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Research Article

Analysis of buserelin in urine by online combination of capillary zone electrophoresis with electrospray mass spectrometry

A fast and precise analysis of the synthetic peptide buserelin in urine using CZE-ESI-MS method has been demonstrated. Formic acid at 50 mmol/L concentration served as backgroud electrolyte in CZE stage and it is compatible with MS detection in positive ionization mode. Two injection modes were tested, *i.e.* pressure (50 mbar for 5 s) and electrokinetic injection (5 kV for 5 s), of which electrokinetic injection provided better calibration parameters. Buserelin LODs were 0.47 μg/mL in water and 0.63 μg/mL in ten times diluted urine samples using pressure injection, while they were 0.32 μg/mL in water and 0.34 μg/mL in ten times diluted urine samples using electrokinetic injection. Repeatability of buserelin migration times was below 6% (pressure injection mode) and 1% (electrokinetic injection mode). Repeatability of buserelin peak area in SIM mode ($m/z = 620.5 \pm 0.5$) was less than 12% (pressure injection mode) and 5.8% (electrokinetic injection mode). In this work, no interferences were observed during the analyses of spiked urine samples.

Keywords:
Buserelin / Capillary electrophoresis / Electrospray / Mass spectrometry / Urine
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1 Introduction

Buserelin is a synthetic analog of natural gonadotropin-releasing hormone and is used to treat prostate or breast cancer. This nonapeptide (5-oxoPro-His-Trp-Ser-Tyr-D-Ser (t-Bu)-Leu-Arg-Pro-NHC_2H_5; $M_w = 1238.66$ g/mol, pI = 10.5) [1] is a white or slightly yellowish hygroscopic powder, sparingly soluble in water and in dilute mineral acids [2]. Buserelin inhibits production of luteinizing hormone from the pituitary gland, which decreases the levels of testosterone. Prostate cancer is often sensitive to testosterone level, and a reduction in its level may influence the rate of cancer growth progression and affect the size of the tumor. Hormone therapy with buserelin cannot cure prostate cancer but may decrease symptoms and improve the quality of life for most patients. Breast cancer may also be treated with buserelin [3].

Buserelin is completely absorbed after subcutaneous injection, with peak plasma concentration occurring after about 1 h. It is accumulated in the liver and kidneys as well as in the anterior pituitary. A high degree of inactivation of the drug occurs by the gastrointestinal enzymes (peptidases), and the proportion of active compound reaching systemic circulation is very low. The main serum metabolite is inactive buserelin (5–9) pentapeptide. Buserelin is excreted in urine and bile as the unchanged drug (66% of dose) and its metabolites (28% of dose in 24 h). Small part of dose (17–32%) is detected in urine after intravenous or subcutaneous administration. Very small amounts are distributed into breast milk. Half-life in plasma is 80 min and protein binding in plasma is approximately 15%. The dose, for example, in prostatic carcinoma, is 500 μg (of the base) applied subcutaneously every 8 h for 7 days, then 200 μg into each nostril every 8 h [2].

Several chromatographic techniques have been applied for the analysis of buserelin, *e.g.* LC [4] and LC-ESI-MS [5, 6]. Optimization of chromatographic and/or electrophoretic separation conditions for analysis of mixture of peptides with buserelin was published in several papers [7–11]. CZE is the mostly used CE mode for peptide analysis. The separation of peptides in the CZE step is based on different charge/mass ratios and the selection of MS compatible BGE at low pH (2.0–3.5) usually consists of formic acid and/or acetic acid [12].

Several combinations of CZE and detection techniques have been applied for the analysis and characterization of

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Abbreviation: TIC, total ion current

buserelin, *e.g.* CZE-UV [1, 8], multiple-injection CZE [13], CZE-ESI-MS [14] and CZE-TOF-MS [15]. Comparison of sheath-liquid and sheath-flow electrospray interfaces for CZE-ESI-MS analysis of mixture of peptides with buserelin was published by Sanz-Nebot *et al.* [14].

CZE-MS was proved to be successful and powerful analytical technique for separation and identification of biologically important compounds, *e.g.* proteins and peptides, as well as for analysis of different complex biological mixtures. The increasing use of CZE-MS in proteomics and peptidomics area can be illustrated by the high number of reviews published in the last years [12, 16–25]. Main advantages of this coupling are its high separation efficiency, short analysis time, high detection selectivity and sensitivity, low sample and electrolyte consumption.

At present, the ESI is the mostly used ionization technique for CE-MS coupling [21, 22]. Main advantages of this ionization technique are (i) direct transfer of analyte molecules from liquid phase to the gas phase *via* one of the three known interfaces (sheathless, liquid junction and sheath flow), (ii) high ionization efficiency and (iii) the multiple charged ions obtainable. Sheath-flow interface is dominating between the interfaces used in commercially available CE-ESI-MS instruments [25].

The high variety of mass analyzers has been used for an analysis of proteins and peptides. Mainly single quadrupole, triple quadrupole, ion trap or TOF mass analyzers have been employed. The mostly used type of mass analyzer is quadrupole, due to its lower price, small size and simplicity of operation. Disadvantages and limitations of using quadrupole in peptide analysis are its low resolution, modest sensitivity and limited mass range [12]. The last disadvantage can be overcame by using ESI ionization technique that can produce multiple charged ions providing the use of quadrupole mass analyzer also in the field of protein and peptide analysis.

This article is dealing with the analysis and quantification possibilities of buserelin present in complex biological matrix (urine) by using CZE-ESI-MS with sheath-flow interface. Main interest was devoted to the study and optimization of important experimental parameters such as composition and concentration of BGE, composition and concentration of sheath liquid, flow rates of drying gas and sheath liquid, temperature of drying gas and electrospray voltage.

2 Materials and methods

2.1 Chemicals

Acetic acid, ammonium acetate, formic acid and sodium hydroxide were purchased from Sigma (St. Louis, MO, USA). Methanol and 2-propanol were obtained from Fluka (Buchs, Switzerland). Buserelin standard as noncommercial sample was obtained from Merck (Darmstadt, Germany). Water for LC-MS was purchased from Honeywell (Burdick and Jackson, MI, USA) and it was used for the preparation of stock and working solutions. Running electrolyte for CZE was also prepared in LC-MS water.

2.2 Standard solution preparation

The stock solutions of buserelin were prepared separately by dissolving of about 1 mg of standard with 1 mL of LC-MS water to obtain the concentration level 1 mg/mL. The stock solution was freshly prepared on each working day. A series of working solutions was prepared by diluting the stock solution with different amounts of LC-MS water to obtain the concentrations within the concentration range 0.5–20 μg/mL. Urine samples used as a matrix in the analyses of buserelin were obtained from five healthy volunteers and they were immediately diluted 10 or 100 times with LC-MS water. Urine-based calibration solutions were prepared by spiking of diluted blank urine samples with the buserelin stock solution to reach the final concentrations.

2.3 Apparatus

CZE-MS analyses were performed on an Agilent CE system HP 3D (Waldbronn, Germany) equipped with an on-column DAD and an Agilent G6130 single quadrupole mass-selective detector. Sheath liquid for an electrospray ionization interface was supplied by an Agilent G1310 LC isocratic pump.

2.4 CZE-MS condition

Running electrolyte was prepared by dissolving an appropriate amount of formic acid in deionized water and pH of the running electrolyte was measured. Fresh running electrolyte solution was prepared daily.

The CZE analyses were performed in uncoated fused silica capillary of 81 cm total length and 50 μm id (MicroSolv Technology, Eatontown, NJ, USA). The effective length to the UV detector was 21.5 cm. The capillary was thermostated to 20°C. The capillary was rinsed for 20 min with NaOH at 1 mol/L concentration, then for 20 min with LC-MS water and finally with the running electrolyte for 20 min in the beginning of each working day. The capillary was washed with running electrolyte for 5 min between the runs. Sample injection was performed either by applying 50 mbar pressure for 5 s (pressure injection) or by applying 5 kV voltage for 5 s (electrokinetic injection). Constant voltage + 27 kV was applied in all CZE-MS experiments in the CZE stage. All measurements were repeated five times if not stated otherwise.

Sheath liquid consists of 50% v/v methanol, 49.95% v/v of LC-MS water and 0.05% v/v formic acid. After a 99:1

splitting, the actual flow rate of sheath liquid was 4 μL/min. The MS conditions were as follows: electrospray capillary voltage +4.0 kV, drying gas flow rate 10 L/min, drying gas temperature 200°C and nebulizer gas pressure 10 psi. The MS detector was working either in scanning mode (total ion current (TIC)) to obtain MS spectrum or in the SIM mode to monitor the ion population at $m/z = 620.5 \pm 0.5$ ($[M+2H]^{2+}$, doubly charged buserelin ion).

3 Results and discussion

3.1 The study of some experimental parameters of CZE-MS

From the buserelin p*I* value [1], it is clear that the separation has to be carried out at low pH value of BGE. Several BGEs were tested for CZE-MS experiments to obtain the best signal in MS stage. Therefore, acetate (pH 4–5.5), 50–200 mmol/L acetic acid (pH 3.1–2.7) and 10–200 mmol/L formic acid (pH 2.9–2.3) were tested. Of these, the best signal in MS stage was obtained with formic acid at 50 mmol/L (pH 2.5) concentration level. Low pH value of running electrolyte was a choice with regard to using volatile electrolytes based on formic acid or acetic only. The presence of any counter ion other than H^+ can preferably lead to ionization of that counter ion and thus to lower sensitivity of the proposed method.

Commercial sheath-flow type of ESI interface [26] was used for the online coupling of CE and MS parts in our work. Selected parameters that have a significant influence on sample ionization, spray stability and the final MS signal intensity [27–32] were studied. Of these, the composition of sheath liquid plays a key role. The best results were obtained when the methanol:water:formic acid (50:49.95:0.05 v/v/v) solution mixture was used. The addition of formic acid led to the better protonization of the analyte (buserelin) in comparison with the situation when no formic acid was added in the sheath liquid.

Temperature of drying gas was another important parameter. The best results were obtained when 200°C of drying gas temperature was used and +4 kV voltage was applied. The final MS conditions are summarized in Table 1.

Table 1. Optimal separation conditions for CZE-ESI-MS

Final optimized conditions	
Sheath liquid composition	Methanol:water 50:49.95 v/v+0.05% v/v formic acid
Sheath liquid flow rate	4 μL/min
Drying gas flow rate	10 L/min
Drying gas temperature	200°C
Nebulizer gas pressure	10 psi
Potential on spraying capillary	4000 V
ESI mode	Positive

3.2 CZE-MS determination of buserelin in urine

Part of applied buserelin dose is reported to be eliminated unchanged in urine [2]. Due to very low doses of buserelin used in therapy, its concentration in urine is very low and, therefore, the analytical methods providing high sensitivity are required in such analyses. MS can be detector of choice due to its high selectivity and sensitivity. CZE is very often used when high separation efficiency is required. We performed all the experiments in uncoated fused silica capillary (50 μm id) of 81 cm length. Constant voltage + 27 kV was used for the separation in CZE stage of CZE-MS runs. Two injection modes were tested, *i.e.* pressure injection mode (applying 50 mbar constant pressure for 5 s) and electrokinetic mode (applying +5 kV for 5 s).

Signal in MS step was monitored either within the $m/z = 100$–1500 range (TIC mode) or at $m/z = 620.5 \pm 0.5$ (SIM mode). The CZE-MS analysis of buserelin standard (0.1 mg/mL) is shown in the Fig. 1, where signal from SIM mode is shown in the upper part and MS spectrum is shown below where doubly charged buserelin ion is dominating.

The potential influence of urine matrix on buserelin signal was tested in series of experiments, where three different amounts of urine matrix (no urine, 10 times diluted urine sample and 100 times diluted urine sample) were added to the buserelin calibration standards. Urine samples used as a matrix were obtained from five healthy volunteers and the reported data in Table 2 and in Figs. 2A–C represent the worst situations. Even though urine contains considerable concentration levels of sodium and potassium, affinity of buserelin to these metals is relatively low and clusters with these metals were not observed in significant intensities. For sodium, ratio of relative intensities of $[M+2H]^{2+}$ and $[M+H+Na]^{2+}$ was 25:1 and for potassium, the corresponding ratio was 50:1. Clusters with two or more metal ions were not observed. Data were obtained for sample containing ten times diluted urine and 0.5 mg/L of target analyte. Calibration parameters of buserelin (measured within the 0.5–20 μg/mL concentration range of buserelin, at six concentration levels) obtained without the addition of urine matrix served as the standard for the evaluation of urine influence (Table 2). Each calibration point was measured three times. It is visible from Table 2 that the intercepts (*a* in Table 2) of all calibration lines are negative independent of the injection mode, indicating that either adsorption phenomenon occurred during the manipulation/separation or lower ionization efficiency of ESI at low concentration level of buserelin. This fact can be responsible for the potential problems with the analysis of low buserelin concentrations using CZE-MS method. All calibration curves are linear with correlation coefficients (*r* in Table 2) from 0.9976 to 0.9996. LOD and LOQ were calculated according to the literature [33], as the ratio of

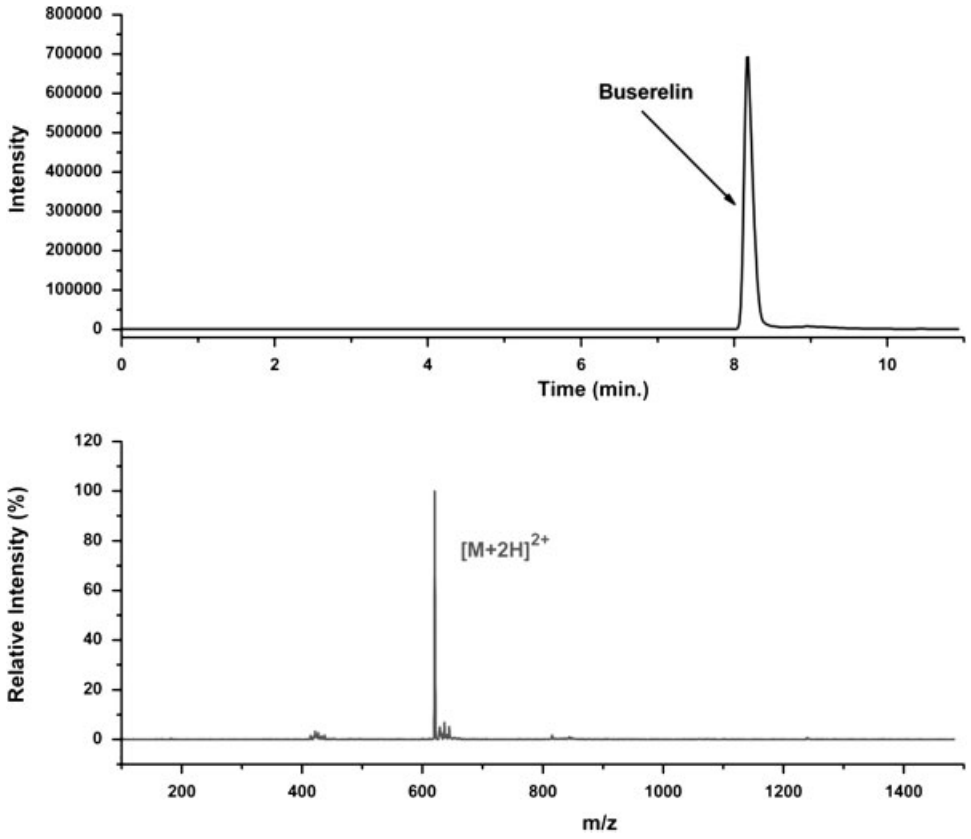

Figure 1. CZE-MS analysis of the buserelin standard in water (concentration was 0.1 mg/mL). SIM mode at *m/z* = 620.5 ± 0.5 (upper trace) and MS spectrum within *m/z* = 100-1500 (lower trace).

Table 2. Calibration parameters of buserelin

Parameter	Buserelin without urine		Buserelin with 100 times diluted urine		Buserelin with 10 times diluted urine	
	PI	EKI	PI	EKI	PI	EKI
a (counts/min)	−204 500	−35 100	−303 700	−26 000	−163 100	−30 200
s_a (counts/min)	24 700	5800	25 100	5100	22 400	5800
b (counts/min mL/μg)	174 200	60 900	172 600	52 300	117 200	57 100
s_b (counts/min mL/μg)	2100	1100	2000	1000	2800	600
r	0.9996	0.9977	0.9993	0.9976	0.9980	0.9992
LOD (μg/mL)	0.47	0.32	0.48	0.32	0.63	0.34
LOQ (μg/mL)	1.4	0.96	1.5	0.98	1.9	1.0

PI, pressure injection; EKI, electrokinetic injection.

standard deviation of intercept (s_a in Table 2) and slope (*b* in Table 2) of the calibration line multiplied by factor 3.3 (LOD) and 10 (LOQ), respectively. LODs of buserelin for pressure injection and electrokinetic injection modes were 0.47 and 0.32 μg/mL, respectively. These values are one-half or one-third of the values published by Sanz-Nebot *et al.* [15], measured on TOF MS detector providing better performance in comparison with single quadrupole MS detector, and they are more than ten times better than the values published by Lodén and Amini [13]. MS signals in SIM mode obtained from blank sample and the sample spiked with buserelin at 1 μg/mL concentration level are shown in Fig. 2A. There is no interference visible in the position of buserelin peak; only the baseline level is shifted to higher values (for about 200 counts/min intensity) and it becomes noisier after 8.5 min. Similar shifts are also documented for buserelin and other peptides in the work of Sanz-Nebot *et al.* [15]. The shift of baseline can be probably interpreted as an electrophoretic system peak generated on the interface of zones of analyte and BGE [34]. Slightly different composition of analyte zone and BGE changes an efficiency of ionization process and thus leads to the change (in this case increase) of MS signal. Deeper difference between these two systems leads to more significant changes in ionization process and thus to more significant change in baseline signal.

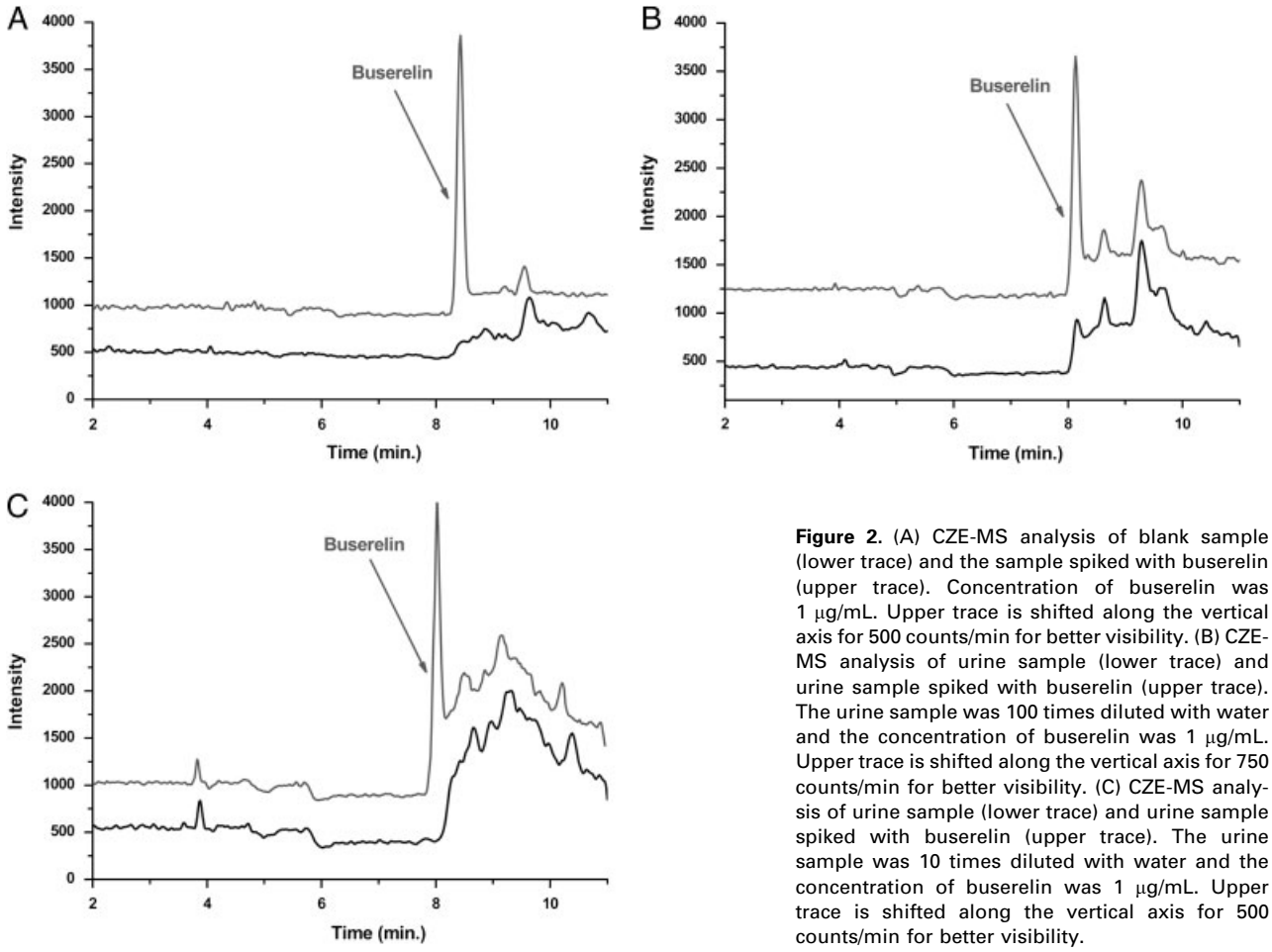

Figure 2. (A) CZE-MS analysis of blank sample (lower trace) and the sample spiked with buserelin (upper trace). Concentration of buserelin was 1 μg/mL. Upper trace is shifted along the vertical axis for 500 counts/min for better visibility. (B) CZE-MS analysis of urine sample (lower trace) and urine sample spiked with buserelin (upper trace). The urine sample was 100 times diluted with water and the concentration of buserelin was 1 μg/mL. Upper trace is shifted along the vertical axis for 750 counts/min for better visibility. (C) CZE-MS analysis of urine sample (lower trace) and urine sample spiked with buserelin (upper trace). The urine sample was 10 times diluted with water and the concentration of buserelin was 1 μg/mL. Upper trace is shifted along the vertical axis for 500 counts/min for better visibility.

The same concentration levels of buserelin were used in the second calibration series and each calibration solution contained 100 times diluted urine sample (Section 2). Calibration parameters of buserelin obtained from the calibration samples containing the addition of 100 times diluted urine matrix are summarized in Table 2. It is visible that in this case the slope of calibration line is only 1% lower for pressure injection, but about 14% lower for electrokinetic injection mode. The values of correlation coefficient are practically the same as they were when no urine was present in the sample. Similar situation is visible in the case of LOD and LOQ, as both values are only slightly worse. MS signals in SIM mode obtained from blank sample and the sample spiked with buserelin at 1 μg/mL concentration level, both containing 100 times diluted urine sample, are shown in Fig. 2B. There is small interfering peak visible in the position of buserelin peak and the baseline level is shifted to higher values (for about 400 counts/min intensity) and there are several peaks, originating from the urine matrix, visible after 8.0 min.

The third calibration series used the same concentration levels of buserelin and each calibration solution contained ten times diluted urine sample (Section 2). Calibration parameters of buserelin obtained with the addition of ten times diluted urine matrix are summarized in Table 2. It is visible that in this case, the slope of calibration line is about 33% lower for pressure injection but only 6.2% lower for electrokinetic injection mode. This can be explained by the fact that during the electrokinetic injection process, the amount of the sample being injected into the capillary is not reflected the original sample composition. Ions with higher mobility are injected preferably. These ions can form isotachophoretic conditions as they can work in the role of leading ions for the rest of the ions injected from the sample. The values of correlation coefficient are practically the same as they were when no urine was present in the sample. The values of LOD and LOQ are about 50% worse for pressure injection but only slightly worse for electrokinetic injection. MS signals in SIM mode obtained from blank sample and the sample spiked with buserelin at 1 μg/mL concentration level, both containing ten times diluted urine sample, are shown in Fig. 2C. There is practically no interfering peak visible in the position of buserelin peak and the baseline level is shifted to higher values (for about 800 counts/min intensity) and there are several high peaks, originating from the urine matrix, visible after 8.0 min.

Table 3. Repeatability of buserelin migration time and peak area

Parameter	Buserelin without urine		Buserelin with 100 times diluted urine		Buserelin with ten times diluted urine	
	PI	EKI	PI	EKI	PI	EKI
t_m (min)	9.376	8.046	9.062	8.080	9.553	8.031
s_{tm} (min)	0.493	0.050	0.090	0.013	0.582	0.060
RSD_{tm} (%)	5.3	0.62	1.0	0.16	6.1	0.75
A (counts/min)	646 500	264 900	560 300	260 100	423 900	255 500
s_A (counts/min)	62 520	14 330	66 950	21 070	30 600	14 700
RSD_A (%)	9.7	5.4	12	8.1	7.2	5.8

PI, pressure injection; EKI, electrokinetic injection; t_m, migration time; s_{tm}, standard deviation of migration time; A, peak area; s_A, standard deviation of peak area.

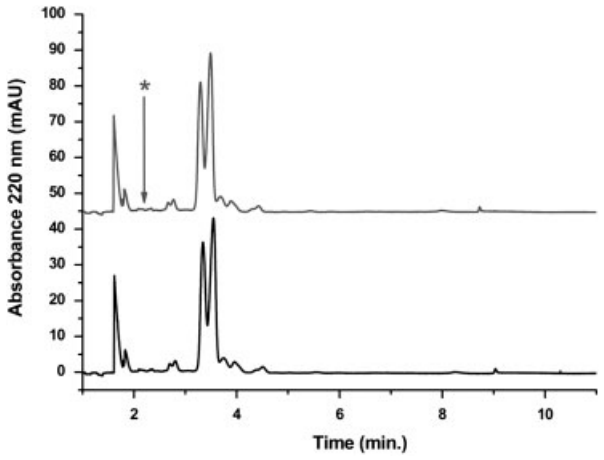

Figure 3. CZE-UV analysis of urine sample (lower trace) and urine sample spiked with buserelin (upper trace). Migration position of buserelin is marked by asterisk. The urine sample was 10 times diluted with water and the concentration of buserelin was 1 μg/mL. Upper trace is shifted along the vertical axis for 45 mAU for better visibility.

Repeatability of the CZE-MS method was performed by ten replicate analyses of the samples containing buserelin at 5 μg/mL concentration and one of the three matrices we studied (water, 100 times diluted urine and 10 times diluted urine) and the results obtained in this study are summarized in Table 3. Relative standard deviations of migration time obtained in the runs when pressure injection mode was applied were within 1–6.1% range and they were below 1% when electrokinetic injection mode was used. Better repeatability of migration times in electrokinetic injection mode can be explained by the fact that during the injection process, the amount of the sample being injected into the capillary is not reflected in the original sample composition. Ions with higher mobility are injected preferably. Relative standard deviations of buserelin peak area obtained in SIM mode ($m/z = 620.5 \pm 0.5$) were between 7.2 and 12%, when pressure injection mode was used and they were between 5.4 and 8.1% when electrokinetic injection mode was used. The worse results were obtained in pressure injection mode in comparison with the results obtained in electrokinetic injection mode. Pressure injection mode is the most widely used injection technique. Its advantages and drawbacks, as well as those of electrokinetic injection technique, are deeply discussed in the literature [35].

The importance of MS detector is shown in Fig. 3, where UV signals (monitored at 220 nm wavelength, where the absorption spectrum of buserelin reaches maximum at low pH values) obtained from blank sample of ten times diluted urine and the ten times diluted urine sample spiked with buserelin at 1 μg/mL concentration level are shown. Clearly, the UV detector can neither detect nor prove the presence of buserelin at this concentration level in urine sample. The same samples analyzed by MS detector are shown in Fig. 2C and buserelin is clearly visible at this concentration level in ten times diluted urine sample.

4 Concluding remarks

In this study, a coupling of CZE with UV and single quadrupole MS detectors was employed for the analysis of buserelin in urine samples. The CZE separation conditions compatible with MS detection were established. Main experimental parameters affecting signal in CZE-ESI-MS were studied to obtain the suitable peak intensities for target compound. Composition of sheath liquid, flow rate of sheath liquid, drying gas temperature, drying gas flow rate and spraying capillary voltage were studied. A sheath liquid with methanol:water:formic acid (50:49.95:0.05 v/v/v) provided the best MS signal intensity and electrospray stability when delivered at 4 μL/min flow rate. The temperature of drying gas was 200°C, its flow rate was 10 L/min and potential applied on electrospray capillary was +4.0 kV. An average analysis time was approximately 2.5 min when UV detector was used and 8 min when MS detector was utilized. CZE-ESI-MS method evaluated during this work showed good linearity within the concentration range 0.5–20 μg/mL. LOD were 0.47 μg/mL for buserelin standard in water and 0.63 μg/mL for buserelin in ten times diluted urine samples using pressure injection, while they were 0.32 μg/mL for buserelin standard in water and 0.34 μg/mL for buserelin in ten times diluted urine

samples using electrokinetic injection. The repeatability of migration times was below 6% in pressure injection mode, while it was below 1% when electrokinetic injection mode was applied. Repeatability of buserelin peak area was less than 12% in pressure injection mode and it was less than 5.8% in electrokinetic injection mode. In this work, small interferences not influencing the determination were observed during the analyses of spiked urine samples.

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