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On-line SPE–CE for the determination of insulin derivatives in biological fluids

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Abstract

An on-line SPE–CE system is described for the determination of insulin derivatives in urine, serum and plasma. By combining techniques based on different separation mechanisms, in this case reversed-phase SPE and CE, a more selective sample clean-up is obtained. The described on-line SPE–CE procedure is able to desalt and clean biological samples, resulting in more repeatable electrophoretic results as well as a good linearity for urine, serum and plasma samples spiked with insulin derivatives, thus proving the elimination of detrimental effects caused by the sample matrix. The on-line SPE–CE system was linear for urine, serum and plasma samples spiked with insulin derivatives between 5 and 80 mg/l. The repeatability in migration time was below 1% relative standard deviation (R.S.D.). The repeatability of the peak was better ($<2.4\%$ R.S.D.) when no off-line precipitation reaction ($<6.2\%$ R.S.D.) was used, proving the beneficial characteristics of on-line sample pretreatment procedures over off-line sample pretreatment procedures which are prone to sample losses and contamination.

Keywords: SPE; On-line; CE; Insulin; Plasma; Serum; Urine

1. Introduction

In addition to liquid chromatography (LC) and gel-electrophoresis, capillary electrophoresis (CE) is frequently used and is still gaining popularity for the separation of large biomolecules. CE combines the high efficiency of gel-electrophoresis with the speed, automation and on-line potential of LC. Additional advantages of CE are its simplicity, its versatility and its small injection volumes. The latter advantage is, at the same time, its main disadvantage. Due to these small injection volumes, the mass sensitivity of CE is high, but the concentration sensitivity is rather low. This especially is a problem when the analytes are detected using concentration-sensitive detection techniques [1,2]. As a result many in-capillary concentration procedures such as isotachophoresis and/or solid-phase preconcentration capillaries (spPC) have been developed to allow injection volumes as large as the capillary volume, several μl, or even some-

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times larger [13]. However, these concentration techniques are only useful when the sample matrix is well defined and has a low ionic strength.

Injection of biological samples in CE often results in irreproducible results. This is partly caused by the, before mentioned, high and often varying salt concentrations in biological samples. Salts will increase the conductivity of the sample plug, and therefore, increase the heat generated in the capillary during the analysis. This heat generation, also called Joule heating, can cause air bubbles, which can result in current breakdowns. Joule heating also results in a decrease in the solvent viscosity in the capillary, resulting in decreasing migration times during consecutive measurements. Irreproducible results in CE, encountered when injecting biological samples, are also caused by secondary adsorption effects. Biological matrices contain quite a number of components (e.g. proteins, lipids) that can be adsorbed to fused silica capillaries. Static or dynamic coating of the capillary wall can solve these secondary adsorption effects [3,4]. Still, it is frequently necessary to remove salts and most of the proteins before injecting a biological sample in CE. Many off-line methods such as (ultra)filtration [15], dialysis [16], centrifugation [17] and precipitation [18] are available to accomplish this. The disadvantage of centrifugation, (ultra)filtration and precipitation is that they are difficult to automate and even more difficult to perform on-line in combination with separation techniques. In addition, they can be rather time consuming when relatively large volumes of solvent have to be evaporated.

Salts and proteins in biological samples can also be removed by using solid-phase extraction (SPE). SPE is a versatile and relatively fast technique which is easy to automate [5]. An additional advantage of using SPE prior to CE is its additional selectivity.

Different approaches have been developed to couple SPE with CE. The majority of these approaches involve off-line SPE using conventional SPE cartridges or micro-devices such as ZipTips© [14]. The off-line coupling of SPE and CE is the most simple but also more susceptible to sample losses and sample contamination during clean-up than at-, on- and in-line techniques. Off-line methods are always more laborious and time consuming than automated at-, on- or in-line systems. With respect to on- and in-line SPE–CE, two different approaches can be distinguished. The most popular are spPC [2] and membrane preconcentration capillaries (mPC) [6]. Both are in-line devices that can be used for biological samples, but are more suitable for the preconcentration of rather clean samples. Using these devices for biological samples can cause problems with clogging, a reversed electro-osmotic flow (EOF) or irreproducible results caused by adsorption of sample matrix components to the capillary wall. The second approach is the coupling of an SPE cartridge to the CE via an interface, the so-called on-line approach [7,12]. Applying this approach, the biological matrix does not enter the CE capillary, in this way avoiding secondary adsorption effects of matrix components. Also the other problems encountered with spPC and mPC are avoided using the on-line approach.

In the present study an on-line SPE–CE procedure has been applied to determine insulin derivatives in different biological matrices [7]. Insulin functions as a test component in our study, because it can be seen both as a large peptide or as a small protein (Fig. 1). As a result, this system may be suitable for both peptide and protein determinations. Insulin derivatives are of interest both from a clinical or pharmaceutical point of view. The on-line SPE–CE system consists of a reversed-phase (RP) SPE cartridge and CE coupled via a home-made interface. In this way, two different separation mechanisms are coupled, adding additional selectivity to the method. The homemade interface can be seen as a micro-injection vial through which the plug eluting from the SPE cartridge passes and then is injected hydrodynamically into the CE capillary. Only part of the sample plug can be injected in this way, the remaining sample is flushed to waste. Although the sensitivity of the described on-line SPE–CE system is limited, the samples are desalted and hardly any of the sample matrix components enter the capillary resulting in reproducible electropherograms. This is especially beneficial for quantitative analyses.

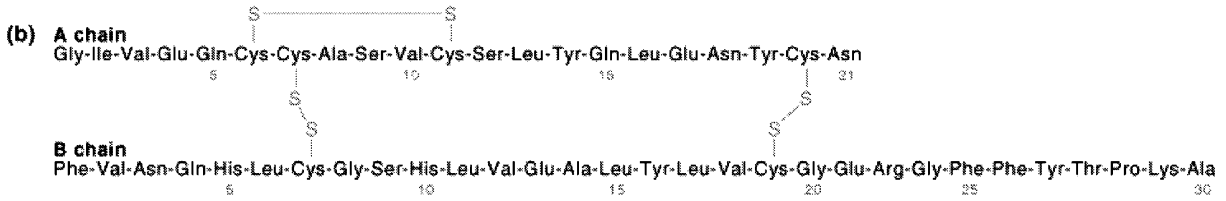

Fig. 1. Amino acid sequence of BI.

2. Experimental

2.1. Chemicals

Bovine insulin (BI), human insulin (HI), Arg-human insulin (AHI), 2-cyclohexylamine ethanesulfonic acid (CHES), bovine serum and human plasma were obtained from Sigma-Aldrich (Zwijndrecht, Netherlands). Disodium hydrogenphosphate, sodium acetate and sodium chloride were purchased from Riedel-de-Haën (München, Germany). Sodium phosphate came from Baker (Deventer, Netherlands). Triethylamine (TEA) and sodium dihydrogenphosphate were obtained from Merck (Darmstadt, Germany). Acetonitrile (ACN) (Baker) was HPLC-grade and filtered (0.45 μm filters, Millipore, Bedford, MA, US) before use. High purity water obtained from a Milli-Q system was used for all experiments (Millipore). Urine samples were volunteered by a 23-year-old healthy male.

Additional information about the used insulin derivatives is given in Table 1. As can be seen, the different insulin derivatives are quite similar and differ only in one to three amino acids. As a result, the molecular weight (MW) and pI are quite similar.

Table 1
Differences in amino acids of insulin derivatives

	MW (u)	Positions in amino acid sequence where differences occur			
		A8	A10	A18	B30
BI	5733.5	Ala	Val	Asn	Ala
HI	5807.6	Thr	Ile	Asn	Thr
AHI	5963.8	Thr	Ile	Asn	Thr

2.2. Set-up of the on-line SPE–CE system

2.2.1. SPE instrumentation

SPE was performed with the aid of three pumps: two model pumps from Applied Biosystems (Foster City, CA, USA) and a high precision pump model 300 from Cynkotek (Separations, H.I. Ambacht, Netherlands). Three six-port valves were used: two automatic MUST switching valves from Spark Holland (Emmen, Netherlands) and one manual home-made six-port valve. Detection was performed using a model 759A absorbance detector from Applied Biosystems. A 20 × 2.1 i.d. mm HP Hypersil MOS column, packed with 5 μm particles (Agilent Technologies, Amstelveen, Netherlands) was used for SPE. All tubing used in the SPE system was blue PEEK (1/16 in. o.d., 0.25 mm i.d., Bester, Amstelveen, Netherlands) except for the tubing between valve 3 (V3) and the CE apparatus, which was red PEEK (1/16 in. o.d., 0.13 mm i.d., Bester).

2.2.2. CE instrumentation

A PrinCE (Lauerlabs, Emmen, Netherlands) CE system was used equipped with a 75 μm i.d./375 μm o.d. untreated fused-silica capillary (BGB Analytic, Adliswil, Switzerland) of 120 cm (60 cm effective length). Detection was performed on-capillary at 200 nm (rise-time 0.5 s), by removing several mm of the capillary outer polyimide coating. Data acquisition was performed using ATLAS software (version 3.59a, Labsystems, Altrincham, UK).

When CE analysis was performed without on-line SPE, the method described in Table 2 was used.

Table 2
Method used for CE analysis

Description	Pressure in mbar	Voltage (kV)	Time (min)
Conditioning of capillary	2000	0	2.0
Injection	−70	0	0.2
Electrophoresis	0	−30	15.0
Flushing capillary	2000	0	2.0

2.2.3. On-line SPE–CE system

The principle of the system used was described previously and is shown in Fig. 2 and Tables 2 and 3. The system consists of an SPE and CE part coupled via a home-made interface [7]. In the SPE system three six-port valves were used. The CE apparatus controlled the automated six-port valves V2 and V3. The manual valve V1 was equipped with a 500 μl green PEEK injection loop (1/16 in. o.d., 0.75 mm i.d., Bester).

On-line SPE–CE was performed using the procedure described in Table 3. After injection (step 0) the contents of the sample loop was transferred to the SPE cartridge at 0.3 ml/min using loading solvent, 10 mM phosphate buffer pH 7.8, using pump 1 (P1). While the sample was

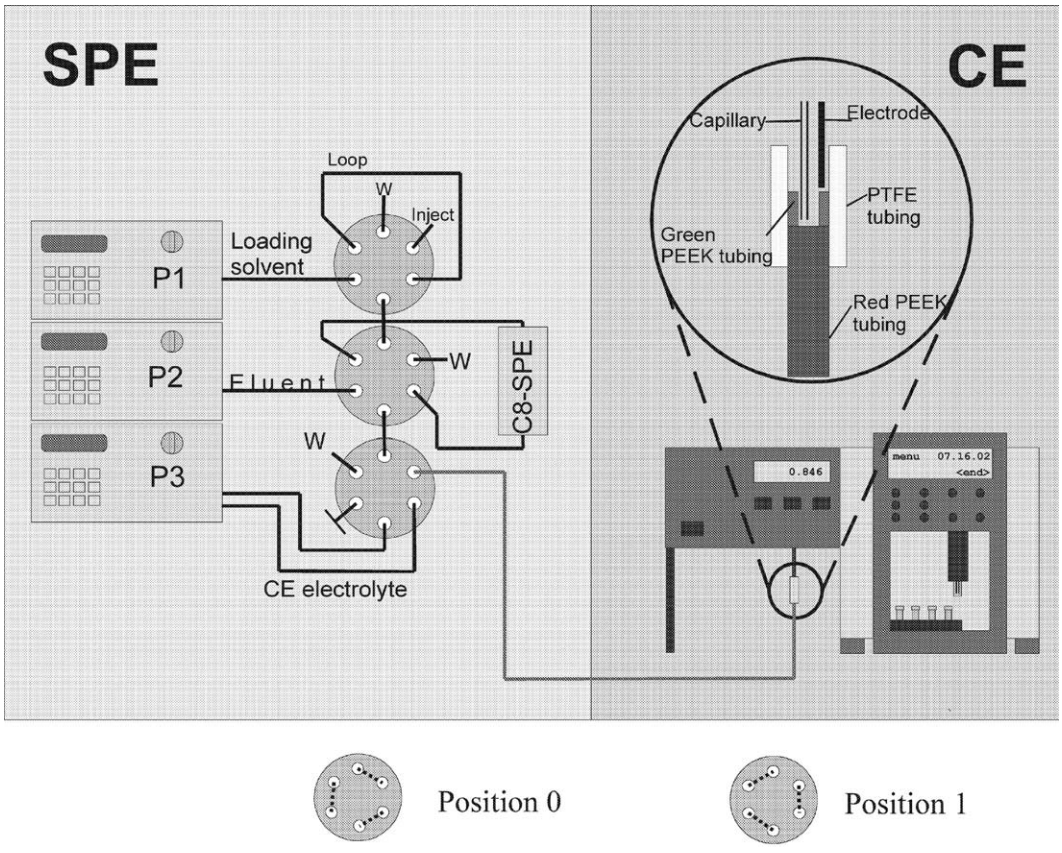

Fig. 2. Set up on-line SPE–CE.

Table 3
An overview of the on-line SPE–CE system

Step	Description	Pressure (mbar)	Voltage (kV)	Duration (min)	Position valves		
					V1	V2	V3
0	Start	0	0	0.00	1	1	0
1	SPE adsorption/conditioning of CE capillary	2000	0	3.00	0	1	0
2	Elution SPE	0	0	0.04	0	0	0
3	Injection of sample plug into CE	−50	0	0.60	0	0	0
4	Flush interface	0	0	1.50	0	0	1
5	CE separation	0	−30	16.0	0	0	1
6	Flushing CE capillary	2000	0	2.00	0	1	0

transferred to the SPE cartridge the CE capillary was flushed and conditioned with 50 mM sodium acetate with 850 mM CHES at pH 7.8 adjusted with TEA, which was used as CE electrolyte.

After loading of the sample on the SPE cartridge and washing of the cartridge with the loading solvent to remove salts from the sample, the sample was desorbed. This was done, in step 2, by switching V2, thus pumping the elution solvent, 60% 10 mM phosphate buffer pH 7.8/40% ACN, at 0.3 ml/min over the SPE cartridge via pump 2 (P2). The elution solvent transferred the sample via V3 to the interface.

When the sample plug passed the micro-injection vial in the interface, a hydrodynamic injection with −30 mbar was made into the CE, step 3. Only part of the SPE eluate was injected and the remaining liquid was flushed to waste. After valve V3 was switched (step 4), the interface was flushed with the CE electrolyte at 0.3 ml/min with the use of pump three (P3), after which the CE separation was started. Finally, during step 7, the capillary was flushed again and the valves were placed in the correct positions for the next injection.

2.3. System solvents

In the SPE system two solvents were used. The loading solvent was used to condition the SPE cartridge, transport the sample from the sample loop to the cartridge and to wash the SPE cartridge. The loading solvent was 10 mM phosphate buffer pH 7.8. The elution solvent used to elute the insulin from the SPE cartridge consisted of 40% ACN/ 60% 10 mM phosphate buffer pH 7.8. A 50 mM sodium acetate buffer containing 850 mM CHES adjusted to pH 7.8 with TEA was used as the CE electrolyte. All solutions were filtered (0.45 μm filters, Millipore) before use.

Insulin derivatives were dissolved in a 10 mM phosphate buffer (pH 11.5) at various concentrations unless stated otherwise.

The repeatability of the SPE procedure ($n = 5$) was determined using a 40 mg/l solution of AHI and BI. The linearity of the SPE procedure was determined performing a triple injection of a mixture of AHI and BI in the concentration range of 7–306 mg/l.

The repeatablity of the CE procedure ($n = 6$) was determined with an 80 mg/l mixture of AHI and bovine. The linearity of the CE method was determined using a triple injection of a mixture of AHI and BI in the range of 10–160 mg/l.

The repeatability of the on-line SPE–CE system ($n = 6$) was evaluated using a 100 mg/l mixture of AHI and BI. The linearity of this system was determined using six standards varying between 6 and 80 mg/l. Each standard was at least measured twice.

When the effect of the concentration of salt in the sample on the CE separation was determined a 27 mg/l solution of AHI and BI was compared with a solution with the same concentration of insulin derivatives and 140 mM of sodium chloride. When the effect of salt on the on-line SPE–CE system was tested, 90 mg/l solutions of AHI and BI in 10 mM phosphate buffer pH 11.5 with

varying salt concentrations between 4 and 340 mM sodium chloride were used.

To spike urine samples a 200 mg/l AHI and BI solution dissolved in 100 mM phosphate buffer pH 11.5 was used. To spike serum and plasma samples, a 200 mg/l solution of bovine and AHI in 25 mM of phosphate buffer pH 11.5 was used, unless stated otherwise.

2.4. Sample pretreatment

Aqueous samples were injected without further sample pretreatment.

2.4.1. Urine

Spiked urine samples were filtered over a 0.2 μm cellulose acetate filter (Schleicher & Schuell, 's Hertogenbosch, Netherlands) before injection. When further sample pretreatment before filtration was performed, the following precipitation procedure was used.

One ml of the earlier described 200 mg/l solution of BI and AHI was added to 9 ml of urine, thus giving the sample a final insulin concentration of 20 mg/l. Next, a precipitation step was performed by setting the pH to 7.8 or 11.5 with a few drops of a 3 M sodium hydroxide. When a blank sample was measured, the insulin solution was replaced with 1 ml of a 100 mM phosphate buffer pH 11.5. The solution was rested half an hour to settle the precipitate and injected after filtration over a 0.2 μm cellulose acetate disposable filter unit (Schleicher & Schuell). Each extract was at least injected three times.

Spiked urine samples with insulin concentrations from 5 to 80 mg/l, without the precipitation procedure, were used to test the linearity of the on-line SPE–CE method. All solutions were injected twice.

2.4.2. Serum and plasma

Sample preparation of serum and plasma samples involved a protein precipitation step with 50 or 60% ACN. The used procedures are listed in Table 4.

The serum or plasma, the 200 mg/l spike solution of the insulin derivatives and the ACN (Table 4) are mixed and divided over several Eppendorf tubes of 1.5 ml. Then, the mixture was centrifuged at 4 °C at 13 000 rpm for 10 min. The supernatant was transferred in 1500 μl portions to fresh Eppendorf tubes and the ACN in the sample was evaporated using a flow of 10 ml/min of dry nitrogen. The residue in each Eppendorf tube was diluted to 1500 μl with 25 mM phosphate buffer 11.5. Each extract was injected at least twice after filtering the sample using a 0.2 μm acetate cellulose filter (Schleicher & Schuell).

Table 4
Used procedures for protein precipitation of serum and plasma samples

	A (ml)	B (ml)	C (ml)
Bovine serum	1.00	1.00	
Human plasma			1.00
200 mg/l spike solution	1.00	1.00	1.00
ACN	2.00	3.00	3.00

3. Results and discussion

3.1. Aqueous samples of insulin derivatives:

Before the on-line system could be used for biological samples the SPE, the CE separation and the interface were optimized for aqueous samples. In the following sections the optimization will be briefly discussed.

3.1.1. SPE of insulin derivatives

Before the SPE could be coupled to the CE, the following parameters were evaluated: breakthrough, memory effects, linearity and repeatability of the elution times. Especially the last parameter was of importance. During the on-line SPE–CE procedure, the sample plug eluting from the SPE cartridge was injected via the microvial of the interface into the CE capillary. Because this was a timed process, the elution time should be as constant as possible.

The best repeatability was obtained using a buffered solvent system (10 mM phosphate pH 7.8) in the SPE procedure. The pH of 7.8 was chosen with respect to the CE separation, which

was performed at the same pH. Using a 10 mM phosphate buffer pH 7.8, all insulin derivatives were completely trapped onto the HP Hypersil MOS cartridge. For elution of the insulin derivatives in a single peak 40% ACN/60% 10 mM phosphate buffer pH 7.8 was used while the cartridge was placed in the backflush mode to reduce peak tailing.

The repeatability (n = 5) of the SPE procedure was established using a 500 μl injection of a 40 mg/l AHI and BI and the relative standard deviations (R.S.D.) were 1.8 and 1.3 for the retention time and peak area response, respectively.

The linearity of the SPE procedure was determined performing a triple injection of a mixture of AHI and BI in the concentration range of 7–306 mg/l. The linearity of the SPE procedure was 0.996 (Table 5).

3.1.2. CE of insulin derivatives

A separation for insulin derivatives has previously been described [8] and was used with some adjustments as described in Section 2. Negative injection pressures and negative voltages were used, because the CE separation was not performed in the standard direction, from sample tray to the outlet electrolyte vial, but in the opposite direction, from the outlet side of the CE apparatus to the sample tray. This was done in order to be able to use the home-made interface.

The repeatability (n = 6) was determined using a 80 mg/l mixture of AHI and BI and was 1.7% R.S.D. and < 3.3% R.S.D. for the migration time and peak area, respectively. The linearity of the CE method was determined using a triple injection of a mixture of AHI and BI in the range of 10–160 mg/l. The linearity for AHI and BI both were 0.999 (Table 5). The limit of detection (LOD) for both bovine and AHI was 10 mg/l.

3.1.3. On-line SPE–CE of insulin derivatives

The SPE and CE systems described in the previous sections could be coupled via a home-made interface (Fig. 2). The injection for the CE was different from the method described in the previous section. To obtain a representative sample from the SPE sample plug, CE injection was performed during the time the sample plug from the SPE passed the interface, which was 0.6 min. Also the injection pressure was changed to −50 mbar, the maximum pressure which could be used without overloading the capillary. Because the capillary encountered some backpressure in the interface, it was uncertain how much nl was injected precisely. In Fig. 3 an example of an electropherogram using the on-line SPE–CE system is shown.

The repeatability (n = 6) of the on-line SPE–CE system was evaluated using a 100 mg/l mixture of AHI and BI. The repeatable was 0.9 % R.S.D. and 4.8% R.S.D. for migration time and peak area response of both insulin derivatives, respectively.

When two systems are coupled the repeatability of the total system normally is dominated by the

Table 5
Linearity parameters of AHI and BI in the different procedures

Set-up	Insulin derivative	R^2	Slope	Standard error in slope	Intercept	Standard error in intercept
SPE	AHI and BI	0.996	113	1.44 (1.2%)	658	215 (3.3%)
CE	AHI	0.999	22.5	0.158 (0.70%)	−17.4	15.9 (91%)
	BI	0.999	25.8	0.259 (1.0%)	38.2	21.5 (56%)
SPE–CE	AHI	0.990	191	6.17 (3.2%)	−765	276 (36%)
	BI	0.991	211	6.18 (2.9%)	−524	283 (54%)
SPE–CE human urine	AHI	0.999	108	1.14 (1.1%)	−136	51.5 (38%)
	BI	0.999	131	0.789 (0.60%)	−106	34.9 (33%)
SPE–CE bovine serum	AHI	0.996	81.6	1.84 (2.3%)	236	88.3 (37%)
	BI	0.994	92.8	2.65 (2.9%)	62.9	139 (221%)
SPE–CE human plasma	AHI	0.990	78.6	2.25 (2.9%)	111	101 (91%)
	BI	0.992	56.9	1.79 (3.1%)	55.9	80.3 (144%)

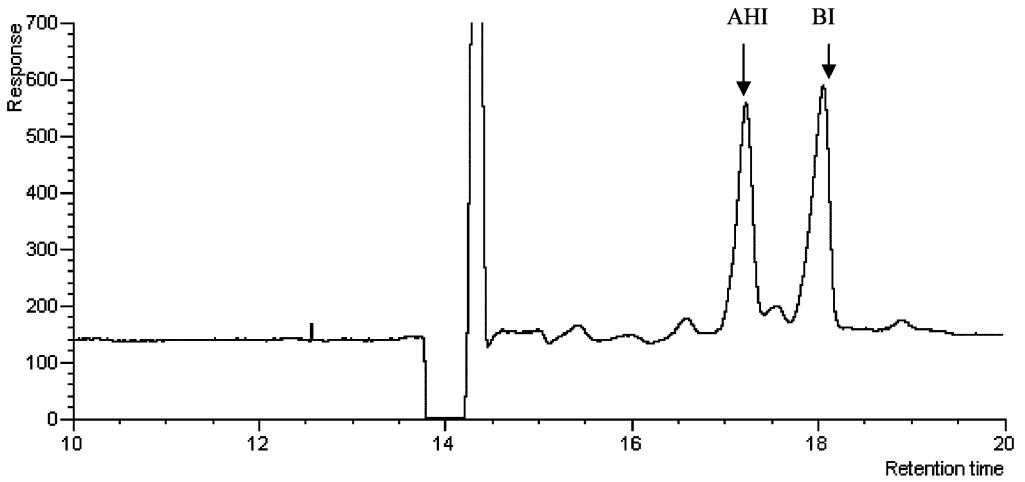

Fig. 3. Electropherogram obtained with the on-line SPE–CE procedure for a 80 mg/l mixture of AHI and BI.

system with the lesser repeatability. Here, the repeatability in retention time of the SPE and the CE separation were worse than the repeatability in migration time of the on-line SPE–CE system.

The improved repeatability of the on-line system compared with the SPE step alone could be explained by the fact that the timed plug, which was used for hydrodynamic injection to the CE system, was somewhat larger in volume than the elution plug of the insulin derivatives from the SPE. As a consequence, a representative part of the total elution plug was always injected into the CE. Therefore, the repeatability in the retention time of the SPE cartridge could be neglected and hardly contributed to the overall repeatability.

The better repeatability of the on-line SPE–CE system compared with the CE system was probably caused by the limited reproducibility of the manual injection, which was used when the CE was optimized compared with the totally automated injection in the on-line system. Injection in CE was made by manually switching the buffer vial for the sample vial and vice versa. The injection was performed in this mode, because CE was performed in a direction opposite to the one most used on the apparatus, see the experimental part, thus prohibiting the use of the sample tray.

The linearity of the system was determined using six standards varying between 6 and 80 mg/l. Each standard was at least measured twice. The linearity for AHI and BI were 0.990 and 0.991 (Table 5), respectively. The LOD (S/N = 3) was 2 mg/l. The total analysis time of the on-line SPE–CE system was 25 min.

3.2. *On-line SPE–CE of insulin derivatives containing various amounts of sodium chloride*

The on-line SPE–CE system among others was developed to be able to desalt biological samples. Therefore, the effect of sample ionic strength on the CE separation with or without an on-line SPE procedure was determined. When CE analysis of a 27 mg/l solution of AHI and BI dissolved in buffer was compared with a 27 mg/l solution of AHI and BI dissolved in buffer with 140 mg/l of sodium chloride several differences can be observed (Fig. 4). The resolution between BI and AHI in the sample containing sodium chloride was 1, while the resolution between these two insulin derivatives in a sample without sodium chloride was 1.25. The peak height of the insulin derivatives in the sodium chloride containing sample was 30% lower than the peak height of the insulin derivatives in a sample without sodium chloride.

When the same experiments were performed using the on-line SPE–CE set-up no differences in response was seen between samples with high

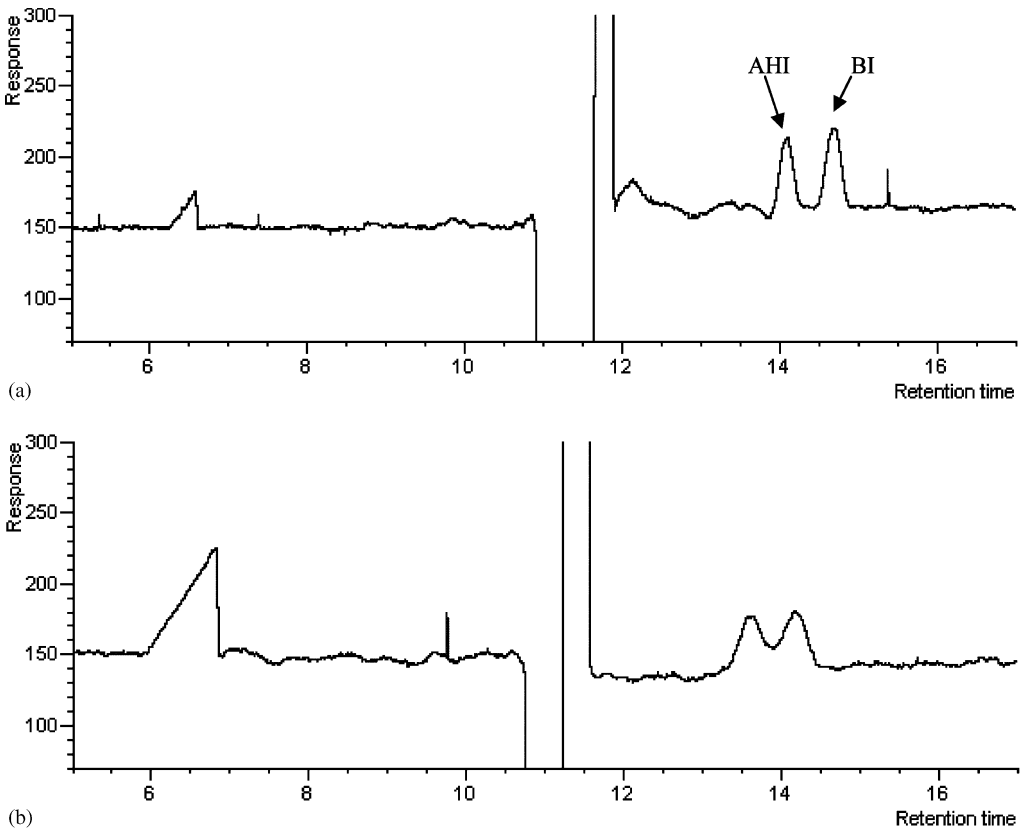

Fig. 4. Effect of salt concentration in the sample solution on the CE separation of two insulin derivatives. Electropherogram of a 27 mg/l solution of AHI and BI. (a) solved in 10 mM phosphate buffer pH 11.5, (b) solved in 10 mM phosphate buffer pH 11.5 and 140 mM sodium chloride.

amounts or low amounts of sodium chloride, proving the desalting ability of the system.

3.3. On-line SPE–CE of diluted urine spiked with insulin

The next step was to determine if the system was suitable for a low-protein containing biological matrix. Urine was chosen, because it contains a wide variety of components, but has a moderate peptide and protein content. Urine is a relevant matrix in this case since ca. 1% of the excreted insulin is present in urine [9].

When biological samples are injected into separation systems without any pretreatment, column clogging frequently occurs. Therefore, urine samples with and without a precipitation step were injected into the on-line SPE–CE system. Precipitation reactions at alkaline conditions were performed as described Section 2. Using a precipitation step before the injection reduced the background, especially when the precipitation was performed at pH 11.5. Instead of a precipitation step a simple filtration also provided acceptable results (Fig. 5). The repeatability ($n = 6$) of a urine sample spiked with 20 mg/l AHI and BI was 0.24% R.S.D. and $< 2.4\%$ R.S.D. for the retention time and peak area response, respectively. The linearity of urine samples spiked with an AHI and BI concentration varying between 5 and 80 mg/l, as described in Section 2, were 0.999 (Table 5) for both AHI and BI.

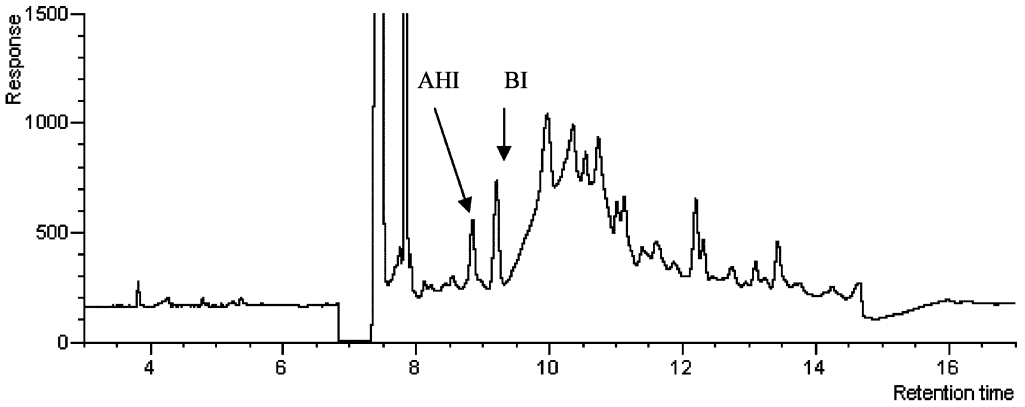

Fig. 5. Electropherogram of 20 mg/l BI and AHI spiked urine sample after on-line SPE–CE.

3.4. On-line SPE–CE of diluted bovine serum spiked with insulin

Serum and plasma contain many different peptides and proteins and as a consequence are a more challenging matrix to test the on-line SPE–CE system. Because serum and plasma are rather viscous, chromatographic analysis is mostly performed with diluted serum or plasma samples.

First of all, 5-fold diluted plasma was filtered and directly injected into the system, which caused blockage of the SPE cartridge. Therefore, it was concluded that an extra sample pretreatment step was necessary. Since ACN precipitation is often used [10], a 50% v/v and 60% v/v ACN precipitation procedure were studied, procedure A and B, respectively. Insulin derivatives do not precipitate using these amounts of ACN [11].

The ACN in the supernatant had to be removed before injection to retain the insulin derivatives onto the SPE cartridge. The ACN was evaporated using a gentle flow of nitrogen at room temperature. When the ACN was evaporated, phosphate buffer was added to retrieve the original sample volume. As can be seen in Table 4, in this way a 5-fold dilution of the plasma or serum was obtained.

Comparing the electropherograms using a 50 and 60% v/v ACN precipitation, (Fig. 6) using 60% v/v ACN clearly resulted in cleaner extracts. To avoid blockage of the SPE cartridge as much as possible further precipitations were conducted with 60% ACN v/v. The use of a precipitation step before injection increased the analysis time with 13 min.

The repeatability ($n = 6$) of the on-line SPE–CE system of a diluted serum sample spiked to 40 mg/l bovine and AHI was 0.64% R.S.D. and $< 6.2\%$ R.S.D. for the migration time and peak area response, respectively.

The linearity of the on-line SPE–CE method using a 5-fold diluted serum sample spiked between 5 and 80 mg/l bovine and AHI was 0.996 and 0.993 for AHI and BI, respectively (Table 5). The LOD ($S/N = 3$) of the system was 5 mg/l for both insulin derivatives.

This slight decrease in repeatability for the peak area response and the slight decrease in linearity compared with the urine samples were probably caused by the precipitation step. The precipitation was an off-line procedure, which is more prone to sample losses and lower repeatabilities than on-line pretreatment methods.

3.5. On-line SPE–CE of diluted human plasma spiked with insulin

Instead of bovine serum, human plasma was also spiked with insulin derivatives. The differences between bovine serum and human plasma are small. Before injection into the on-line SPE–CE system precipitation with 60% v/v ACN was conducted, via procedure C, as described in Section 2 (Fig. 7).

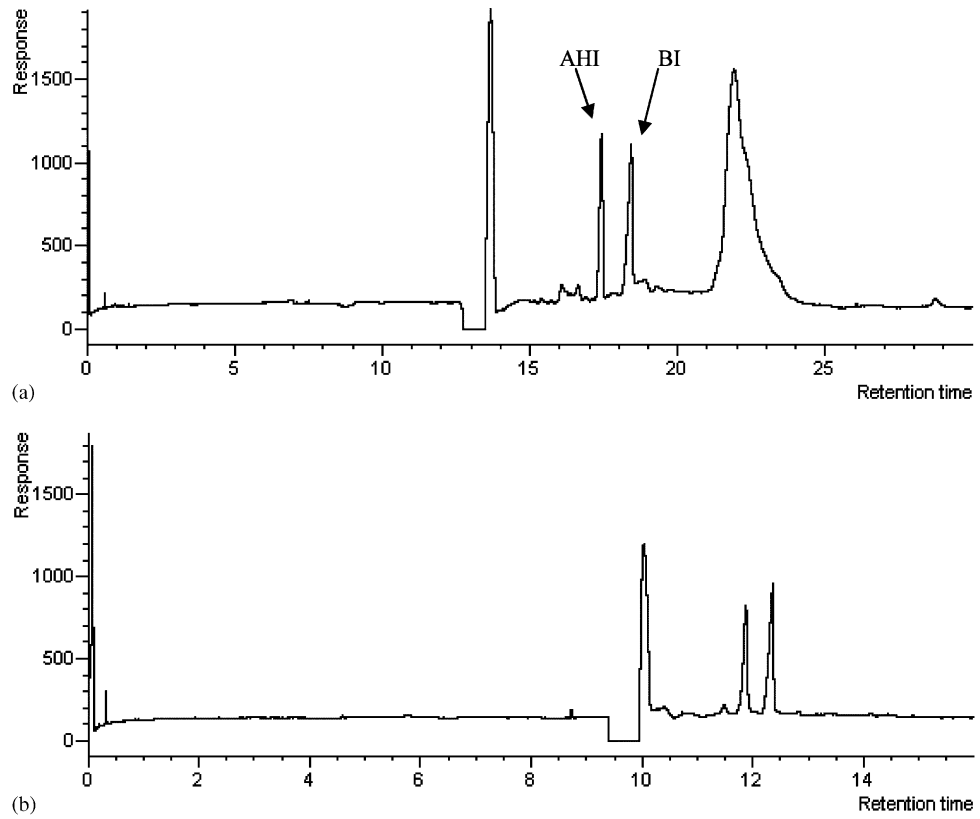

Fig. 6. Electropherogram of 40 mg/l BI and AHI spiked bovine serum sample after precipitation with (a) 50% ACN v/v, (b) 60% ACN v/v and on-line SPE–CE.

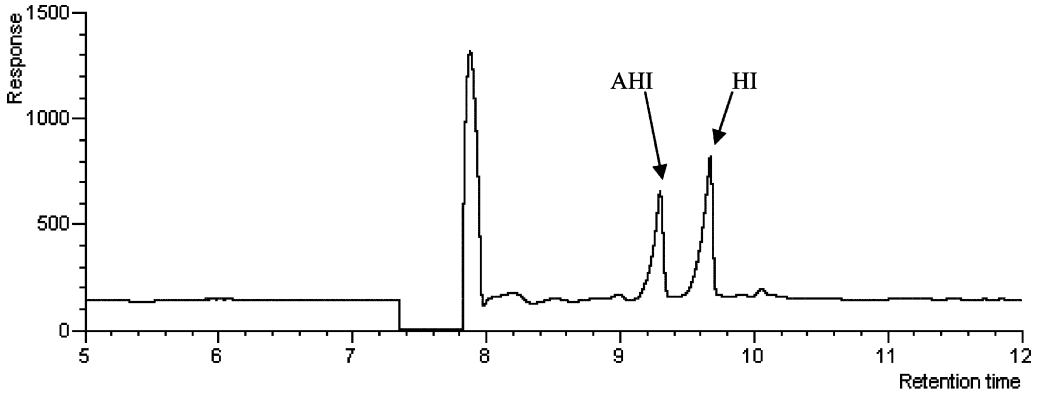

Fig. 7. Electropherogram of 40 mg/l HI and AHI spiked human plasma sample after precipitation with 60% ACN v/v and on-line SPE–CE.

The repeatability (n = 6) of a 5-fold diluted human plasma sample spiked to 40 mg/l AHI and BI was 0.65% R.S.D. and < 6.82% R.S.D. for migration time and peak area response, respectively, for both insulin derivatives.

The linearity of the system for 5-fold diluted human plasma samples was determined using 10–80 mg/l solutions of HI and AHI. HI was chosen instead of BI, because it was a better representation of a 'real life' sample. The precipitation reaction was performed via procedure C described in Section 2 with one exception. The insulin stock consisted of a 200 mg/l solution of HI and AHI in 10 mM phosphate buffer pH 11.5. The linearity was 0.989 and 0.992 (Table 5), respectively, for AHI and HI, respectively. The LOD (S/N = 3) was 5 mg/l for both insulin derivatives.

4. Conclusions

The described on-line SPE–CE could be used for the analysis of insulin derivatives in various biological matrices. When spiked urine samples were analyzed, a simple filtration sufficed before injection into the system, but when serum and plasma samples were analyzed, an off-line precipitation step had to be used before injection to prevent blockage of the SPE-cartridge. This precipitation step increased the total analysis time with 13 min.

Detection limits were 5 mg/l for HI, AHI and BI in urine, serum and plasma. The system was linear ($R^2 > 0.99$) (Table 5) from the LOD to 80 mg/l. The repeatability of the migration times were always good (< 1.0% R.S.D.). The repeatability of the peak area was better, < 2.4 versus < 6.2% R.S.D., when no precipitation reaction was used. The precipitation was an off-line procedure, which is more prone to sample losses and had a lower repeatability than on-line pretreatment methods.

Because the SPE device can be handled separately form the CE system, salts and other interfering sample compounds would not enter the capillary during SPE, as is the case with spPC-CE and mPC-CE. As a result, interfering compounds would not contaminate the capillary using the described on-line SPE–CE system. Consequently, the repeatability of the migration time and peak area of the described on-line SPE–CE system were superior over other on-line SPE–CE systems.

Better detection limits probably can be obtained by using the SPE not only for clean-up, but also for preconcentration. Miniaturizing the SPE system would lead to smaller elution volumes, and therefore, to the possibility for preconcentration. On the other hand would the maximal amount of material to be loaded onto the SPE cartridge decrease, increasing the possibility of breakthrough. The best chance to improve detection limits, therefore, lies in injecting a larger percentage of the SPE sample plug into the CE. Our focus will lie in this direction in further research.

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