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Quantitative capillary electrophoresis assay for the proteolytic stability of luteinizing hormone-releasing hormones

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Abstract

A rapid and simple capillary electrophoresis (CE) assay for measuring the stability of luteinizing hormone-releasing hormone (LHRH) analogues in the presence of intestinal enzymes has been developed and validated. Buffer pH and sample stacking were important factors in controlling resolution and reproducibility. The CE assay for human (h) and salmon LHRH analogues between 0.05 and 0.25 m*M* was linear for peak height versus concentration (r^2>0.99). Analysis of hLHRH at 0.1 m*M* had an intra-day relative standard deviation of 1.25% and an inter-day relative standard deviation of 5.0%. The method was applied to the stability of LHRH analogues in salmon intestinal digests.

Keywords: Proteolysis; Stability; Luteinizing hormone-releasing hormones

1. Introduction

Analogues of LHRH are used for the treatment of prostate and breast cancer [1–3] and for the control of animal reproduction [4]. The oral route is considered to be the most convenient route for the delivery of drugs, however the proteolytic activity of the gastrointestinal tract is a major barrier to successful oral delivery of peptides. Orally delivered peptides can be hydrolysed by the pancreatic endopeptidases: chymotrypsin, trypsin and elastase and by the large number of endo- and exopeptidases associated with the intestinal brush-border [5]. There are several high-performance liquid chromatographic (HPLC) assays for monitoring the metabolic stability of LHRH analogues in vitro [6–8]. However, analysis by HPLC is time consuming, as separation requires gradient elution followed by re-equilibration of the column; furthermore HPLC analysis requires the purchase and disposal of organic solvents.

Capillary electrophoresis (CE) offers a number of potential benefits including, high resolving power, inexpensive materials, small sample volumes and rapid analysis times [9]. CE has been applied in many areas of peptide analysis [10] and has been shown to be a powerful tool for resolving peptides [11]. However, there are few reports in the literature that describe the use of CE for monitoring the proteolytic stability of peptides in vitro. Huang and Wu [12] used CE to monitor the degradation of thyrotropin releasing hormone and insulin during

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iontophoretic transdermal delivery in vitro but they did not report the CE assay conditions or assay reproducibility. The lack of reports using CE for studying the proteolytic stability of peptides is in part due to problems associated with the analysis of peptides in aqueous solutions containing proteins and electrolytes. Proteins and peptides can interact with the negatively charged surface of the capillary wall, resulting in peak broadening and changes in the electroosmotic flow (EOF) [13]. Changes to the EOF have been shown to dramatically reduce the reproducibility of an assay system [13]. Electrolytes present in the sample can result in uneven migration and zone spreading due to poor sample stacking [14]. Sample stacking causes the sample to be concentrated into a smaller zone, which then migrates along the capillary under the electrophoresis conditions of the run buffer [15]. Sample stacking requires significantly lower concentrations of electrolytes in the sample relative to the run buffer. When an electrical field is applied under such conditions a proportionally greater field will develop across the sample zone, causing the ions to migrate faster. Once the ions reach the run buffer boundary the electrical field decreases and they migrate slower, thereby concentrating the sample into a small zone [15].

There are various approaches by which interaction with the capillary wall and poor sample stacking may be overcome. High-ionic-strength buffers suppress the ion-exchange effects between analytes and the capillary surface [16]. Furthermore the use of high ionic strength buffers can also increase the conductivity difference between the sample buffer and the capillary run buffer resulting in more efficient sample stacking [15]. The cost of using high-ionic-strength buffers is longer analysis times due to suppressed EOF and Joule heating [17]. Joule heating causes a temperature gradient within the capillary, creating greater electrophoretic mobilities at the centre of the capillary resulting in band broadening, lower theoretical plates and reduced reproducibilities.

Using electrophoretic buffers at low pH (2–3) can also reduce capillary surface interaction [11]. At low pH the silanol groups of the fused-silica are essentially protonated and uncharged, thereby reducing the ionic interaction between the positively charged peptides and capillary. Again the cost is the reduction in EOF, which increases the analysis time.

For our studies on the metabolic stability of human (hLHRH) and salmon (sLHRH) analogues, we wished to develop an assay for these peptides in biological extracts. Human LHRH is composed of 10 amino acids, pyroGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2, while salmon sLHRH differs by two amino substitutions, Trp^7 and Leu^8.

We report the development of a quick, simple CE assay to measure the proteolytic stability of the human and salmon LHRH analogues in the presence of α-chymotrypsin and intestinal homogenates from salmon. Sample stacking and buffer pH were shown to be important for reproducibility and efficiency of the CE assay.

2. Experimental

2.1. Materials

Human and salmon luteinizing hormone-releasing hormones were purchased from Bachem (CA, USA). Bovine serum albumin fraction V was purchased from Serva (Feinbiochemica, Heidelberg, Germany). All other chemicals were analytical grade purchased from either Ajax (Auburn, Australia) or BDH (Poole, UK) unless otherwise specified. Dimethylchlorosilane was obtained from Pierce (Rockford, IL, USA). Light mineral oil with a specific gravity of 0.875–0.885 at 25 °C and α-chymotrypsin (EC 3.4.21.1, TLCK treated Type VII) were purchased from Sigma (St. Louis, MO, USA). Deionised–distilled water was produced with a Millipore Milli-Q Reagent Water System (Continental Water Systems, USA). Micro-vials C.E.S. (0.5 ml) were purchased from Dionex (Sunnyvale, CA, USA). These were silanised as follows; Dionex sample vials were placed in a beaker, a 5% (v/v) solution of dimethylchlorosilane in heptane was added to the beaker and the beaker placed in a desiccator. The desiccator was evacuated until the dimethylchlorosilane had evaporated (1–2 h). The sample vials were rinsed once with methanol, several times with deionised water then dried in an oven at 60 °C. Fused-silica capillary tubing (50 μm I.D.×360 μm O.D.) was purchased from Supelco (Bellefonte, PA, USA). Nylon filter

membranes (0.45 μm) were purchased from Alltech (Auckland, New Zealand).

2.2. Methods

2.2.1. Instrumentation and standard conditions

The CE system was a Dionex CES I interfaced to a computerized data handling system (Dionex AI-450 Chromatography Automated Software v 3.3.2). Data were collected at the rate of 5 Hz and electrophoresis was carried out towards the cathode. A fused-silica capillary column of 60 cm×50 μm I.D. was prepared and installed as described in the Dionex User's Manual [18]. A cooling jacket covered 33 cm of the capillary. Neslab RTE-111 (NESLAB Instruments, Portsmouth, NH, USA) recirculating bath maintained the temperature of the water in the capillary cooling jacket at 20±0.1 °C. CE was carried out in 10 m*M* phosphate buffers made up by mixing solutions of H_3PO_4 (10 m*M*), NaH_2PO_4 (10 m*M*) and Na_2HPO_4 (10 m*M*) appropriately to give pH values of 2.5, 6.5 and 7.5. For electrophoresis at pH 4.0, 10 m*M* sodium acetate was adjusted to pH 4.0 with glacial acetic acid. NaCl was added to the buffer stocks prior to pH adjustment. All buffers were passed through a 0.45 μm filter before use. Samples were transferred to silanised Dionex microvials (0.5 ml) and covered with approximately 1 mm layer of light mineral oil to prevent evaporation. Sample vials containing the capillary inlet were elevated to 150 mm higher than the destination capillary outlet then lowered to the height of the destination outlet; the loading process took 60 s to complete. Electrophoresis was carried out at constant current by ramping the current from 0 to 60 μA over 1 min and holding the current at 60 μA for the remainder of the run. A current of 60 μA is the maximum recommended current before Joule heating of the run buffer becomes significant [18]. The peptides were detected by a UV detector set at 215 nm. At the start of each day the installed capillary and the source and destination reservoirs were washed with 0.1 *M* NaOH, deionised water and the run buffer. The rinse time at 6 p.s.i. for the capillary was 120 s, the rinse and refill times for the source and destination vial was 6 s (1 p.s.i.=6894.76 Pa). Prior to each run the capillary, source and destination reservoirs were rinsed with the run buffer as described above.

2.2.2. LHRH chymotryptic digests

Chymotryptic digests of human and salmon LHRH were prepared by incubating LHRH analogues (0.2 m*M*) with chymotrypsin at 2.5 μg/ml in 50 m*M* sodium phosphate buffer, pH 8.0 at 15 °C (total volume 250 μl). After 2 min incubation 250 μl of 0.2 *M* HCl was added to the incubate to stop the reaction and the mixture centrifuged at 20 000 *g* for 5 min at room temperature to remove particulates. Samples (50 μl) of the supernatant were transferred to 0.5-ml sample tubes and stored at −20 °C for analysis by CE.

2.2.3. Assay validation

Triplicate standard curves for solutions of hLHRH and sLHRH in 50 m*M* sodium phosphate buffer, pH 8.0 analysed by CE were plotted. For hLHRH three independently prepared stock solutions (1.5, 1.6, 1.6 mg/ml) were diluted with 50 m*M* sodium phosphate buffer, pH 8.0 to give final concentrations of 0.05, 0.07, 0.1 and 0.2 m*M*. Three independently prepared stock solutions of sLHRH (1.3, 1.5, 1.6 mg/ml) were prepared and diluted in 50 m*M* sodium phosphate buffer, pH 8.0 to give final concentrations of 0.06, 0.12, 0.15, 0.2 and 0.25 m*M*. LHRH standards were then diluted with equal volume of 0.2 *M* HCl to give a total volume of 40 μl and centrifuged at 20 000 *g* for 5 min. Supernatants (20 μl) were placed in silanised Dionex sample vials containing 60 μl of deionised water and mixed. The solutions were immediately covered with approximately 1 mm layer light mineral oil to prevent evaporation and analysed by CE. Samples were electrophoresed in 10 m*M* sodium acetate buffer at pH 4.0 containing 75 m*M* NaCl at a constant current of 60 μA. Linear regression analysis was carried out using the computer program Minitab (Minitab10Xtra, Minitab, PA, USA). The intra-day variation of the assay for hLHRH at 0.1 m*M* and was determined by injecting each of the three independently prepared standards six times. Inter-day variation was also determined at 0.1 m*M* by injecting one of the standard solutions a further six times on 2 subsequent days.

2.2.4. Intestinal lumen and mucosal homogenates

Juvenile chinook salmon (*Oncorhynchus tshawytscha*; 234±34 g, $n=6$) were killed with a blow to the head followed by cervical dislocation. The lower intestinal tract of the salmon was removed and the posterior intestine, defined as the section from the ileorectal valve to the anus [19] was ligated and cut. The lumenal contents of the posterior intestine was carefully squeezed into a centrifuge tube (10 ml), the intestinal section was cut longitudinally and the remaining contents were washed into the centrifuge tube with the minimum volume of ice cold 50 m*M* sodium phosphate buffer, pH 8.0. Lumenal contents were diluted with cold 50 m*M* sodium phosphate buffer, pH 8.0 to give a final volume of 5 ml, homogenised (homogeniser; Ystral, Dottingen, Germany) at 4 °C for 2 min and centrifuged at 3000 *g* for 10 min at 4 °C. The supernatants were collected and pooled 100 μl samples were dispensed into 0.5-ml sample tubes then stored at −80 °C. The mucosal surface of the posterior intestine was washed with cold 50 m*M* sodium phosphate buffer, pH 7.4 and gently cleaned with a soft moist tissue. The mucosa were removed from the intestine immediately by scraping off the epithelial cell layers with a microscope cover slip. The scrapings of the posterior intestine were pooled and homogenised in 5 ml of 50 m*M* phosphate buffer, pH 7.4 at 4 °C as above. The homogenate was centrifuged at 3000 *g* for 10 min at 4 °C to remove cellular and nuclear debris. Samples of the supernatant (100 μl) were transferred to 0.5-ml sample tubes and stored at −80 °C. The protein concentration of the supernatants was determined using the method of Lowry as modified by Peterson [20] using bovine serum albumin as the standard.

2.2.5. Degradation of LHRH analogues

Lumenal and mucosal homogenates (1.3 and 12.5 μg of protein, respectively) diluted in 50 m*M* sodium phosphate buffer at pH 8.0 and 7.4, respectively (total volume 200 μl) were incubated at 15 °C for 10 min. To start the reaction, 50 μl of LHRH (1 m*M*) was added and at predetermined times, 30 μl samples were withdrawn from the incubates and mixed with 30 μl of 0.2 *M* HCl to precipitate tissue proteins and terminate the reaction. The resulting mixture was centrifuged at 20 000 *g* for 10 min at 4 °C to remove precipitated protein and particulates and 40 μl of the supernatant was stored at −80 °C for analysis by CE as specified in Section 2.2.3.

3. Results

Sample stacking required the run buffer to have a greater conductivity than the sample buffer. Electrophoresis was carried out in 10 m*M* sodium phosphate, pH 2.5 containing 25, 50, 75 and 100 m*M* NaCl to investigate the effect of run buffer ionic strength on peak shape and resolution. When human and salmon LHRH chymotryptic digests were electrophoresed in the run buffer at the different NaCl concentrations they migrated as non-symmetrical peaks (data not shown). This was presumably due to poor sample stacking due to the small difference in ionic strength between the sample and run buffer. Increasing the ionic strength of the run buffer resulted in longer migration times of the analytes. At the highest ionic strength (100 m*M* NaCl) the hLHRH and sLHRH analogues eluted after 32.0 and 46.5 min, respectively. These elution times were considered too long for our purposes.

To increase the ionic strength difference between the sample and run buffer, LHRH chymotryptic digests were diluted 2.5-, 4- and 6-fold in deionised water. When the LHRH digests were diluted sixfold in deionised water and electrophoresed in run buffer containing 25 m*M* NaCl there was insufficient stacking of the analyte bands for elution as symmetrical peaks (data not shown). Peak-shape distortion (asymmetry) also occurred when LHRH digests were diluted 2.5-fold in deionised water and electrophoresed in run buffers containing 50 and 75 m*M* NaCl. However, when digests were diluted fourfold in deionised water and electrophoresed under the same conditions the analytes eluted as symmetrical peaks and the resolution of the peaks was improved.

To determine whether peak height or peak area gave better reproducibility, hLHRH (0.2 m*M*) was injected 12 consecutive times and electrophoresed in 10 m*M* sodium phosphate buffer, pH 2.5 containing 75 m*M* NaCl. The relative standard deviation (RSD) values for peak height and area were 2.5 and 3.0%, respectively. It was concluded that under these

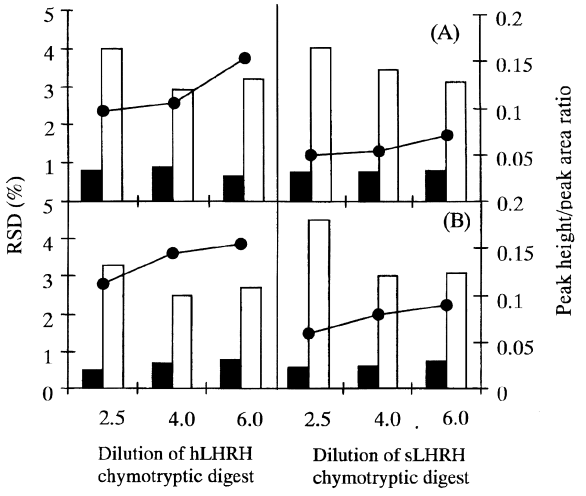

Fig. 1. Effect of diluting chymotryptic LHRH digests in deionised water on RSD (%) of the migration time (closed bars), peak height (open bars) and peak height/peak area ratios (closed circles) when electrophoresed in 10 m*M* sodium phosphate buffer, pH 2.5 containing 50 m*M* NaCl (A) and 75 m*M* NaCl (B) (n=6).

electrophoresis conditions peak height gives better precision than peak area. In the subsequent experiments peak heights only were used.

Diluting the chymotryptic LHRH digests from 2.5 to 4-fold in deionised water improved sample stacking (height/area ratio) and the RSD of peak height when electrophoresed in buffer containing 50 and 75 m*M* NaCl (Fig. 1). However there was little improvement in RSD values when the dilutions of the digests were increased from four- to sixfold (Fig. 1). Stacking was greatest for digests electrophoresed in buffer containing 75 m*M* NaCl.

Under these electrophoresis conditions it would appear that the optimal stacking conditions for resolution and reproducibility require the digests to be diluted fourfold in deionised water and electrophoresed in run buffer containing 75 m*M* NaCl. In subsequent experiments these stacking conditions were used.

The precision of the migration times of neither analogue was affected by the difference in ionic strengths between run and sample buffer; all RSD values were less than 1%. Increasing the NaCl concentration in the run buffer resulted in longer migration times. The retention times of hLHRH and sLHRH electrophoresed in run buffer containing 50 m*M* NaCl were 18 and 23 min, and in 75 m*M* were 23 and 32 min, respectively.

To reduce analysis time, the pH of the run buffer was progressively increased from 2.5 to 4.0, 6.5 and 7.5 to increase the EOF. The LHRH chymotryptic digests in Fig. 2 illustrate this. At pH 2.5, 4.0 and 6.5 both LHRH analogues were resolved from their chymotryptic metabolites, while neither LHRH analogue was resolved from its chymotryptic metabolites at pH 7.5 (data not shown). For both analogues the RSDs of the migration times were less than 1% for CE between pH 2.5 and pH 6.5. The RSDs of the

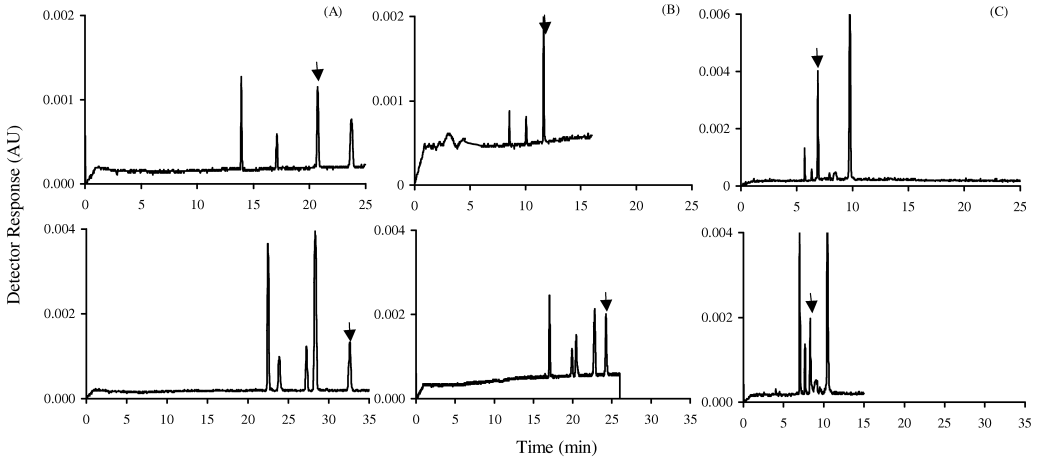

Fig. 2. Capillary electrophoretic separation of chymotryptic digests of hLHRH (top) and sLHRH (bottom) in run buffer at, pH 2.5 (A), 4.0 (B) and 6.5 (C) containing 75 m*M* NaCl. Arrows indicate hLHRH and sLHRH. A, B and C are different chymotryptic digests.

peak heights were ≤3.3% between pH 2.5 and pH 4 but rose to 6% at pH 6.

Under the optimum conditions for sample stacking the optimum pH for reproducibility and separation appeared to be at low pH, either pH 2.5 or 4.0. Since analysis time at pH 4.0 was shorter than at pH 2.5, with similar assay reproducibility, pH 4 was chosen as the optimum pH for analysis of LHRH digests. Under these conditions (Fig. 2), hLHRH and sLHRH migrated at 12.5 and 18.5 min, respectively. Regression analyses of the calibration curves of peak height (PH) versus concentration of hLHRH (C_h) were linear over the concentration range 0.05 to 0.2 m*M* [PH=0.1363 (±0.0035) C_h −77.4 (±9.4), r^2= 0.993, n=12, P<0.0001]. Intra- and inter-day relative standard deviations for the assay were 1.25 and 5.0%, respectively, at 0.1 m*M*. Regression analysis of the calibration curves of peak height versus concentration of sLHRH (C_s) showed that it was linear over the concentration range 0.06 to 0.25 m*M* [PH=0.2283 (±0.0053) C_s −226 (±11), r^2=0.99, n=15, P<0.001].

The optimised CE assay for the analysis of LHRH chymotryptic digests was applied to the analysis of the metabolic stability of the LHRH analogues in the presence of homogenates from the salmon posterior intestine. Fig. 3A shows the capillary electrophoretic separation of hLHRH and sLHRH analogues after incubation with the lumenal contents of the posterior intestine. The rate of the disappearance of the human and salmon LHRH (0.2 m*M*) from electropherograms of the digests followed pseudo-zero kinetics for 2 h with half-lives of 58.4±5.0 and 52±3.6 min (mean±S.D., n=3), respectively. Capillary electropherograms of the posterior mucosal digests of human and salmon LHRH analogues showed the formation of multiple metabolic fragments (Fig. 3B). The degradation of both LHRH (0.2 m*M*) analogues also followed pseudo-zero order kinetics. The half-lives of the human and salmon LHRH analogues in the posterior mucosal homogenate were 111±10 and 101±8 min (mean±S.D., n=3), respectively.

4. Discussion

Analysis of LHRH during proteolytic digestion required analysis to relatively low concentrations of peptide. In order maintain assay sensitivity this required the injection of relatively large samples with a consequent need for sample stacking. Conditions favourable to sample stacking were found to enhance peak shape, resolution and reproducibility. Electrophoresis of LHRH chymotryptic digests in run

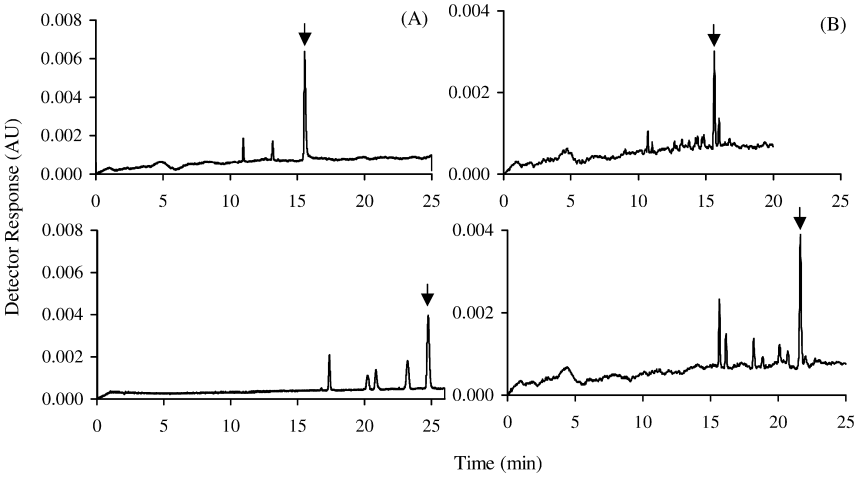

Fig. 3. Capillary electrophoretic separation of a posterior intestinal lumenal digest (A) of hLHRH (top) and sLHRH (bottom) after 120 min incubation and a posterior intestinal mucosal digest (B) of hLHRH (top) and sLHRH (bottom) after 180 min incubation when electrophoresed in 10 m*M* sodium acetate, pH 4.0 containing 75 m*M* NaCl. Arrows indicate hLHRH and sLHRH.

buffer, pH 2.5 containing 25 to 100 m*M* NaCl resulted in peak-shape distortion, illustrating poor sample stacking. To improve sample stacking samples were diluted 2.5-, 4- and 6-fold in deionised water. Run buffer containing 25 m*M* NaCl led to insufficient stacking of the diluted digests for elution of symmetrical peaks. Electrophoresis in buffer containing 50 and 75 m*M* NaCl resulted in the elution of symmetrical peaks after digests were diluted fourfold in deionised water. Digest diluted fourfold in deionised water gave improved resolution and reproducibility (Fig. 1) compared to the digest diluted 2.5-fold.

When LHRH digests were diluted fourfold in deionised water and electrophoresed in run buffer, pH 2.5 containing 75 m*M* NaCl, hLHRH and sLHRH eluted after 23 and 32 min, respectively. The large difference in migration time was expected as the EOF is suppressed at low pH and analytes migrate according to electrophoretic velocity, which is proportional to their charge-mass ratio. The N- and C-termini will not be ionised in either LHRH analogue, they are blocked. At pH 2.5, hLHRH will carry two positive charges due to the ionisation of His^2 and Arg^8, while salmon LHRH will carry a single positively charged His^2. However the effect of the proline residue was far more pronounced than we anticipated. The partition coefficient of hLHRH was higher than we expected, the pK_a of the arginine seems to be lower, and the stability to trypsin higher. Similarly, we found sLHRH to be more resistant to elastase than expected. We plan to publish this and other data elsewhere.

For electrophoresis at pH 2.5, peak height was a more precise measure of concentration than peak area for both hLHRH and sLHRH. This is in agreement with Wätzig [21] who observed that the precision of peak height was often better than for peak area and concluded this was due to peak height being less influenced by migration times and integration errors. The improvement in RSD of peak height when using high ionic strength run buffer was further evidence for the utility of sample stacking (Fig. 1). Dilution of digests in deionised water resulted in greater peak height/area ratios (Fig. 1). The RSD of migration time was less than 1% under all ionic strengths indicating no analyte wall interaction (Fig. 1). This could be attributed to the combination of low pH, high NaCl concentration in the run buffer and the removal of the protein component (chymotrypsin) by acid precipitation prior to analysis.

In an attempt to reduce analysis time the pH of the run buffer was increased; however as the EOF increased the resolution and reproducibility of the assay decreased (Fig. 2). At pH 7.5, human and salmon LHRH analogues were not resolved from their chymotryptic metabolites. It would appear that although the separation time decreased with increasing EOF the resolution of the LHRH from its metabolites also decreased. Similarly, Sutcliffe and Corran [22] observed that for the analysis of neurohypophyseal peptides and analogues, high EOF conditions (pH>6.0) resulted in shorter migration times with reduced resolution. The best separation of neurohypophyseal peptides and analogues was achieved at pH 2.5 [22].

Decreasing the elution time by increasing the pH of the run buffer also decreased the peak heights of the LHRH analogues. This was most likely due to the higher velocities of the analytes passing the detector [13]. The LHRH migration times and peak heights were not as reproducible at pH 6.5 as they were at pH 2.5 and 4.0. This is presumably because at higher pH (>4.0) the capillary surface becomes negatively charged resulting in higher EOF and greater analyte–wall interaction. Adsorption of analytes to the wall surface will alter the surface charge resulting in a change in the EOF [13]. Washing the capillary with acid or alkali between runs could reduce this problem; however this would increase analysis times and since good results were obtained at lower pH (2.5 and 4.0) this was not investigated.

From these results it was concluded that the optimum conditions for stacking and separation of the chymotryptic digests of LHRH analogues require the digest to be diluted fourfold in deionised water and electrophoresed in run buffer of 10 m*M* sodium acetate run buffer at pH 4.0 containing 75 m*M* NaCl. Under these conditions, hLHRH and sLHRH migrated at 12.5 and 18.5 min, respectively.

Under the optimised CE conditions, standard curves for peak heights versus concentrations for hLHRH and sLHRH were linear within the concentration range of 0.05 to 0.2 m*M* and 0.06 to 0.25

m*M*, respectively. Using this assay we were able to show that there was no significant difference ($P > 0.05$) between the rate of digestion of hLHRH and sLHRH in either lumenal or mucosal extracts of chinook salmon.

The results of this work demonstrate the usefulness of CE for following the proteolytic stability of peptides in vitro. The most attractive features of the CE assay are its simplicity and low cost. A single inexpensive capillary is used, the run buffer is aqueous eliminating the purchase and disposal of organic solvents, and the same CE assay can be used for the analysis of both LHRH analogues. We have used this assay to investigate the metabolic stability of human and salmon LHRH analogues to pancreatic endopeptidases [23] and to demonstrate the inhibition of proteolysis by polyacrylates [24]. This assay is currently being used to investigate the metabolic stability of LHRH analogues in the presence of intestinal extracts from salmon.

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