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RESEARCH ARTICLE

Online large volume sample stacking preconcentration and separation of enantiomeric GHRH analogs by capillary electrophoresis

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Abstract

A capillary electrophoresis method is proposed to analyze the four most well-known growth hormone-releasing hormone (GHRH) analogs that are misused by athletes. Dimethyl- β -cyclodextrin used as a chiral selector allowed, for the first time, the separation of those basic peptide analogs, including enantiopeptides (sermorelin and CJC-1293) that differ by the chirality of only one amino acid. To increase the method sensitivity, electrokinetic preconcentration methods have been investigated. The large volume sample stacking with polarity switching (PS-LVSS) method with an injected sample volume corresponding to 80% of the capillary one was found superior to the sweeping in terms of signal enhancement factor (SEF). Acid and organic solvent addition to the sample (0.1 mM phosphoric acid with 30% methanol) led to a twofold signal improvement, when compared to water as a matrix. We increased capillary dimensions to provide a signal enhancement through the injection of a larger sample volume. Finally, using a combination of the optimized PS-LVSS preconcentration with the chiral capillary zone electrophoresis (CZE), the GHRH analogs were separated and limits of detection between 75 and 200 ng/mL were reached. This method was successfully applied to urine after a desalting step. An optimized C18 SPE was used for that purpose in order to provide low sample conductivity ($<130 \mu\text{S}/\text{cm}$) and preserve the efficiency of LVSS preconcentration. SEF of 640 was obtained with desalted urine spiked with sermorelin by comparison to the CZE (without preconcentration) method.

Abbreviations: κ , conductivity; μ_{ep} , electrophoretic mobility; CZE, capillary zone electrophoresis; DEA, diethylamine; EDA, ethylenediamine; EK, electrokinetic; GH, growth hormone; GHRH, growth hormone releasing hormone; L_{eff} , effective length; L_{tot} , total length; LVSS, large volume sample stacking; M-LVSS, multiple-LVSS; MRPL, minimum required performance level; PS-LVSS, LVSS with polarity switching; SEF, signal enhancement factor; TEA, triethylamine; WADA, World Anti-Doping Agency.

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KEYWORDS

capillary electrophoresis, doping, electrokinetic preconcentration, enantioseparation, peptide

1 | INTRODUCTION

Peptide hormones are produced endogenously and regulate many endocrine functions, and growth hormone-releasing hormone (GHRH) is among the most well-known. GHRH stimulates the endogenous secretion of growth hormone (GH), which has an anabolic effect. For therapeutic use, several GHRH analogs have been developed from the bioactive part of GHRH (1–29 GHRH) with amino-acid modifications or the addition of chemical groups, allowing peptide stabilization and a longer half-life time [1, 2]. Due to their biological activity, some of them could also be misused by athletes to improve their performances [3, 4]. Indeed, GH and its releasing factors can induce ergogenic activity and accelerate injury recovery [5]. These two factors motivated the World Anti-Doping Agency (WADA) to introduce those peptides into the prohibited list of compounds for athletes [6]. The four most well-known GHRH analogs are sermorelin, CJC-1293, CJC-1295, and tesamorelin. They are derived from the amino-acid sequence of GHRH, which creates very basic peptides (pI between 10 and 10.4) with close molecular masses (three between 3355 and 3365 Da and one at 5132 Da).

Different techniques can be used to analyze peptide hormones, such as immunoassays [7–12] and separation techniques [13, 14]. Although radioimmunoassays [15, 16] have been used in the past to detect and quantify those peptides, they have been replaced by other assays like enzyme-linked immunosorbent assay [7, 8] as well as chemiluminescence and fluorescence immunoassays [9]. Even though such tests provide good selectivity and allow a picomolar limit of detection (LOD), they nevertheless induce cross-reactivity and cannot allow distinction between analogs [11, 17–19]. Liquid chromatography techniques coupled with mass spectrometry (MS) have therefore been often used to analyze the GHRH analogs [20]. The current method to analyze GHRH analogs for doping detection is a reverse-phase nano-UHPLC coupled to HRMS/MS [21–24]. This method, combined with an immunoaffinity purification step at the forefront, allows a sensitive LOD of 5 pg/mL in urine or 0.2 ng/mL in blood [22]. These LOD measurements satisfy the minimum required performance level (MRPL) defined by WADA of 1 ng/mL [25]. However, two GHRH analogs, sermorelin and CJC-1293, are not separated by this method due to their nearly identical sequence that only differs by the enantiomeric nature of their C-terminal second amino acid (Lys2).

Capillary electrophoresis (CE), possibly coupled to MS, can be a good alternative to these chromatography techniques [26, 27]. In addition to its various possibilities of coupling detection, CE is a high resolution technique, particularly for enantiomers [28]. With the addition of chiral selectors in the background electrolytes (BGE), the literature highlights efficient enantiomer separations primarily for small peptides [29–32]. However, fewer studies investigate large peptides such as the GHRH one (29 AA). Furthermore CE is frequently reported for peptide analysis, doping peptide hormones have rarely been exploited, mostly due to CE's lack of sensitivity [33].

In addition to CE high resolution power, electrokinetic (EK) preconcentration offers a powerful approach for increasing detection sensitivity in CE [34, 35]. Some studies already successfully performed peptide analysis in biological fluids using dynamic techniques based on the variation of velocity analytes. These studies explore different modes of EK preconcentration for peptides, such as field amplified sample stacking methods (FASI) [36–38], transient isotachopheresis [39, 40], dynamic pH junction [41, 42], large volume sample stacking (LVSS) [43–48], and sweeping [49–51]. Sweeping could be advantageous, especially when combined with other EK preconcentrations showing signal improvements up to 200. However, it has been rarely used to preconcentrate peptides, possibly due to a random concentration factor that depends on micelle-analyte affinity. Preconcentration modes based on electric field amplification are the most widely used for peptides. In FASI, the analyte accumulation and stacking are caused by the conductivity difference between the sample zone and the BGE, which leads to a signal enhancement. When electric field amplification occurs, a large sample volume can be injected to increase the signal intensity with no resolution loss [52]. Several studies have compared EK preconcentration techniques of peptides and shown that LVSS allows the best signal enhancement factor (SEF) compared to other electric field amplification-based method, reaching a factor of 100 [44, 46]. However, LVSS requires two main criteria: (i) a high conductivity BGE compared to a low conductivity sample matrix, which may be a problem when dealing with urine or other biological fluids, and (ii) opposite electroosmotic flow and electrophoretic mobilities to be able to extract the sample matrix. During the sample matrix extraction, the analytes get stacked at the frontier between the BGE and the sample. Thus, for small positively charged peptides, some LVSS preconcentrations have been realized with a cationic coating [43, 44]. To

the best of our knowledge, the present polybrene coating LVSS application for large and basic peptides combined with enantioseparation has been explored for the first time.

In this study, we developed a CE-UV method for separating four unresolved GHRH analogs. Different cyclodextrins (CDs) were tested in order to determine the best chiral separation of sermorelin and CJC-1293. We then investigated several online EK preconcentrations in order to improve the detection sensitivity of the method. The chiral CE-UV method combined with the EK preconcentration was then applied to these four analogs in urine after an optimized desalting sample treatment.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

HPLC grade methanol, 1 N sodium hydroxide, 1 N hydrochloric acid, HPLC grade acetonitrile, and citric acid were purchased from VWR (Fontenay-sous-Bois, France). HPLC grade trifluoroacetic acid was obtained from Fisher Scientific (Illkirch Graffenstaden, France). Sodium dodecyl sulfate, lithium hydroxide monohydrate, ortho-phosphoric acid (85%), hexadimethrine bromide (polybrene) (MW: 374 g/mol), formic acid, acetic acid, sodium hydroxide, lithium hydroxide monohydrate, trizma base, ethylenediamine, triethylamine, diethylamine, α -Cyclodextrin, hydroxypropyl- β -Cyclodextrin, methyl- β -Cyclodextrin, heptakis (2,6-di-O-methyl)- β -cyclodextrin, and 6-aminocaproic acid were obtained from Sigma (Saint-Louis, MO, USA). β -Cyclodextrin and γ -Cyclodextrin were purchased from Beckman Instrument (Fullerton, CA, USA). Sulfobutylether- β -CD was obtained from Cyclolab (Budapest, Hungary). Sermorelin and CJC-1293 were purchased from Bachem (Bubendorf, Switzerland). Tesamorelin and CJC-1295 were provided by the French Anti-Doping Laboratory (LADF, Chatenay-Malabry, France).

2.2 | Apparatus and materials

Deionized water used in all experiments was purified using a Direct-Q3 UV purification system (Millipore, Milford, MA, USA). Conductivity and pH values of buffers and solutions were acquired with a SevenCompact pH meter (Mettler Toledo, Schwerzenbach, Switzerland). Selections of BGE and buffer ionic strength (IS) calculations were based on simulations using the computer program Phoebus (Analisis, Suarlée, Belgium). All solutions were filtered through a 0.22 μ m membrane (Millipore, Bedford, MA,

USA) before use except for the BGE, which was filtered before the addition of CDs.

All experiments were performed using an MDQ system (Sciex Separation, Brea, CA, USA) equipped with photodiode array detection (DAD). Data acquisition and instrument control were carried out using Karat 9.1 software (Sciex Separation, Brea, CA, USA). Fused-silica capillaries of 375 μ m od were from Polymicro (TSP075375, CM Scientific, Silsden, UK).

2.3 | Capillary electrophoresis methods

Prior to the first use, the fused-silica capillary (id of 75 μ m, effective length [L_{eff}] of 100 cm, and total length [L_{tot}] of 110 cm) was flushed under 137.9 kPa with the following sequence: MeOH, water, NaOH 1 M, water, HCl 0.1 M, and water for 15 min each. This preconditioning was followed by capillary conditioning for polybrene coating with the following sequence: water for 7 min, 1 M NaOH for 12 min, and water for 7 min under 137.9 kPa. Then, the polybrene coating was made by applying 0.1 M NaOH for 5 min and 0.2% w/v polybrene solution water for 5 min under 137.9 kPa.

For capillary zone electrophoresis (CZE), the BGE was composed of 100 mM lithium phosphate buffer containing 15 mM of heptakis (2,6-di-O-methyl)-Beta-CD (called dimethyl- β -CD) at pH 3.5. After the BGE flushing (20 psi, 7 min), peptide hormones solubilized in water were injected (34.5 kPa for 10 s), and the separation was performed at -30 kV (-70 μ A). The inter-run capillary rinsing and polybrene coating regeneration were carried out with the following sequence: 50 mM sodium dodecyl sulfate (SDS) for 7 min, water for 11 min, 0.1 M NaOH for 5 min, and finally 0.2% w/v polybrene solution water for 5 min under 137.9 kPa.

For LVSS with polarity switching (PS-LVSS-chiral CZE), the same BGE and inter-run capillary rinsing as in CZE were used. The sample diluted with a 0.1 mM phosphoric acid solution containing 30% MeOH (LVSS sample matrix) was hydrodynamically injected to fill 80% of the total capillary volume (68.9 kPa for 81 s). The PS-LVSS-chiral CZE analysis is divided into two steps: the matrix removal and the separation (SI 1). A voltage of 30 kV was applied to electroosmotically pump out the sample matrix toward the injection end, whereas analyte stacking occurred. At a time (called the polarity switching time) considered necessary to remove the maximal matrix volume while keeping the stacked analytes inside the capillary, the normal polarity was automatically switched for separation (-30 kV). The measurement of migration times starts when the polarity is switched. Note that before performing PS-LVSS-chiral CZE analyses, the polarity switching time

was determined by measuring the time needed to extract the maximum of LVSS matrix out of the capillary. To do so, a volume of matrix corresponding to 80% of the capillary volume was introduced into the capillary. The voltage (30 kV) was applied to remove the matrix, this removal was considered complete when the current reached 95% of the current plateau. This time was considered the polarity switching time and was then used in the PS-LVSS-chiral CZE method, making automatic the polarity switching. This polarity switching time was estimated for each newly prepared LVSS sample matrix (0.1 mM phosphoric acid with 30% MeOH).

The temperature of the capillary cartridge was maintained constant at 25°C. Data were collected at 200 nm.

The value of SEF was calculated by comparing the peak height obtained under LVSS conditions with that in the chiral CZE considering the dilution factor.

The Mann–Whitney statistical test at a 95% probability was used to analyze for differences between all BGE and sample matrix comparison for LVSS development.

2.4 | Urine sample preparation method

Urine samples collected from donors were centrifuged at 500 g for 2 min, and the supernatant was acidified to pH 2.6 with trifluoroacetic acid (TFA) before use. Before the PS-LVSS-CZE-UV analysis, acidified urine samples were desalted using the sorbent AttractSPE Disk Spin C18 from Affinisep (Le Houlme, France). SPE disks were first conditioned with acetonitrile and water, 2 mL each, under centrifugation (500 g, 2 min). A volume of 1 mL acidified urine was percolated on the C18 disk, which was then washed with 16 mL of a solution containing ACN/water: 20/80. The elution step was performed with 2 mL of ACN/water: 80/20 containing 0.1% TFA. The freeze-dried eluate, reconstituted in 120 μ L of LVSS sample matrix, was spiked with sermorelin at 1 μ g/mL for PS-LVSS-chiral CZE analysis.

3 | RESULTS AND DISCUSSION

3.1 | Chiral CZE-UV of four GHRH analogs

The first step of our methodology developed a CZE method to separate the four GHRH analogs. As three GHRH-analog peptides exhibit very close molecular masses and all four are highly basic ($pI \approx 10$ – 10.4) (Table 1), we worked at pH <9 to easily tune their (positive) charge in favor of their separation. The preliminary study that we performed focused only on sermorelin and explored different BGE

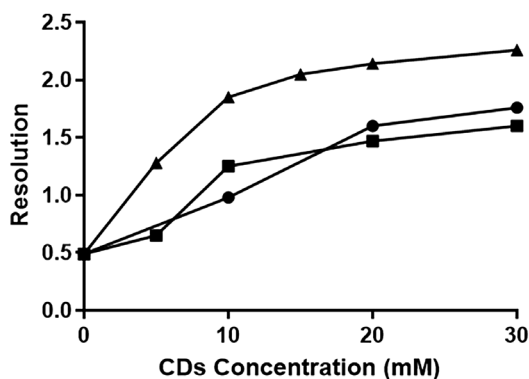
pHs (3.5–9.8). No peak was detected at pH 8.1, whereas the peak broadness at pH 5.2 indicated massive adsorption of the analyte on the capillary wall. To solve this adsorption issue, BGE pH was lowered to 3.5, which reduced electrostatic interactions between the negatively charged wall and positively charged analytes. We then compared lithium acetate, ammonium formate, and lithium phosphate in order to choose the best BGE composition. Because formic and acetic acid-based electrolytes induce lower peak area and intensity, we selected the phosphate-based electrolyte as BGE. We then tested various counterions while keeping the IS constant (60 mM), such as classical counterions with different sizes (Na^+ , Li^+ , and Tris) and alkylamines expected to provide a dynamic capillary coating (ethylenediamine [EDA], triethylamine [TEA], and diethylamine [DEA]). Several parameters (peak asymmetry, relative standard deviations [RSDs] for peak area, and migration time) were used to choose the best counterion. Contrary to our expectations, the dynamic coating reagent did not reduce the peptide's adsorption. Indeed, EDA showed a high peak asymmetry (2.5), TEA showed a high RSD for peak area (5.6%), and DEA showed an unacceptable RSD for migration time (11.5%). Among all counterions tested, Tris led to the best peak asymmetry but lower intensity. Balancing all criteria, we finally chose BGE, the phosphoric acid with LiOH as the counterion.

Sermorelin and CJC-1293 differ only in the second amino-acid (alanine) enantiomery. Sermorelin contains the L and CJC-1293, the D form. This little difference in their peptide chain makes them difficult to separate. We first tested various acidic pHs (from 2.5 to 4) for the lithium phosphate BGE. We observed a resolution improvement from pH 2.5 to 3.5 (0.56 to 0.86), attaining a plateau from 3.5 to 4. We selected pH 3.5, which exhibits a higher buffer capacity for the development of the method.

To increase the resolution, we added CDs into the BGE, as CDs and their derivatives have previously been established as major chiral selectors in CE [28, 54]. We compared native CDs (α , β , and γ) at various concentrations to select the most suitable CD cavity size at the optimal concentration for separating the two enantiomeric peptides. A better separation was observed with 10 mM β -CD than with α - or γ -CDs, even at 20 mM. Enantiomeric separation is possible by forming an inclusion complex that allows interaction with chiral centers at the CD rim. Because the enantiomeric alanine is at the second position of the amino-acid sequence, the Tyr1 has to enter the cavity to allow the Ala2 recognition. The para-substituted aromatic ring Tyr1 fits better to a β -CD explaining the better resolution. However, the enantio-separation was not satisfactory. Different alkylated β -CDs as dimethyl- β -CD, methyl- β -CD, and hydroxypropyl- β -CD were then studied. As shown in Figure 1, dimethyl- β -CD allowed better resolutions than

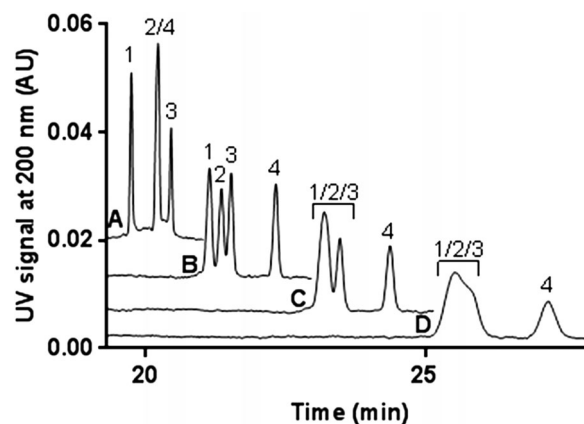
TABLE 1 Characteristics of growth hormone–releasing hormone (GHRH) analogs.

Name	Amino-acid sequence [22]	Monoisotopic mass (Da) [22]	pI [53]
Sermorelin	YADAIFTNSY RKVLGQLSAR KLLQDIMSR-NH ₂	3355.8	9.99
CJC-1293	YdADAIFTNSY RKVLGQLSAR KLLQDIMSR-NH ₂	3355.8	9.99
CJC-1295	YdADAIFTQSYRKVLAQLSAR KLLQDILSR-NH ₂	3365.9	10.4
Tesamorelin	C6H9O-YADAIFTNS YRKVLGQLSA RLLQDIMSR QQGESNQERG ARARL	5132.7	10.5

**FIGURE 1** Effect of the concentration of hydroxypropyl-β-CD (●), methyl β-CD (■), and dimethyl-β-CD (▲) on the resolution between sermorelin and CJC-1293. Conditions: bare fused-silica capillary of 60 cm × 50 mm id (50 cm as L_{eff}); injection for 10 s at 34.5 kPa; 100 mM lithium phosphate at pH 3.5; 200 nm; 30 kV; 25°C. Sample concentration of 0.1 mg/mL.

the two others, even at low concentrations in the BGE. According to Chankvetadze [55], methyl group substitution makes the β-CD cavity more hydrophobic, increases the CD cavity depth, and renders it more flexible by reducing the hydrogen bond among hydroxyl groups at the CD rim. Those modifications facilitate the peptide inclusion and, by extension, the differentiation between D and L-alanine of the two peptides is improved after the inclusion of the Tyr1 in the cavity. Using a 15 mM dimethyl-β-CD, we successfully developed for the first time a chiral CE method to separate Sermorelin and CJC-1293 with a resolution of 2.

We also studied the effect of anionic β-CD on this enantio-separation using sulfobutylether-β-CD. Identical CE profiles were obtained for each of the enantiomers with two peaks of equal sensitivity and migration times (0.6 and 1.1 min) observed for each of them. This anionic β-CD thus did not allow the distinction of the GHRH enantiomers. This lack of distinction was possibly caused by the positively charged peptides interacting with the negatively charged CDs' surfaces by electrostatic interactions instead of inclusion. In this case, two peptide/CD complexes could be formed, one with a stoichiometry of 1:1 and another with 2:1, which could then lead to the two distinct peaks observed for each enantiomers profile.

**FIGURE 2** Separation of 4 growth hormone–releasing hormone (GHRH) analogs by chiral capillary zone electrophoresis (CZE) according to the dimethyl-β-CD concentration in the background electrolytes (BGE). All conditions as in Figure 1 except for the dimethyl-β-CD concentration of (A) 0 mM, (B) 15 mM, (C) 20 mM, and (D) 30 mM. GHRH analogs: 1 = tesamorelin, 2 = CJC-1293, 3 = CJC-1295, and 4 = sermorelin.

Finally, dimethyl-β-CD was considered the best chiral selector to separate sermorelin and CJC-1293. Three dimethyl-β-CD concentrations (15, 20, and 30 mM) that allowed a satisfactory resolution (>2) between these enantiomers were then tested for the separation of the four GHRH analogs. Over 15 mM, the resolution between tesamorelin, CJC-1295, and CJC-1293 was altered with co-migrations (Figure 2C,D). Using 15 mM dimethyl-β-CD, the separation of the four GHRH analogs was achieved (Figure 2B). The optimal BGE for separating the four GHRH analogs was 100 mM lithium phosphate BGE containing 15 mM dimethyl-β-CD (Figure 2B).

3.2 | Electrokinetic preconcentration of GHRH analogs

The developed chiral CE method allowed the separation of the four GHRH peptides, but with a LOD of 0.01 mg/mL, which is too high in comparison with the 1 ng/mL MRPL defined by WADA. The small amount of injected sample combined with the small optical pathlength leads to limited LODs. To increase the detection sensitivity, we

evaluated two online EK preconcentration techniques as sweeping and LVSS, taking advantage of the large injected sample volumes up to 100%.

3.2.1 | Preconcentration using a sweeping method

Sweeping is based on the modification of analyte electrophoretic mobilities via their affinity toward a pseudo-stationary phase. Sweeping is performed by supplementing additives (as surfactants, microemulsions, and polymers) into the BGE to form this pseudo-stationary phase. By applying a voltage, the additives cross over the sample zone and sweep analytes, narrowing the analytes band and therefore allowing their preconcentration [56, 57]. Sweeping also presents the advantage of not requiring sample desalting and being applicable to samples exhibiting high conductivity, such as urine and blood.

The sweeping of GHRH peptides was carried out using SDS as additives as it is frequently used for sweeping [49]. For the method development, a large sample volume of CJC-1293 solution was injected, which corresponded to 30% of the capillary volume. Two configurations of SDS addition were first investigated. In comparison to that observed by adding 25 mM SDS into the BGE (100 mM lithium phosphate, pH = 3.5), the set-up with a plug composed of SDS in BGE that was immediately introduced after the sample injection (Figure 3A) allowed a better peak intensity (data not shown). Different parameters were then optimized, such as the SDS concentration in the plug (5–35 mM) and the SDS plug size (1%–10% of the total capillary volume). The peak height of CJC-1293 peptide was used to evaluate the preconcentration efficiency.

Figure 4A shows the optimum SDS concentration at 15 mM, when keeping constant the SDS plug size at 5% of the capillary volume. The peak height increases when the plug size is up to 5% of the capillary volume. A lower peak height was observed when the plug size was increased and the repeatability was improved. Because the highest peak height is the most important criteria for a good sweeping preconcentration of GHRH peptides, we selected a SDS plug size of 5% (15 mM SDS in BGE). Under these conditions, we obtained an acceptable SEF of 30 for CJC-1293.

Unfortunately, applying this method to a mixture of sermorelin and CJC-1293 failed to separate the enantiomers even when adding dimethyl- β -CD to the BGE, the sample, or both. The affinity between GHRH peptides and SDS micelles probably prevents the peptide from entering the CDs' cavity. A combination of enantioseparation with this sweeping preconcentration was thus impossible.

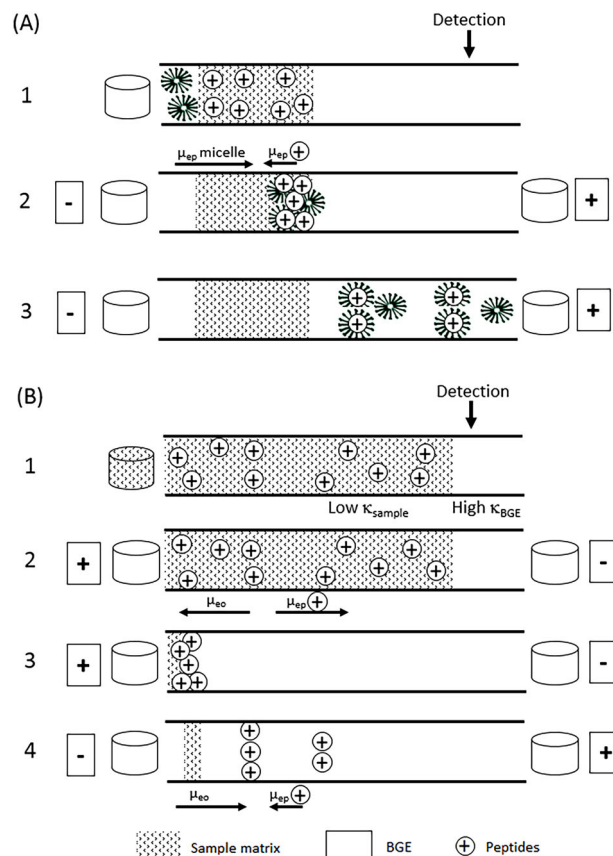


FIGURE 3 Principle of the used electrokinetic (EK) preconcentration techniques to separate positively charged peptides. (A) Several steps of the sweeping carried out in a silica capillary using acidic background electrolytes (BGE): (1) hydrodynamic injection of the sample (30% of the capillary volume) followed by a sodium dodecyl sulfate (SDS) plug; the BGE used as sample matrix ensures the only impact of the sweeping mechanism, (2) the voltage application induces the migration of SDS micelles through the sample zone to sweep the peptides in a narrow band, and (3) the strong affinity micelles/peptides combined to the high micelle μ_{ep} lead to the peptide migration toward the detector at the cathode. (B) Different steps of the large volume sample stacking with polarity switching (PS-LVSS) performed in a PB coated capillary: (1) hydrodynamic injection of a large sample volume (80% of the capillary volume); the sample matrix and BGE respectively exhibiting low and high conductivity, (2) the voltage application generates a reverse electroosmotic flow (EOF) extracting the sample matrix toward the inlet, whereas peptides were concentrated at the sample/BGE boundary due to the decrease of the peptide velocity induced by the high conductivity difference, (3) the matrix removal is complete, whereas peptides are kept inside the capillary, and (4) the voltage was switched from the normal to reverse polarity, initiating the peptide separation.

3.2.2 | Preconcentration using a PS-LVSS method

PS-LVSS is an EK preconcentration method compatible with injection of a very large sample volume. The analyte

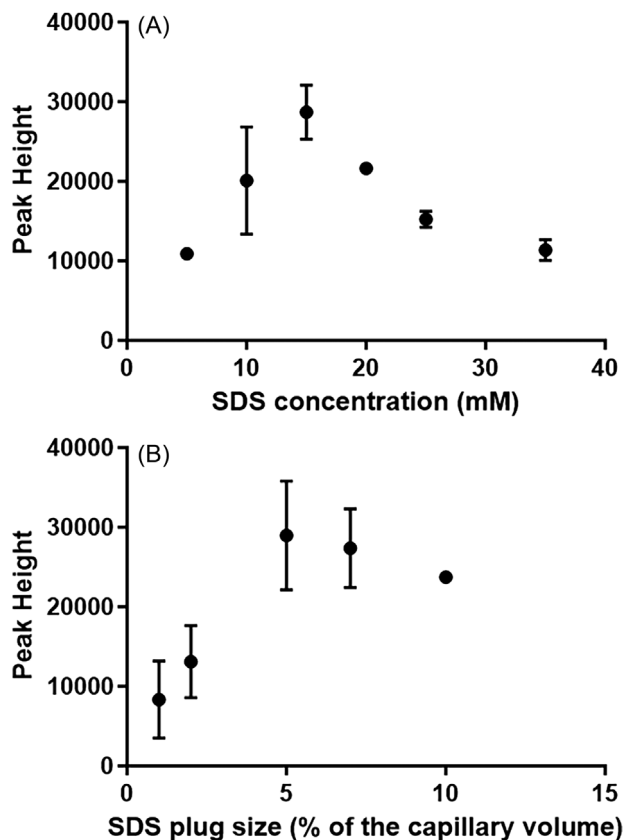


FIGURE 4 Influence of the sodium dodecyl sulfate (SDS) concentration in the plug kept at a constant size (5% of the capillary volume) (A) and the SDS plug size at a constant SDS concentration of 15 mM (B) on the CJC-1293 peak height. Data shown as mean \pm standard deviation of triplicate analyses. Conditions as in Figure 1 except for the hydrodynamic injection (3 psi, 68 s) of CJC-1293 at 10 μ g/mL.

preconcentration is based on a field amplification process that occurs when a conductivity (κ) difference between the sample (low κ) and BGE (high κ) zones happens. This difference induces a decrease of the analyte velocity at the sample/BGE boundary, creating the analyte stacking. The large injected sample volume is possible via the sample matrix removal process thanks to the electroosmotic flow (EOF) moving toward the inlet. When most of the matrix is eliminated, the polarity is switched, and the CZE separation consequently starts (Figure 3B).

The stacking combined with the matrix removal is possible when the EOF and the analyte electrophoretic mobilities are similar in intensity but in opposite direction. The EOF must be high enough to pump the matrix out of the capillary but sufficiently low to let the stacking occurs. Using a fused-silica capillary and the acidic BGE optimized for chiral separation creates a cathodic EOF that is too low to extract the sample matrix, preventing the PS-LVSS process from happening. Moreover, the EOF must be opposite to the peptide electrophoretic mobilities (μ_{ep}) to remove

the sample matrix during PS-LVSS process requiring the use of a cationic coating. To facilitate its implementation in anti-doping laboratories, an easy-to-use and reproducible non-covalent coating was selected. Based on our previous study [58], a polybrene coating was used to reverse the EOF and also avoid peptide adsorption.

To improve the efficacy of the PS-LVSS process, we first optimized the conductivity difference between the BGE and the sample matrix, which maximized the CJC-1293 stacking. We then varied the IS of the selected BGE (lithium phosphate pH 3.5). To observe the impact of the IS variation, the peptide was dissolved in deionized water as low conductivity matrix and injected at 80% of capillary. The obtained SEF for the 50 mM BGE was significantly lower than that obtained for 100 and 150 mM BGE (Figure 5A). To avoid the Joule heating effect, 100 mM BGE at pH 3.5 was chosen, which led to a SEF of 61.

We then investigated the impact of sample acidification (by adding phosphoric or acetic acid) on the preconcentration efficiency. Even if acidification slightly increased the conductivity of the sample zone (detrimental to the stacking), acidification was also expected to influence the analyte μ_{ep} by increasing their positive charge. The μ_{ep} was increased in the acidified sample matrix so that the stacking will be more efficient at the sample/BGE boundary. The matrix acidification with both acids increased the signal by more than 50% compared to the water matrix (Figure 5B). The nature of the added acid did not significantly impact the result. Phosphoric acid was therefore chosen to maintain a unique anion inside the capillary (BGE and sample matrix) to avoid dispersion and peak tailing.

We also studied the role of organic solvents in the sample matrix with or without acidification. The organic solvent can impact the electroosmotic mobility by its viscosity or dielectric constant [59]. In an LVSS process, a sample matrix removal that is too fast due to a high EOF may lead to an incomplete stacking during the time in which the entire matrix is removed, thus lowering the preconcentration efficiency. Knowing that methanol has a smaller ϵ/η ratio than water, we tried to add it to the sample to decrease the EOF during the PS-LVSS process. The addition of 30% methanol induced a similar SEF improvement to the acidification (no significant difference). The combination of sample acidification and methanol addition significantly improved the SEF by 100% reaching 123, compared to a water matrix.

We then attempted to further increase the signal intensity by increasing the injected sample volume by processing multiple LVSS (M-LVSS), as multiple injections of a large sample volume induce successive LVSS cycles [60]. For LVSS, the injected volume represented 80% of the capillary volume. The realizations of three LVSS cycles (with

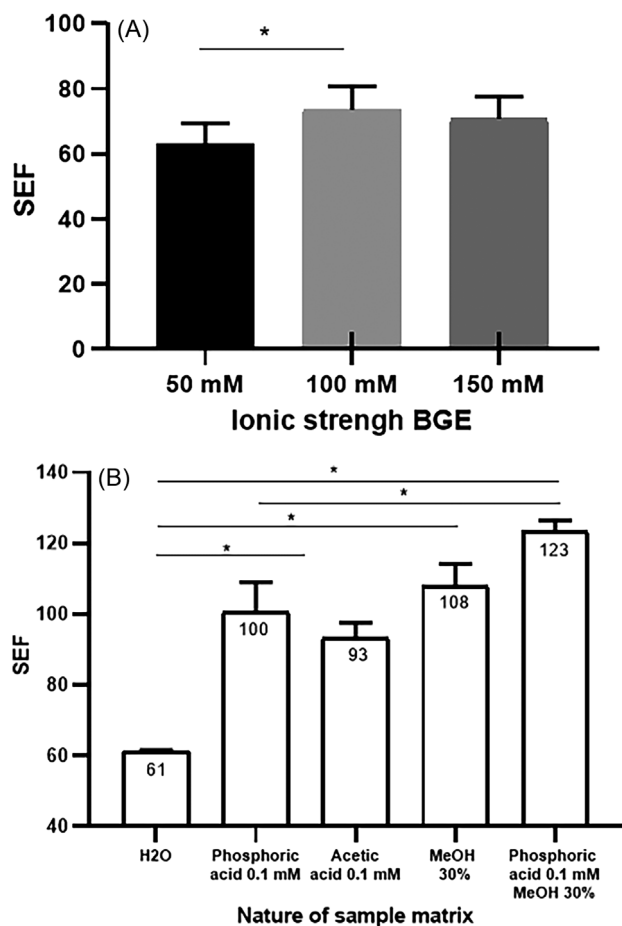


FIGURE 5 Effect of (A) the background electrolytes (BGE) ionic strength keeping the sample in water and (B) the sample matrix keeping the same BGE (100 mM lithium phosphate, pH 3.5), on the signal enhancement factor (SEF) of CJC-1293 ($n = 3$). Capillary electrophoresis (CE) conditions: bare fused-silica capillary of 90 cm \times 50 μ m id (80 cm as L_{eff}) with polybrene coating; large volume sample stacking (LVSS) with a hydrodynamic injection of 80% of total capillary; UV detection at 200 nm; applied voltage, 30 kV than -30 kV at 25°C. Sample concentration, 0.01 mg/mL. Data shown as mean \pm standard deviation of triplicate analyses; significant differences ($p < 0.05$ with Mann–Whitney test) are indicated by an “*.”

an injected volume of 80% of the capillary volume each) were attempted. In those conditions, the injected volume reached 240% of the capillary volume. Unfortunately, no signal improvement was obtained with the selected sample matrix (0.1 mM phosphoric acid solution containing 30% MeOH) and BGE (100 mM lithium phosphate, pH 3.5). No increase of peak height was observed, but a peak broadening (with a pronounced front tailing) was seen. The peak width at 10% height went from 0.27 min for a single LVSS to 0.71 min for three cycles. This broadening suggests a stacking not proportional to the injected volume compared to the single LVSS. We hypothesized that the conductivity difference between sample and BGE zones could have been

disturbed after the first LVSS cycle. This disturbance could have interfered with the stacking effect and induced analyte diffusion at the boundary. To limit the diffusion, we first considered increasing the BGE conductivity; increasing it from 5.5 mS/cm (100 mM IS BGE) to 10.1 mS/cm (200 mM IS-BGE) did not increase the signal. However, the increase induced a loss of peak height. Then, a plug of very high conductivity BGE (16.5 mS/cm with 400 mM IS) was inserted between the low conductivity sample and the BGE (100 mM). Two plug sizes were considered (5% and 10% of capillary volume), but no higher signal was observed. Last, the buffer capacity of the BGE was increased, which is relatively low for the chosen 100 mM lithium phosphate BGE (8.3 mmol/L, pH) to avoid peak tailing. This BGE was compared to a 100 mM lithium aminocaproic phosphate BGE (13.3 mmol/L, pH) and a 100 mM lithium citrate phosphate BGE (22.6 mmol/L, pH), but no signal improvement was observed. Finally, the BGE inlet vial was replaced by a sample matrix vial to ensure a unique sample matrix zone without disturbance; this test was convenient as some BGE could interleave between the concentrated analytes and the newly injected sample volume during multiple cycles, whereas the sample matrix was dropped into the BGE inlet vial during the LVSS process. Still, no peak height increase was observed.

Because none of our conditions resulted a successful M-LVSS, we then returned to the developed single PS-LVSS-chiral CZE (Figure 5B). We modified the capillary dimensions to increase the injected volume. This injected volume was doubled to 3.9 μ L using a 110 cm \times 75 μ m capillary. The sermorelin SEF was then increased to 170. The LODs (related to peak heights and calculated as three times the signal-to-noise ratios) were individually determined for each peptide to be 200 ng/mL for CJC-1295, 100 ng/mL for sermorelin and tesamorelin, and 75 ng/mL for CJC-1293.

Modifying the capillary length increased the analysis time by extending the matrix removal duration by 4.2 min. This length capillary modification influenced the analyte migration time taking 15 min longer. Moreover, the larger pathlength led to a loss of resolution of the three less resolved GHRH analogs (Figure 6). Even if, the migration time was impacted by the modification of the capillary dimensions, its RSD ($n = 3$) was acceptable, below 5% for each GHRH analog. The combination of the optimized sample matrix with the increase of injected sample volume induces a good SEF improvement from 61 to 170.

3.3 | Application to urine sample

Then, we used the optimized single PS-LVSS-chiral CZE method to detect sermorelin in four different real urine samples (from three females and one male donors),

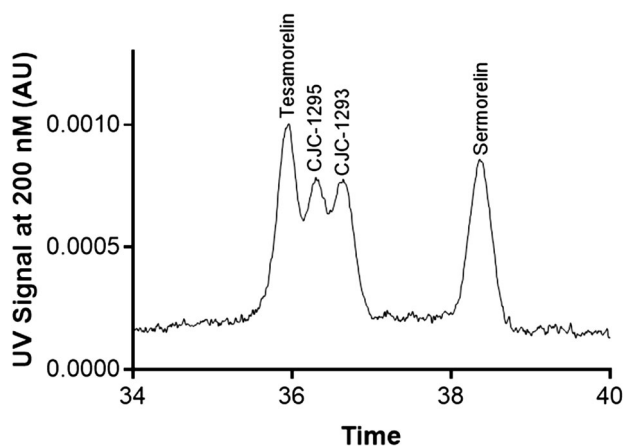


FIGURE 6 Online large volume sample stacking with polarity switching (PS-LVSS) preconcentration and chiral capillary zone electrophoresis (CZE) separation of 4 growth hormone-releasing hormone (GHRH) analogs. Capillary electrophoresis (CE) conditions: polybrene coated capillary: 110 cm $L_{\text{tot}} \times 75 \mu\text{m}$ id (L_{eff} : 100 cm); background electrolytes (BGE): 100 mM lithium phosphate pH 3.5; injection: 87 s at 68.9 kPa; UV detection: 200 nm; temperature: 25°C; voltage, 30 kV then inversion of the polarity when current intensity reached 95% of the current plateau. Sample concentration: 100 ng/mL of sermorelin, CJC-1293, CJC-1295, and tesamorelin. AU, absorbance unit.

obviously exhibiting different pHs and conductivities as measured before SPE treatment. Urines were first acidified with TFA and then desalted using a C18 SPE column. A large washing step with 20% ACN was followed by a final elution using 80% ACN containing 0.1% TFA. After a freeze-drying step, the residues were solubilized with the optimized LVSS sample matrix (0.1 mM phosphoric acid with 30% MeOH) and then spiked with sermorelin at 1 $\mu\text{g/mL}$. The obtained average SEF was 685 with a satisfactory RSD of 6.2%. The SEF was surprisingly higher than that determined with the sermorelin in the non-urinary matrix (SEF 170) (SI 2). This more favorable SEF might be a consequence of the presence of other urine-containing molecules such as peptides, proteins, and hormones retained by the C18 phase and having a positive impact during the PS-LVSS process. For instance, those ionized molecules could create a simultaneous isotachopheresis to the PS-LVSS process improving the preconcentration performances. Then, we estimated the LOD to be 70 ng/mL for sermorelin. However, we have to consider that the C18 SPE step can be used not only for desalting but also to lower this LOD by preconcentrating the target molecule. When using 1 mL of urine, we can expect having at least an additional 7–10 fold preconcentration factor based on final volume, lower than the starting one (typically 90–120 μL). This improvement could lead to a

LOD lowdown to a few ng/mL which is close to the WADA MRPL requirement (1 ng/mL).

4 | CONCLUDING REMARKS

We developed here a chiral CZE method to separate four GHRH analogs (sermorelin, CJC-1293, CJC-1295, and tesamorelin) with the benefit of a cationic coating of the silica capillary using polybrene. To our knowledge, it the first time an analytical protocol allows the separations of sermorelin and CJC-1293 that are structurally very close and not discriminated by conventional HPLC-MS methods used by anti-doping laboratories. An excellent resolution ($R_s \approx 2$) of two enantiopeptides was possible by adding dimethyl- β -CD as a chiral selector in the BGE. An online PS-LVSS preconcentration was also developed to overcome the low sensitivity of chiral CZE-UV method. SEF of 170 for CJC-1293 solubilized in 0.1 mM phosphoric acid solution containing 30% MeOH could be obtained. LODs between 75 and 200 ng/mL were determined with acceptable migration time repeatability below 5%. This method was applied to desalted urine spiked with sermorelin and showed surprisingly a much better SEF than that obtain with the sermorelin in water prepared in the LVSS matrix. We estimated a LOD of 70 ng/mL for sermorelin and even a 10 ng/mL LOD by exploiting C18 SPE as a first concentration step. Such a result constitutes a good proof of concept for the anti-doping community as this reaches level of concentrations near the WADA requirements and proposes a solution for the separation and thus the identification of the two analogs that cannot be distinguished so far.

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CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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