 17 µL of $Na^{125}I$ (= 1 mCi), and 30 µL of CAT solution (1 mg/mL). 40 seconds incubation was stopped by addition of 30 µL of a 2 mg/mL $Na_2S_2O_5$ solution. The radiolabelling of $SDP_{167-175}$ was performed according the Bolton Hunter (BH) method: 10 µL of a 4.5 mg/mL NaI solution is added to 15 µL of BH solution, 17 µL of $Na^{125}I$ and 15 µL of CAT solution (4 mg/mL in 25 mM phosphate buffer pH 7.5). After 60 seconds, the reaction was stopped by addition of 15 µL of a 8 mg/mL $Na_2S_2O_5$ solution. Then 50 µL of a 1 mM $SDP_{167-175}$ peptide solution is added to the I-labeled BH and the mixture is incubated overnight at room temperature. The mono-iodinated peptide fractions were isolated on a radio-HPLC. The radio-HPLC apparatus consisted of a LaChrom Elite L-2130 pump with degasser (flow rate: 1 mL/min), a LaChrom L-2300 column oven (40°C), a LaChrom Elite L-2400 UV-detector set (215 nm) (all Hitachi Tokyo, Japan), a Theodyne 7725i manual injector (Theodyne, Rohnert Park, CA, USA), a Berthold LB500 HERM radioactivity detector (Berthold Technologies, BadWilbad, Germany). For separation, a Vydac Everest C18 (250 x 4.6 mm, 5 µm particle size) column (Grace, Lokeren, Belgium) was coupled to the HPLC system. Mixtures of 95/5 (V/V) H_2O/ACN supplemented with 0.1% (V/V) TFA (A) and 5/95 (V/V) H_2O/ACN supplemented with 0.1% (V/V) TFA (B) were used as mobile phases. The mono-iodinated peptide fractions were isolated and concentrated by nitrogen drying. The appropriate peptide concentrations (i.e. 30 000 cpm/µL for multiple-time regression analysis (MTR) and 10 000 cpm/µL for capillary depletion (CD)) were prepared using Lactated Ringer's solution containing 1% of BSA (LR/BSA). The labelling of the negative control BSA and positive control dermorphin is described in (Werle and Bernkop-Schnürch 2006).

3. Results

Figure 3 shows the radioactivity (corrected for injected dose) versus time. The activity in serum decreased in function of time designating the tissue uptake or elimination of the ^{125}I-labeled peptides. An estimation of the elimination rate constant was made, based on a one compartment model (***Table 2***). $SDP_{167/175}$ showed no elimination, whereas $SDP_{101-121}$ has an estimated serum half-life of 5.2 min.

Table 2: Overview of the pharmacokinetic elimination results.

Peptide	**$SDP_{167-175}$**	**$SDP_{101-121}$**
k_e^a (min^{-1})	0.026	0.134
$T_{1/2}$ (min)	26.670	5.161

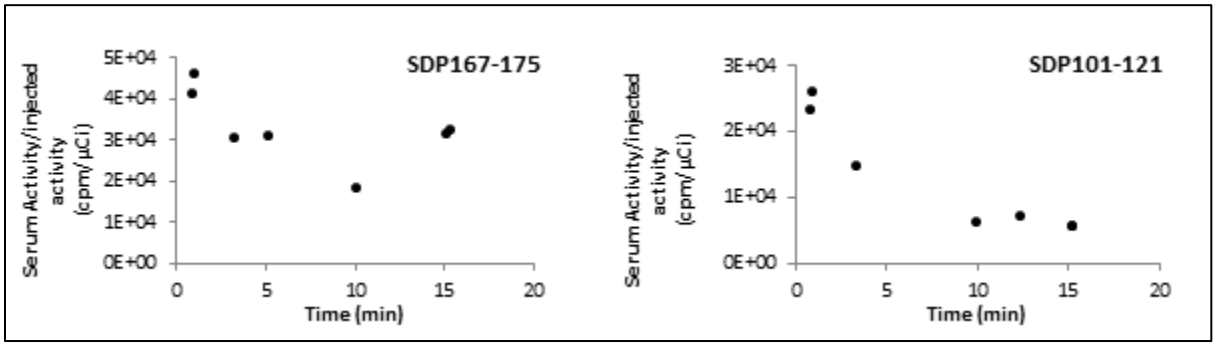


Figure 3: ^{125}I-serum levels during multiple time regression (left: linear y-scale; right: ln y-scale).

Figure 4 visualizes the distribution of $SDP_{167-175}$ and $SDP_{101-121}$ in the brain. The capillary depletion study (at 10 min after injection) indicated a clear influx into the brain parenchyma: 82% for $SDP_{167-175}$ and 87% for $SDP_{101-121}$. The *in vitro* metabolic study demonstrated a long stability in the brain for these peptides. Also in serum, the peptides are stable during the time of the BBB experiment. Moreover, the amount of $SDP_{101-121}$ entering the brain in a time span of 10 minutes is 10 times higher compared to $SDP_{167-175}$.

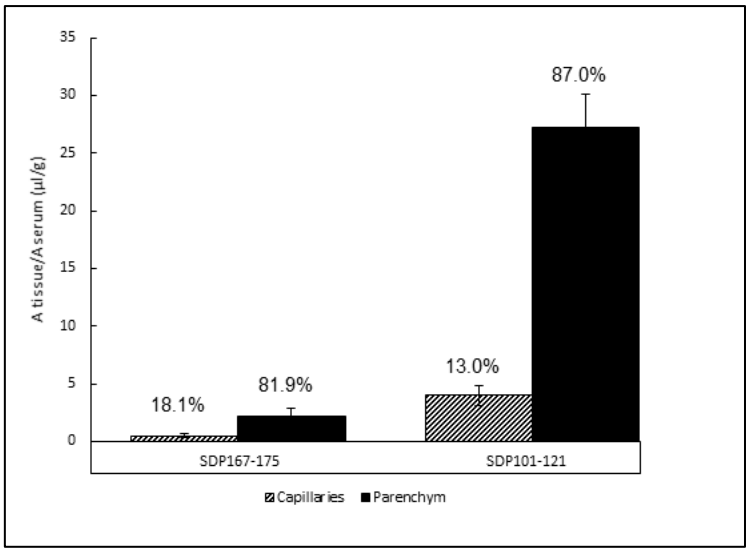


Figure 4: Compartmental distribution of $SDP_{167-175}$ and $SDP_{101-121}$ normalized to brain weight (mean absolute amounts in µL/g ± SEM, n = 2) at 10 minutes post injection.

SI 3. Metabolisation of the SDPs.

Table 3 shows chemical stability (*i.e.* recovery of ≥ 90%) calculated with equation 1 of all peptides during a time period of 2 hours in buffer except for peptide $SDP_{165\text{-}177}$ which recovery was only 87%. Because no degradation peaks were detected above the reporting threshold (0.5%), also $SDP_{165\text{-}177}$ was considered to be chemical stable as well.

$$\%\, recovery = \frac{area\ reference\ t_{165\ min}}{area\ sample\ t_{0\ min}} * 100 \qquad (1)$$

Table 3: Chemical stability results.

Peptide	Recovery (%)	Degradation peaks observed
$SDP_{39\text{-}70}$	93.14	No
$SDP_{167\text{-}175}$	102.70	No
$SDP_{165\text{-}177}$	87.08	No
$SDP_{1\text{-}18}$	106.30	No
$SDP_{M+1\text{-}18}$	110.17	No
$SDP_{101\text{-}121}$	101.83	No

SI 4. Surface Acoustic Wave.

In an additional experiment, the binding characteristics of the SDPs towards the hGH were evaluated by surface acoustic wave (SAW). The results are given in the section below. Two SDPs, *i.e.* $SDP_{39\text{-}70}$ and $SDP_{165\text{-}177}$ showed an significant interaction with the antibody.

1. Materials and methods

SAW experiments were performed with a SAM®5 BLUE from SAW Instruments GmbH (NanoTemper Technologies, Bonn, Germany). Sensor chips, covered with a thin layer of gold, were also purchased at SAW Instruments GmbH.

The golden sensor chips were chemically modified with carboxymethylated dextran hydrogel, as described by Bracke *et al.* (Bracke et al. 2015). Ligand (*i.e.* peptide) immobilization was done in an offline approach. The carboxylated dextran layer was activated by adding 25 µL of 1:1 N-ethyl-N'-(3-dimethylaminopropyl)carbidiimide/N-hydroxysuccinimide (EDC/NHS) solution in all channels. After 10 minutes, the solution was aspirated and each channel was washed three times with 25 µL water. An amount (depending on the MW) of SDPs was weighted and solved in 100 µL HBS to obtain a concentration of 1 mg/mL. From this solution, a peptide solution with a final concentration of 100 µg/mL in sodium acetate was made and put on the chip channel. The ligand was immobilized for 45 min. Five minutes before aspirating the ligand solutions, 3.5 µL of a 10% trehalose solution was added to each channel. Next, the ligand solutions were aspirated and the surface was washed for 3 times with a 1% trehalose solution. The immobilization was terminated by adding 25 µL of the ethanolamine solution. After 5 minutes, 3.5 µL of the 10% trehalose solution was added in each channel for 5 more minutes to protect the ligand. Finally, the trehalose capping solution was aspirated and each chamber was washed again with 1% trehalose solution. The chip was dried with N_2 in the coating device before insertion into the biosensor. The specific binding of the analyte (*i.e.* hGHAb) to the peptide-immobilized surface was studied with a 50 nM hGHAb injection and compared to a 50 nM bovine serum albumin (BSA) injection. The non-specific binding (NSB%) of the interactions was expressed as a ratio of the response of BSA versus hGHAb analyte to the peptide-immobilized surface:

$$NSB_{\%} = {}^{\varphi_{BSA}}/_{\varphi_{analyte}} \times 100 \qquad (2)$$

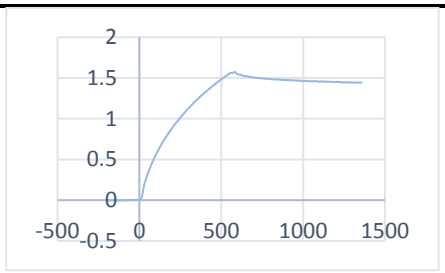
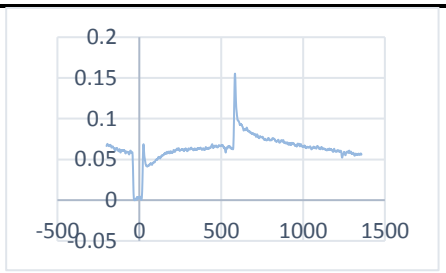
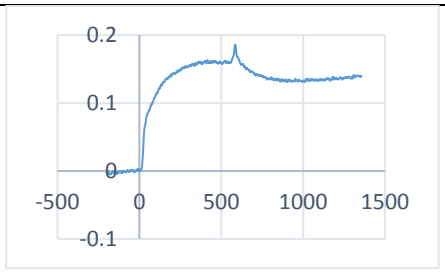
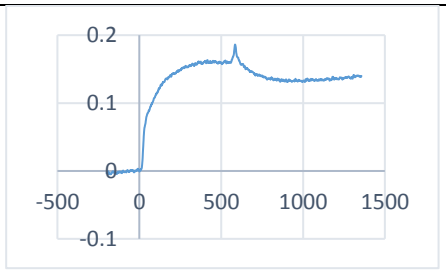
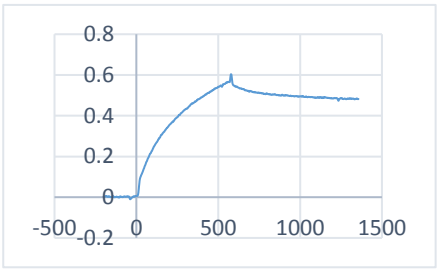
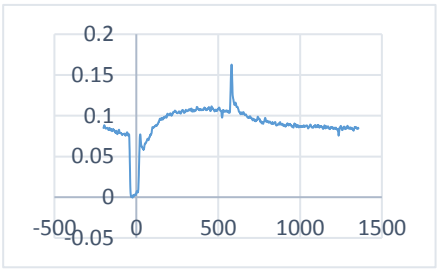
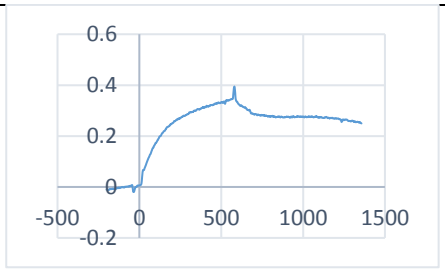
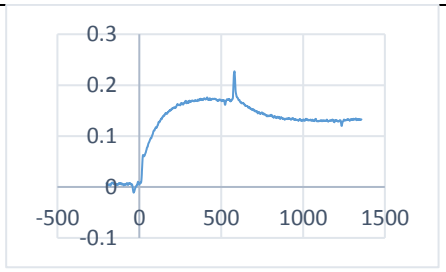
Detection of phase shifts were taken at -200 s prior to analyte injection and at 500 s post analyte injection. Low NSB indicates a specific binding between ligand (*i.e.* SDP) and analyte (*i.e.* hGHAb).

2. Results

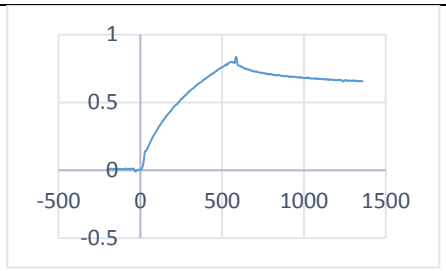
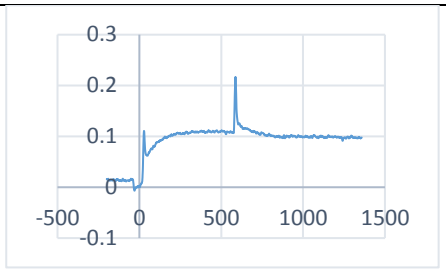
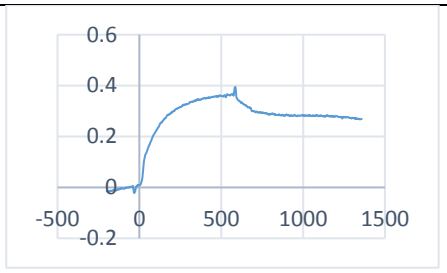
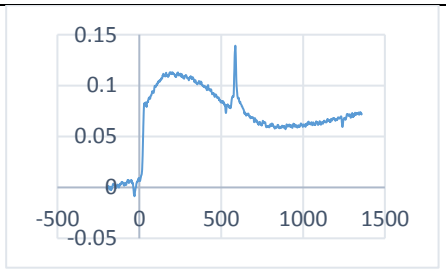
Table 4 represents the phase shift detected after hGHAb and after BSA (representing the non-specific binding) injection.

$SDP_{39\text{-}70}$ showed a remarkably low non-specific binding (0.18%) meaning that the phase shift is due to specific binding of the peptide to hGHAb. Also for $SDP_{165\text{-}177}$, a low non-specific binding of 3.93% was found. Peptides $SDP_{1\text{-}18,M+1\text{-}18,101\text{-}121}$ showed NSB between 10% and 50%, while $SDP_{167\text{-}175}$ showed a very high NSB of 66%.

Table 4: Phase shift after hGHAb- and BSA injection of all SDPs and corresponding %NSB.

Peptide	50 nM hGHAb[1]	50 nM BSA[3]	% NSB
$SDP_{39\text{-}70}$			**<u>0.18</u>**
$SDP_{167\text{-}175}$			66.00
$SDP_{165\text{-}177}$			**<u>3.93</u>**
$SDP_{1\text{-}18}$			47.16

[1] X-axis: time (in seconds), Y-axis: phase shift (in °)

SDP$_{M+1-18}$			12.38
SDP$_{101-121}$			20.68

SI 5. The natural existing fragments of the growth hormone.

Two native occurring hGH fragments as a result of GH catabolism are hypothesized to exist in pituitary and/or serum: fragments 1-43 and 44-191 (***Figure 5***) (Baumann 2009). However, some critics assign them as a results from extraction artifacts in the process of preparation from pituitary gland or serum.

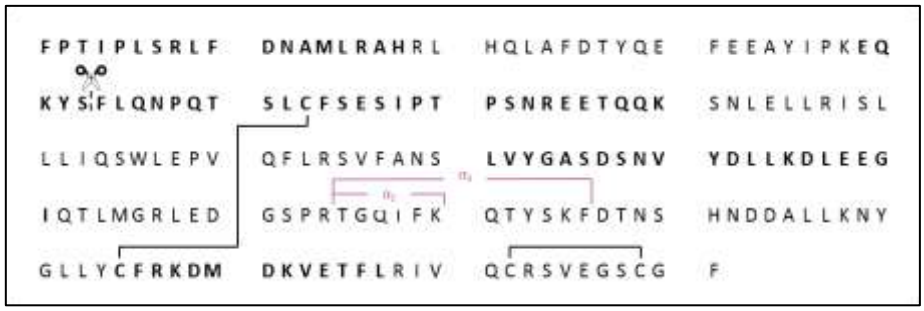


Figure 5: Catabolic site on GH for the generation of the 1-43 and 44-191 fragments, with the somatropin-derived peptides indicated in bold. In purple, the deleted fragments for the generation of the α2 and α3 two-chain forms.

Fragment 1-43:

- 5215.9 Da or 5 kDa fragment
- Isolation from pituitary extracts and detected in serum (Singh et al. 1983; Lopez-Guajardo 1998)
- No binding to hGHR detected (Rowlinson et al. 1996)
- Function: insulin potentiating effect

Fragment 44-191:

- 17 kDa fragment
- Blood circulation and pituitary
- Binding with low affinity to the hGHR
- Function: diabetogenic activity (Lewis, Sinha, and Lewis 2000)

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