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On-line SPE–RP-LC for the determination of insulin derivatives in biological matrices

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Abstract

An automated and on-line solid-phase extraction (SPE)–liquid chromatography (LC) procedure is described for the determination of insulin in biological matrices. The total procedure consists of two SPEs in series, followed by RP-LC separation. During the first SPE a strong anion-exchange (SAX) cartridge (ISOLUTE, 40–90 μm, 10 × 4 mm i.d.) is used, followed by a RP-cartridge (Luna C_8, 4 × 2.0 mm i.d.). The second SPE cartridge contains the same material as the LC column and is used to transfer the sample from the SAX cartridge to the LC column. The developed system can detect 100 nmol/l insulin in aqueous samples and 200 nmol/l insulin in spiked plasma samples using UV. When electrospray ionization (ESI)–mass spectrometry (MS), was coupled with the developed system, the LODs were lowered by a factor two to 50 and 100 nmol/l for aqueous and spiked plasma samples, respectively.

Keywords: SPE–LC; On-line; Plasma; Insulin

1. Introduction

Sample preparation techniques for peptides and proteins, such as precipitation and extractions, are frequently laborious and time consuming and, as a result, normally are the bottleneck in an analytical method. This especially is a disadvantage in clinical and pharmaceutical analysis where high-throughput analysis is a requirement. Therefore, significant effort is put into the development of automated sample pretreatment techniques, such as solid-phase extraction (SPE), which can reduce the analysis time considerably while, at the same time, they are less laborious.

In addition to automated sample pretreatment techniques, the emphasis is on integrating, preferably automated, sample pretreatment techniques and separation systems such as liquid chromatography (LC) and capillary electrophoresis (CE). In most cases a robotic arm or a transfer line is used to connect the two systems. The first type of system is called at-line the latter is called on-line. When no robotic interface or a transfer line is used, and the two systems are fully integrated, they are coupled in-line.

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The highest selectivity is obtained when the sample pretreatment and the separation system are based on different separation mechanisms, resulting in fewer problems with interfering components. Examples of different types of separation mechanisms, which are regularly used for peptide and protein research, are size (i.e. size-exclusion chromatography (SEC)), mass to charge (i.e. ion-exchange chromatography, CE, iso-electric focusing), affinity (i.e. affinity chromatography) and hydrophobicity (i.e. RP-LC).

In addition to extra selectivity and reducing cost and analysis time, hyphenated systems also reduce sample losses and minimize contamination problems. This is particularly important when the analytes are present in low concentrations.

Many different combinations of, automated, sample pretreatment and separation techniques are possible, but SPE–LC is the most popular due to its simplicity and versatility [14]. Many different sorbents for both SPE and LC are available, resulting in a wide range of different separation mechanisms which can be combined.

In this case, strong anion-exchange (SAX) SPE is coupled on-line to RP-LC to determine insulin derivatives in biological matrices. During the SPE procedure only negatively charged molecules will be retained while uncharged components are flushed away. Therefore, to retain insulin derivatives on a SAX cartridge the proteins need to possess an overall negative charge. Consequently, sorption onto the SAX cartridge should be performed above the isoelectric point (pI), the pH at which a solute has no net electric charge, of the insulin derivatives. The retained insulin derivatives will be desorbed when the net negative charge on the molecules is removed, which can be brought about by lowering the pH to the pI of the insulin derivatives, or even to a lower pH, which will result in net positively charged insulin derivatives. At that point, the insulin derivatives can be separated on differences in hydrophobicity using a reversed-phase LC column.

Insulin, see Fig. 1, is chosen as a test component for different reasons. First, it is a clinically and pharmaceutically interesting compound. Secondly, insulin can be considered both as a very large peptide and as a very small protein, making it an ideal test component to when developing automated sample pretreatment methods for peptides and proteins in biological matrices.

Many papers have been published about the LC determination of insulin or insulin related compounds, such as Arg-insulin [1], desamido-insulin [2,3] and *N*-carbomoyl-insulin [3], but only a few of these publications discuss the determination of insulin in 'real' samples such as serum, plasma, urine or cell extracts [4–7]. The reasons for this are that insulin is present in high pmol/l to low nmol/l concentrations in 'real' samples [4–7,13] and that as a consequence it is difficult to measure using the normally applied detection methods. Instead, to solve this limitation radioactivity detection is used, but even more frequently insulin in biological samples is determined by means of a radioimmunoassay [13]. The disadvantages of immunoassays are the cross-reactivity for several other insulin derivatives and they are time consuming. Secondly, insulin is also interesting from a more industrial point of view, i.e. impurity research of insulin containing drugs. In this case, the concentrations are significantly higher, often in the mg/ml range, and UV absorbance frequently suffices.

Most LC separations of insulin derivatives are performed on a RP column using acidic conditions with a pH of about 2.0. Buffer systems normally contain either a phosphate buffer [2–4] containing sodium sulfate or 0.1% trifluoroacetic acid (TFA) [1,7,11,12] as an ion-paring agent, both in combination with acetonitrile (ACN).

Besides RP-LC, immuno-affinity separations have been published by Muller et al. [8–10] using a phosphate buffer containing sodium chloride to desorb insulin from the column.

When biological samples containing insulin are analyzed, several sample pretreatment methods have been used: SEC [7], immuno-affinity extraction [5] and RP-SPE [4,6]. Sample clean up is usually combined with a sample concentration step. So far only off-line procedures have been described.

In the present manuscript an on-line system using ion-exchange SPE and RP-LC to determine insulin will be described using UV absorbance and mass spectrometry (MS) as a means of detection.

Fig. 1. Amino acid sequence and disulfide bridges of BI.

2. Experimental

2.1. Chemicals

Bovine insulin (BI), porcine insulin (PI), human insulin (HI), Arg-human insulin (AHI), human plasma and TFA were purchased from Sigma–Aldrich (Zwijndrecht, the Netherlands). Ammonium hydroxide was obtained from Baker (Deventer, the Netherlands). Sodium dihydrogen phosphate came from Merck (Darmstadt, Germany). Perchloric acid, isopropanol, sodium chloride, sodium hydroxide and disodium hydrogen phosphate were purchased from Riedel-de-Haën (Seelze, Germany). ACN and methanol (Baker) were HPLC-grade and filtered (0.45 μm filters, Millipore, Bedford, MA, USA) before use. High purity water obtained from a Milli-Q system was used for all experiments (Millipore).

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 also quite similar.

Table 1
Differences in amino acid of insulin derivatives

	MW (u)	Positions in amino acid sequence where differences occur			
		A8	A10	A18	B30
BI	5733.5	Ala	Val	Asn	Ala
PI	5777.6	Thr	Ile	Asn	Ala
HI	5807.6	Thr	Ile	Asn	Thr
AHI	5963.8	Thr	Ile	Asn	Thr

2.2. System set-up

The on-line system, shown in Fig. 2, consists of two SPEs and a LC separation connected in series. The two SPE cartridges are part of the sample preparation system, the second SPE cartridge and the LC column are part of the separation system.

The sample preparation system consists of a 234 autoinjector (Gilson, Villiers-le-Bel, France), with a 100 μl PEEK (Bester, Amstelveen, the Netherlands) sample loop, and a Prospekt II on-line SPE system (Spark Holland, Emmen, the Netherlands).

The Prospekt II system contains a high-pressure dispenser (HPD) and an automated cartridge exchanger (ACE) in which 96 well plates containing 10×4.0 mm i.d. Prospekt II cartridges packed with 40–90 μm ISOLUTE SAX material (Separations, H.I. Ambacht, the Netherlands) are placed.

The HPD is connected via the autoinjector (V1) to valve two (V2) of the ACE, which is connected with the clamp in which the SAX cartridge is placed. This valve is also connected to valve three (V3), which, in its turn, is connected to the second 4×2.0 mm i.d. RP-SPE cartridge (Luna C_8 guard column, Phenomenex, Bester) and the LC system as can been seen in Fig. 2. All tubing used in the SPE system is blue PEEK (1/16 in. o.d., 0.25 mm i.d., Bester) except for the tubing between V3 and the second SPE column, which is red PEEK (1/16 in. o.d., 0.13 mm i.d., Bester).

The second system is a gradient LC system in which insulin derivatives are separated and detected. The second SPE cartridge is part of both the SPE and the LC system. After the sample is loaded onto this RP-SPE, the RP-SPE is conditioned and is switched in-line with the gradient LC-system. The LC system operates with two Shimadzu LC10AD*VP* pumps with mixer ('s-

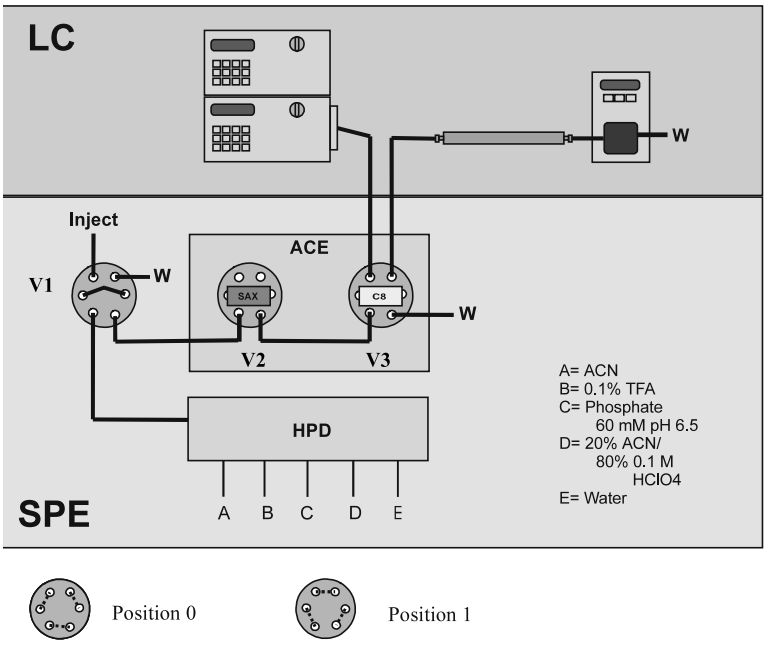

Fig. 2. Schemetically set-up of on-line SPE–RP-LC system.

Hertogenbosch, the Netherlands), a 100 × 2.0 mm i.d. Phenomenex Luna 3 μm C8(2) column (Bester) kept at 30 °C in a Shimadzu CTO-10AS*VP* column oven ('s-Hertogenbosch, the Netherlands) and a Spectroflow 757 Kratos absorbance detector set at 200 nm (Separations). All tubing used in the LC system is red PEEK.

The accompanying method used for this set-up is shown in Table 2 and will be described in the following sections.

Before the analysis was started, 0.7 ml polypropylene vials containing the sample solution were placed in the autoinjector. Prior to injection the injection valve and the syringe were flushed with 500 μl 20% ACN/ 80% 60 mmol/l phosphate buffer pH 6.5 at 0.3 ml/min to make sure no insulin was adsorbed onto the autoinjector tubing and syringe.

When the method was started, first the RP-SPE was conditioned with 2 ml ACN using a flow of 1

Table 2
An overview of the on-line SPE–LC method

Step	Description	Valves			Solvent	ml
		Injector	SAX	C_8		
1	Conditioning RP-SPE	0	0	1	ACN	2
2	Conditioning SAX	0	1	0	60 mmol/l phosphate pH 6.5	3
3	Injection	1	1	0	60 mmol/l phosphate pH 6.5	3
4	Wash SAX	0	1	0	Water	0.5
5	Elution SAX, begin gradient	0	1	1	20% ACN/80% 0.1 mol/l $HClO_4$	0.5
6	Wash RP-SPE	0	0	1	0.05% TFA	1.5
7a	Elution RP-SPE, LC analysis	0	1	0	LC gradient	–
7b	Reconditioning SAX	0	1	0	60 mmol/l phosphate pH 6.5	2
8	Preparation for next analysis	0	0	1	–	–

ml/min. During this step no solvent was flushed over the SAX cartridge. While the SAX cartridge was conditioned with 3 ml 60 mmol/l phosphate buffer pH 6.5 using a flow of 1 ml/min, step 2, the RP-SPE was switched in-line with the LC-system. The LC-system was pumping at the initial conditions of the gradient, 95% 0.05% TFA/5% ACN with a flow of 0.2 ml/min. As a result, the RP-SPE was conditioned to these initial conditions. When both cartridges had been conditioned, 100 μl sample could be injected onto the SAX cartridge at 1 ml/min with 3 ml 60 mmol/l phosphate pH 6.5 as carrier solvent. While injection took place, the RP-SPE stayed in-line with the LC-system to make sure non-retained sample components were flushed to waste instead to the second SPE cartridge. The larger part of phosphate buffer was flushed away by washing the cartridge, step 4, with a 500 μl plug of water using a flow of 1 ml/min after which the RP-SPE was switched back to the SPE-system. After eluting the SAX cartridge with 500 μl 20% ACN/80% 0.1 mol/l perchloric acid using a flow of 0.2 ml/min, step 5, the insulin was trapped on the RP-SPE cartridge. Next, the RP-SPE cartridge was washed and conditioned with 1.5 ml 0.05% TFA using a flow of 1 ml/min, step 6. This 1.5 ml 0.05% TFA was not flushed over de SAX cartridge. After the RP-SPE had been conditioned, it was switched in-line with the LC-system followed by LC separation, using the gradient described in the next section, and detection. During LC analysis, the SAX cartridge was reconditioned for the next injection by flushing it with 2 ml phosphate buffer using a flow of 1 ml/min. Finally, the valves were switched to their initial positions for the next analysis. Data acquisition was performed using ATLAS (version 3.59a, Labsystems, Altrincham, UK).

2.3. On-line SPE–RP-LC–UV-MS

A single quadrupole MS (LCMS-2010, Shimadzu, 's-Hertogenbosch), was coupled to the system described in the previous section. It was interfaced with the LC system via an electrospray ionization (ESI) interface. The probe voltage was set to 4 kV, nitrogen nebulizer gas flow rate was 4.5 l/min, the temperature of the curved desolvation line (CDL) and the block was set to 250 °C, a CDL voltage of 10 V was used and, finally, the Q-array voltages were set as optimized in a tuning experiment. To control the LCMS-2010, for data handling and acquisition the Shimadzu LCMSSOLUTIONS software was used (Shimadzu, 's-Hertogenbosch) When the LCMS was operated in scan mode, the system scanned with 1000 mass units/s between m/z values of 1100 and 2000. Single ion monitoring (SIM) was conducted on eight channels with the following m/z values: 1147.20, 1156.00, 1162.00, 1192.90, 1433.70, 1444.70, 1452.30 and 1491.40.

2.4. Solutions

A gradient using a flow of 0.2 ml/min was formed using the following solutions: solvent A, which consisted of 95% 0.05% TFA/5% ACN, and solvent B, which consisted of 95% ACN/5% 0.05% TFA. The used gradient is depicted in Table 3. In the third column the actual percentage of ACN has been given. The gradient was started in the beginning of step 5 (see Table 2) to make sure the column was properly conditioned at 24% B before the RP-SPE was switched on-line with the LC column. The separation took place in the isocratic part of the gradient between 1 and 14 min.

Before 0.05% TFA was used, three other buffer systems were tried: a 40 mmol/l phosphate buffer pH 2.5, 0.1% TFA pH 2.5 adjusted with ammonium hydroxide and 0.04% TFA, all mixed with ACN. The used gradient was similar to the gradient described in Table 3 with two differences: instead of 24% B, 27% B was used and the gradient was started at 27% B instead starting the gradient at 0%B when 0.1% TFA pH 2.5 was used. When

Table 3
Gradient used in the on-line SPE–LC method

Time (min)	B (%)	ACN (%)	Flow (ml/min)
0.01	0	5	0.2
1.01	24	26.6	0.2
14.01	24	26.6	0.2
17.01	50	50	0.2

0.04% TFA was used, instead of 24%B, 23.5%B was used in the gradient.

During the SPE procedure five different solvents were used, as can be seen in Table 2. ACN was used to clean the RP-SPE column and 0.05% TFA was used to condition or wash the RP-SPE column. For the SAX column 60 mmol/l phosphate buffer pH 6.5 was used to condition the cartridge and load the sample onto the column, water was used to wash the cartridge before elution took place with 20% ACN/80% 0.1 mol/l $HClO_4$.

All insulin solutions used were dissolved in 20% ACN/ 80% 60 mmol/l phosphate buffer pH 6.5 unless stated otherwise. An insulin mix containing 1.25 μmol/l of BI, HI, AHI and PI, solved in 20% ACN/ 80% 60 mmol/l phosphate buffer pH 6.5, was used for spiking plasma samples.

2.5. Sample preparation

Aqueous samples were injected into the system without any pretreatment. Direct injection of plasma samples was performed with human plasma spiked with a 1.25 μmol/l insulin mix (BI, AHI, HI and PI), solved in 40% ACN/60% 60 mmol/l phosphate pH 6.5, to obtain a final ACN concentration of 20% (1:1 v/v).

Sample preparation of plasma samples before injection involved protein precipitation with 60% ACN. Four different procedures where used (Table 3), one for a higher, 625 nmol/l (A), and three for a lower, 300 nmol/l (B), 200 nmol/l (C) and 100 nmol/l (D), spiking concentration of insulin derivatives. Insulin spiked plasma samples (100 nmol/l) (D), were only used in the MS experiments. In all procedures the human plasma was 8-fold diluted.

The in Table 4 described amounts of plasma, the 1.25 μmol/l mix of all four insulin derivatives, 20% ACN/ 80% 60 mmol/l phosphate buffer pH 6.5 and ACN were mixed in an Eppendorf tube of 1.5 ml. Then, after shaking vigorously the mixture was centrifuged at 4 °C at 13 000 rpm for 10 min. The supernatant was transferred to a new Eppendorf tube and was evaporated with the aid of a nitrogen flush at room temperature. The residue was reconstituted in 500 μl 20% ACN/80% 60 mmol/l phosphate pH 6.5. All precipitations were performed in duplo and each extract was injected twice.

3. Results

3.1. LC-system

With respect to the data described in the literature, a reversed-phase column was chosen for the separation of insulin derivatives. The Phenomenex Luna C_8 was chosen because of its excellent endcapping, which is necessary to avoid poorly reversible adsorption of insulin to the free silanol groups resulting, its wide pH range and its suitability for biomolecules.

BI, PI, HI and AHI, were separated from each other using two different buffer systems, phosphate and 0.1% TFA both at a pH around 2.5, which gave comparable results. BI could be baseline separated from the other three insulin, which were only partly separated.

Although detection was performed with an UV absorbance detector during the optimization of the SPE–LC system, eventually MS detection will be used. Therefore, the chosen buffer system needs to be MS compatible. Phosphate is not compatible with LC–MS. TFA is a volatile compound, but on the other hand it is known as an ion-suppressing reagent and is not preferred in MS. Still, the ion-pairing abilities of TFA combined with its acidity are necessary to obtain a separation of the insulin derivatives. The TFA buffer was chosen for further method development. The repeatability ($n = 6$) was: $< 1.7\%$ R.S.D. and $< 5\%$ R.S.D. for retention time and peak area, respectively. The limit of detection ($S/N = 3$) for all four insulin derivatives was around 1 μmol/l (ca. 6 μg/ml of insulin).

To avoid problems with ion-suppression in MS, lower amounts of TFA had to be considered. In literature [6] good results were obtained with 0.04% of TFA, but the separation of the four insulin derivatives turned out to be insufficient. An increase of 0.01 to 0.05% TFA gave a workable resolution between BI and AHI and a reasonable resolution between HI and PI (Fig. 3). This

Table 4
Amounts of human plasma, insulin derivatives, phosphate buffer and ACN used in protein precipitation procedure

	Human plasma (μl)	1.25 μmol/l insulin mix (μl)	20% ACN/80% 60 mmol/l phosphate (μl)	ACN (μl)	Insulin concentration in plasma solution (nmol/l)
A	62.5	250	187.5	750	625
B	62.5	120	317.5	750	300
C	62.5	80	357.5	750	200
D	62.5	40	397.5	750	100

concentration was used in all further experiments. Separation took place in the isocratic part of the gradient between 1 and 14 min. Using an increasing percentage of ACN in this part of the gradient decreased the resolution of the insulin derivatives. The repeatability ($n = 7$) was: $< 3.6\%$ R.S.D. and $< 6\%$ R.S.D. for retention time and area, respectively. The limit of detection ($S/N = 3$) for all four insulin was around 0.3 μmol/l (ca. 2 μg/ml of insulin).

3.2. SPE-system

For the SPE system a Prospekt II consisting of a HPD and an ACE, as described in the Section 2, was used. The HPD is a syringe pump, which has an inlet valve for six different solvents and, therefore, can mimic an off-line SPE procedure in which often a number of different solvents are used. The ACE consists of four automated six-port switching valves of which two are connected to clamps in which a robotic arm can place cartridges from two 96-well plates placed in the ACE.

Cartridges containing different RP materials, from cyano to polymeric hydrophobic materials, were tested. Unfortunately, even the RP material with the lowest affinity for the insulin derivatives, the C_{18} cartridge, had a higher affinity than the Luna C_8 column. In other words, more ACN was needed to desorb insulin derivatives from the SPE cartridges than was needed to separate them on the LC column, resulting in elution of the insulin derivatives with the dead volume of the column. The high affinity is probably due to the strong adsorption of insulin to the free silanol groups, which was supported by the observed tailing when eluting the RP-SPE cartridges and by observed memory effects.

Using a SPE that is based on a different separation mechanism than the LC, adds extra selectivity into the system as described. Therefore, a SAX material was tested. The LC separation was performed at a low pH and when a SAX cartridge was used, elution was also performed at low pH. As a result, a better compatibility exists between the SPE and the LC system when SAX and LC are coupled as when cation-exchange SPE and the LC

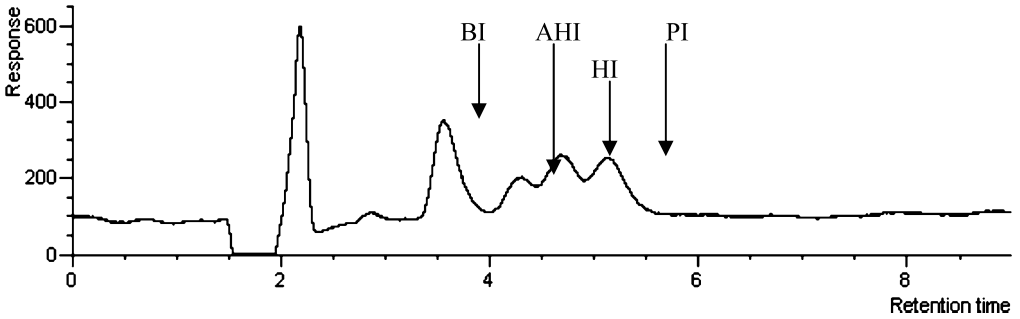

Fig. 3. RP-LC separation of a 1.25 μmol/l mix of four insulin derivatives (BI, AHI, HI and PI) using a 0.05% TFA/ACN gradient. Insulin derivatives were solved in 0.1% TFA pH 2.5.

system are coupled. When a cation-exchange SPE would be used, sorption would be performed at a low pH and desorption would be performed at a high pH. As a consequence, the analytes would be transferred to the LC column in a high pH environment. When the LC separation is performed at low pH, the differences between the pH in the sample plug and the gradient pH are very large, resulting in irreproducible separations.

As is generally known, the loadability of ion-exchange materials is lower than the loadability of RP materials. The amount of insulin that could be loaded onto the anion-exchange material without detecting breakthrough was 0.2 nmol corresponding to 1.2 μg insulin, which was considerably less than the amount which could be loaded onto the RP-SPE. This limits the upper concentration range that can be reached in this method.

When optimizing the SAX procedure several parameters were varied: elution pH, percentage of acetonitrile in the elution solvent, sorption pH and the ion strength of the sorption solvent.

First, the elution pH was varied while retaining insulin derivatives at a high pH onto the SAX cartridge. A 0.1 mol/l perchloric acid solution (pH 1.3) was diluted with water to pH 1.5, 2.0 and 2.5 for this experiment. When the pH of the elution solvent was 2.5 hardly any insulin was recovered from the cartridge and the highest recovery was obtained using 0.1 M perchloric acid (Fig. 4a). A 1 μmol/l solution of BI in sodium hydroxide (pH 11.5) was used in these experiments and a sodium hydroxide solution of pH 9.5 was used to retain the insulin onto the SAX cartridge.

Next, the following amounts of ACN were added to the elution solvent to study the recovery: 0, 20, 40 and 60% v/v. The highest recovery was obtained when between 20 and 40% ACN was added to the elution solvent (see Fig. 4b). Since the SAX had to be coupled to the LC column, a 20% ACN/ 80% 0.1 mol/l perchloric acid v/v solution was used to elute the insulin derivatives from the cartridge. A blank chromatogram was obtained if, after elution of insulin under these optimal conditions, a second elution step was performed. This result indicated complete elution of insulin from the cartridge and the absence of memory effects. Again, a 1 μmol/l solution of BI in sodium hydroxide at pH 11.5 was used as the sample and a sodium hydroxide solution pH 9.5 was used to retain the insulin derivatives on the SAX cartridge.

Thirdly, the pH and ion strength of the sorption solution were varied. To ensure a constant pH and a large usable pH range, a phosphate buffer was chosen between pH 5 and 11 and between 20 and 60 mmol/l. At first, a 1 μmol/l solution of BI in sodium hydroxide at pH 11.5 was used as sample. Elution was performed with the optimal elution solvent determined in the previous section: 20% ACN/80% 0.1 mol/l perchloric acid.

The highest and most repeatable peaks were obtained with 60 mmol/l phosphate buffer of pH 6.5, but the repeatability was not sufficient probably due to secondary adsorption effects of insulin to tubing and valves, which was concluded from the observed decreasing peak areas and heights during consecutive measurements. Still, the insulin was solved in a 60 mmol/l phosphate pH 6.5,

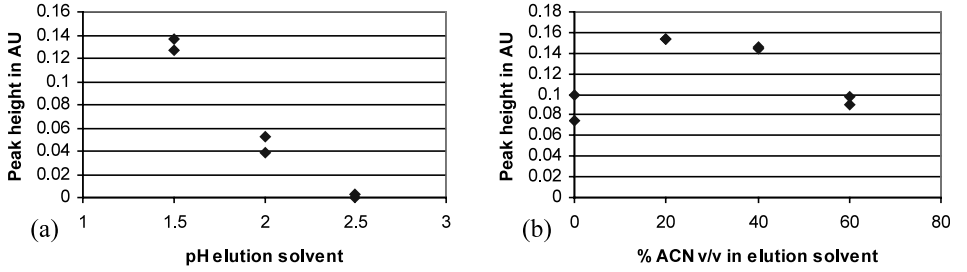

Fig. 4. Optimization of the elution solvent for SAX. (a) pH of the elution solvent vs. peak height. (b)% ACN mixed with 0.1 mol/l perchloric acid vs. peak height. Sample: 1 μmol/l BI in sodium hydroxide pH 11.5.

instead of a sodium hydroxide solution at pH 11.5, from this point on. The sample solvent was changed to obtain a more simple and, accordingly, more stable system.

As mentioned in the previous section, using a 60 mmol/l phosphate buffer at pH 6.5 as the sorption solvent resulted in decreasing peak heights during consecutive measurements. To obtain stable peak areas during consecutive measurements two techniques were explored: adding salt or adding organic modifier to the 60 mmol/l phosphate pH 6.5 buffer. Adding various amounts, up to 400 mmol/l, of sodium chloride did only increase the repeatability slightly. Adding small amounts, 5% v/v, of organic modifiers such as isopropanol, methanol and ACN also improved repeatability slightly but still not to acceptable values. Only after the addition of 20% ACN to the phosphate buffer the repeatability was satisfactorily. When 20% of ACN was added to the sample, ACN was no longer necessary in the sorption buffer to obtain repeatable results. Therefore, it is assumed that secondary adsorption affects of insulin to tubing and valves are eliminated when 20% ACN is added. From this moment on 60 mmol/l phosphate pH 6.5 was used to retain insulin to the SAX cartridges and the insulin was solved in 20% ACN/60 mmol/l phosphate pH 6.5.

Also, to avoid adsorption of insulin to the tubing and stainless steel valve of the autoinjector, the autoinjector was washed with 20% ACN/60 mmol/l phosphate pH 6.5 between injections. When no ACN was used, and the autoinjector was flushed only with phosphate buffer, peak areas and heights decreased during consecutive measurements, as was discovered in the optimization of the SPE procedure.

To remove most of the, involatile, phosphate before elution of the insulin, a short washing step was performed before the elution. No breakthrough of insulin was seen during this washing step (data not shown).

The elution volume of the optimum elution solvent (20% ACN/80% 0.1 M perchloric acid) was varied between 250 and 1250 μl. The optimum elution volume was 500 μl. Increasing the elution volume did not significantly increase the area response, but increased the analysis time. The optimized solutions, as determined and described in the previous sections, were used in the SAX procedure.

3.3. On-line SAX-LC

The optimized SPE method could, unfortunately, not be coupled directly to the LC system. The low pH of the elution plug resulted in elution of the insulin with the dead volume of the column. The following approach solved this problem: the guard column of the LC column was used as a second RP-SPE device, coupled on-line to the SAX cartridge. This was accomplished by washing the RP-SPE, after the elution plug of the SAX cartridges was flushed over it, with 1.5 ml of 0.05% TFA. This was performed to wash away the perchloric acid, but to retain the insulin. After this washing step, the RP-SPE cartridge is switched in-line with the LC column where the insulin will be desorbed from the RP-SPE during the gradient. Using this approach a more versatile SPE–LC is realized, which could be used to couple SPE and LC procedures, which are not directly compatible.

First, it was studied if insulin in 20% ACN/ 80% 0.1 mol/l perchloric acid was indeed trapped completely onto the RP-SPE by testing this for BI. The maximal amount of ACN mixed with 0.05% TFA the C_8 SPE could be washed with before insulin was desorbed, was circa 20%. BI was, in this experiment, solved in 0.1 mol/l perchloric acid. Although, these conditions are not completely similar to the conditions used in the SAX-LC procedure, it may be assumed that the same applies for insulin solved in 20% ACN/ 80% 0.1 mol/l perchloric acid. The latter represents the condition in which insulin is sorbed onto the RP-SPE.

BI, solved in 20% ACN/80% 0.1 mol/l perchloric acid, was completely trapped onto the column. When a calibration curve between 0.4 and 4 μmol/l was measured, the linearity was good. Therefore, the RP-SPE could be used to transfer the insulin from the SAX cartridge to the LC.

3.4. On-line SPE–LC system for insulin in aqueous solutions

The total system was tested for repeatability and linearity using the optimum conditions. Insulin derivatives (BI, AHI, HI and PI) were dissolved and were sorbed onto the SAX cartridges conditioned with 60 mmol/l phosphate buffer (pH 6.5). After sorption, the SAX cartridge was washed with a small plug of water. Next, the insulin derivatives were desorbed with 20% ACN/80% 0.1 mol/l perchloric acid. After starting the elution step with perchloric acid was, the gradient was also started, even though the insulin were not loaded onto the RP-SPE cartridge yet. The reason for starting the gradient before switching the RP-SPE cartridge was to condition the LC column to the isocratic separation conditions, 24% B, before the separation was started to obtain a good repeatability. The perchloric acid plug was transferred over the RP-SPE, where the insulin derivatives were sorbed. Afterwards, the RP-SPE was conditioned to 0.05% TFA. Finally the insulin were desorbed during the gradient and separated on the LC column. An example of a chromatogram obtained using the developed method can be seen in Fig. 5.

The repeatability ($n = 6$) at a insulin concentration of 500 nmol/l of this method was $< 3\%$ and $< 6\%$ for the retention time and area response, respectively. The linearity between 100 and 1250 nmol/l was $R^2 > 0.99$, except for HI, which had a linearity of $R^2 > 0.98$. The limit of detection ($S/N = 3$) of the system was 100 nmol/l (ca. 0.6 μg/ml of insulin).

3.5. On-line SPE–LC system for insulin spiked human plasma

The same conditions as described in the previous section were applied for these experiments. When human plasma spiked with a 1.25 μmol/l insulin mix, solved in 40% ACN/60% 60 mmol/l phosphate (pH 6.5), (1:1 v/v) was injected directly into the system a recovery of only 10% was obtained. Several explanations for this low recovery can be given. First, as mentioned before, the capacity of an ion-exchange cartridge is relatively low. When insulin in plasma is injected the total amount of peptides and proteins present is much higher than the total amount of material that can be sorbed to the cartridge. As a result, only part of the total amount of protein and peptides, and thus only part of the insulin, is trapped onto the column, while the greater part is breaking through and flushing to waste. Secondly, insulin will bind to other proteins in plasma and consequently will not bind to the SAX material, resulting in lower recoveries. Both explanations point to the same solution of the problem: removing the larger part of the plasma proteins. This can be achieved by performing an extra sample pretreatment step prior to injection in the SPE–LC system.

It was decided to use protein precipitation to remove a large part of the plasma proteins. Insulin is known not to precipitate in ACN/water solutions up to 66% v/v ACN. Many other proteins will precipitate under these conditions, though, and therefore, a precipitation step using high amounts of ACN can be used in this case to

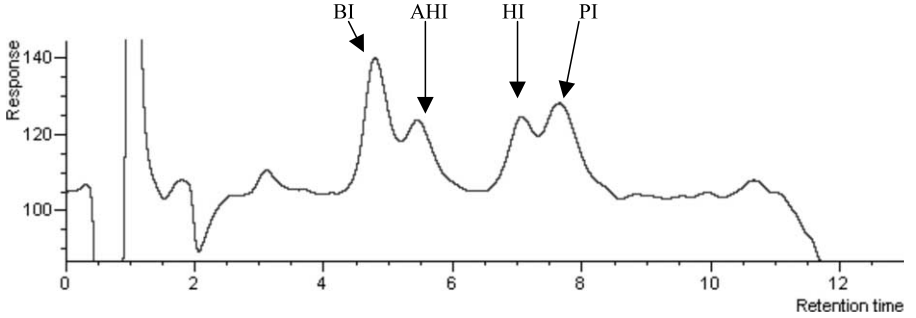

Fig. 5. On-line SPE–RP-LC chromatogram of a 500 nmol/l mix of four insulin derivatives: BI, AHI, HI and PI insulin.

remove a large part of the plasma proteins from insulin.

Most of the plasma proteins could be removed via precipitation with 60% ACN. Insulin was spiked to a diluted plasma solution in three concentrations, 625; 300 and 200 nmol/l. The recovery of the insulin derivatives after protein precipitation was around 52%. These low recoveries were probably due to protein interactions. This assumption was supported by several results. First, when the plasma was diluted 8-fold higher recovery of the insulin derivatives was obtained. When relatively a smaller part of the plasma solution consists of plasma, a lesser degree of protein interactions is to be expected. Secondly, the recoveries of the three spiked concentrations were different as can be seen in Table 5. Lower recovery generally was obtained at a lower insulin concentration. At a lower insulin concentration a relatively larger part of the insulin present will participate in protein interactions, hence reducing the relative amount, i.e. the recovery, of insulin measured. Thirdly, performing the precipitation in an alkaline or acidic medium did not increase the recovery, again indicating secondary adsorption or protein–protein interactions were an issue. Chromatograms, measured after protein precipitation using plasma samples spiked to a 625 and 200 nmol/l level of the insulin derivatives, can be seen in Fig. 6. As can be seen, the resolution between the four insulin derivatives is somewhat less than obtained using aqueous solutions. This is presumably caused by irreversible adsorption of plasma matter to the column material. The limit of detection ($S/N = 3$) of this system was 200 nmol/l (1.2 μg/ml). No interferences were seen in the separation window in which the four insulin derivatives were determined.

3.6. On-line SPE–RP-LC–UV–MS

The developed system was coupled to MS to obtain more qualitative information and to reach lower LODs. Before the system could be coupled to the MS, some MS parameters needed to be optimized and the m/z values for SIM needed to be determined.

MS parameters such as CDL temperature, CDL voltage, block temperature, probe voltage and nebulizer gas flow were optimized during infusion experiments of a 500 nM BI solution in 25% ACN/75% 0.05% TFA. The latter solvent was used to mimic the LC separation. The used optimized parameters can be found in the Section 2. Also MS spectra between m/z 1100 and 2000 were obtained using these conditions. Two ions were most abundant, $(M+4H)^{4+}$ and $(M+5H)^{5+}$, which were used for further SIM experiments. M represents the molecular mass of the measured molecule and the superscript outside the brackets depicts z, the charge on the molecule caused by the take up of protons during ionization.

It was decided to use SIM instead of scan data, because the sensitivity of SIM is higher. As described in the previous section the $(M+4H)^{4+}$ and $(M+5H)^{5+}$ ions were used for further optimization of SIM. The m/z values of these ions, these m/z values increased with 0.5 mass units and these m/z values decreased with 0.5 mass units were used in three separate SIM experiments to obtain m/z values giving the highest MS response for each of the insulin derivatives. The optima of these three experiments were used for further MS experiments and are listed in Table 6. Differences between the theoretical m/z values and the used SIM channels are caused by slight deviations in mass originating from the tuning of the MS.

Table 5
Recoveries of spiked insulin samples in the on-line SAX–RP-LC system after protein precipitation

Insulin concentration in plasma sample (nmol/l)	BI (%)	AHI (%)	HI (%)	PI (%)
625	53	51	53	52
300	41	25	34	38
200	31	32	30	30

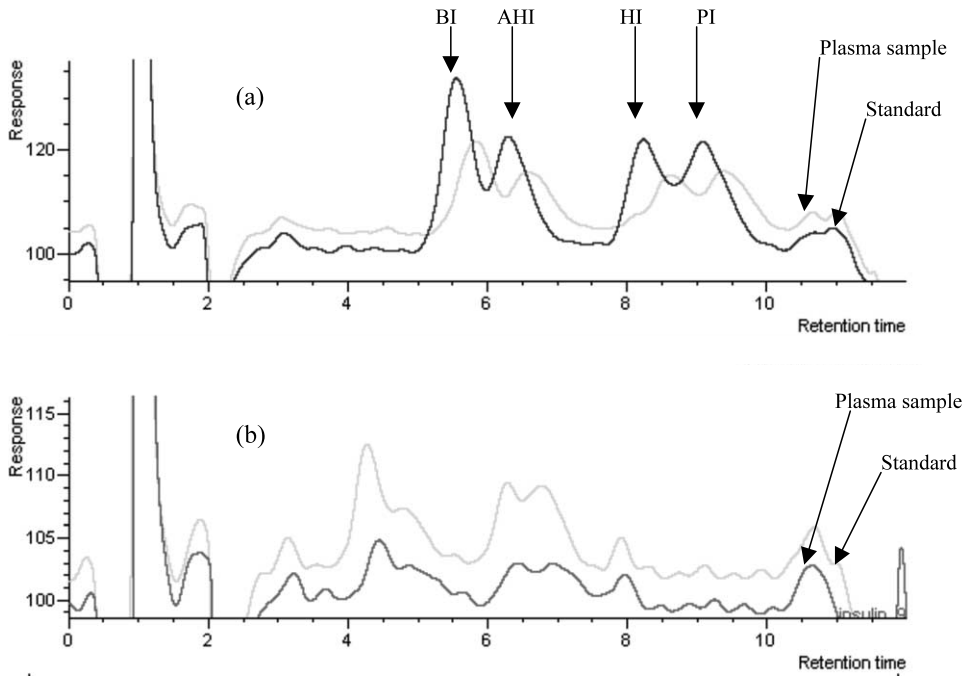

Fig. 6. On-line SPE–RP-LC chromatogram of insulin spiked human plasma after 60% v/v ACN protein precipitation compared with a standard insulin injection of the same insulin concentration. (a) Spiked human plasma sample (625 nmol/l); (b) 200 nmol/l spiked human plasma sample.

On-line SPE–RP-LC–UV–MS experiments were performed using the eight optimized SIM values, while the whole system remained unchanged except for the washing solvent of the RP–SPE and the percentage of ACN used at the start of the gradient. The RP–SPE was washed with 1500 μl 15% ACN/85% 0.05% TFA. This was changed to decrease the solvent peak in MS, which also gave a high response in SIM measurements. The LC gradient was, as a consequence started at 11.1% B, which corresponds to 15% ACN in the eluens. Since the LC separation was performed after the column had been conditioned to the used isocratic conditions, these changes had no effect on the separation.

In Fig. 7 a chromatogram of a 500 nmol/l mix of the four insulin derivatives can be seen. Although, BI and AHI, and HI and PI are not baseline separated, using MS in the SIM mode the four insulin derivatives can be selectively detected separately. Also, the four insulin derivatives can be identified directly using MS. The LOD using MS was not lowered much; instead of a LOD of 100 nmol/l in the on-line SPE–RP-LC–UV sys-

Table 6
Theoretical m/z values of 5+ and 4+ charged insulin molecules and the optimized SIM m/z values used for SIM measurements

	$(M+4H)^{4+}$		$(M+5H)^{5+}$	
	Theoretical	Optimized SIM value	Theoretical	Optimized SIM value
BI	1434.4	1433.7	1147.7	1147.2
AHI	1491.9	1491.4	1193.8	1192.9
HI	1452.9	1452.3	1192.5	1162.0
PI	1445.4	1444.7	1156.5	1156.0

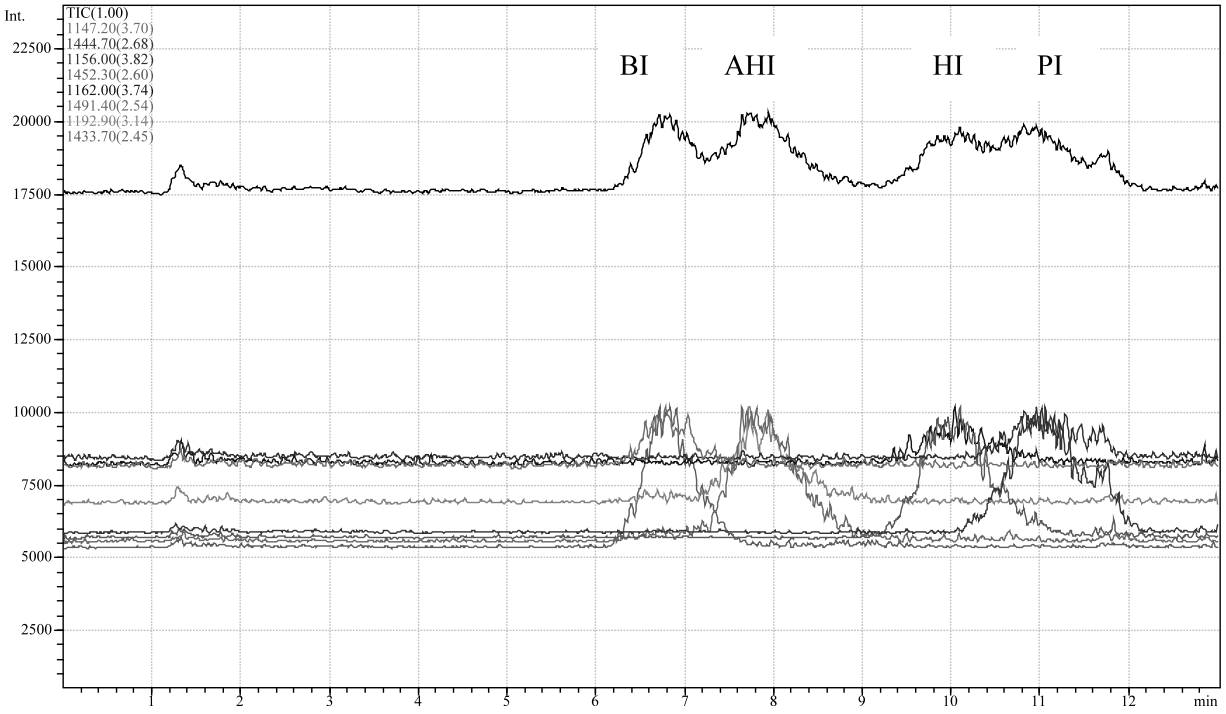

Fig. 7. On-line SPE–RP-LC–UV–MS chromatogram of a 500 nmol/l mix of BI, AHI, HI and PI. ESI-MS in SIM mode at the following m/z values:1147.20, 1156.00, 1162.00, 1192.00, 1433.70, 1444.70, 1452.30 and 1491.40.

tem, now a LOD of 50 nmol/l ($S/N = 3$) was reached.

The performed experiments using insulin spiked human plasma sampled were also performed using MS detection. Again, most of the plasma proteins were removed via precipitation with 60% ACN as described in Section 3.5. Insulin was spiked to a human diluted plasma solution in three concentrations: 300, 200 and 100 nmol/l as described in the Section 2. Plasma samples spiked to 300 and 200 nmol/l were also measured in the UV experiments. A 100 nmol/l spiked plasma sample was not used in the UV experiments because this concentration was just below the LOD. Since the LOD using MS was decreased by a factor two, here a 100 nM spiked plasma sample could be measured.

The recoveries of these extractions are listed in Table 7 and are somewhat higher than in the previous experiments. These differences in recovery are more likely due to variations in the precipitation procedure than to the use of MS. Again, as was determined and explained in Section 3.5, recoveries generally decreased when lower concentration were used to spike plasma samples. As can be seen, the improved LOD of two and the improved detection selectivity, lowered the LOD ($S/N = 3$) of this system to 100 nmol/l (0.6 μg/ml). In Fig. 8 both a 100 nmol/l reference and a plasma

Table 7
Recoveries of spiked insulin samples in the on-line SAX–RP-LC–UV–MS system after protein precipitation

Insulin concentration in plasma sample (nmol/l)	BI (%)	AHI (%)	HI (%)	PI (%)
300	42	53	36	44
200	41	38	35	38
100	36	41	38	35

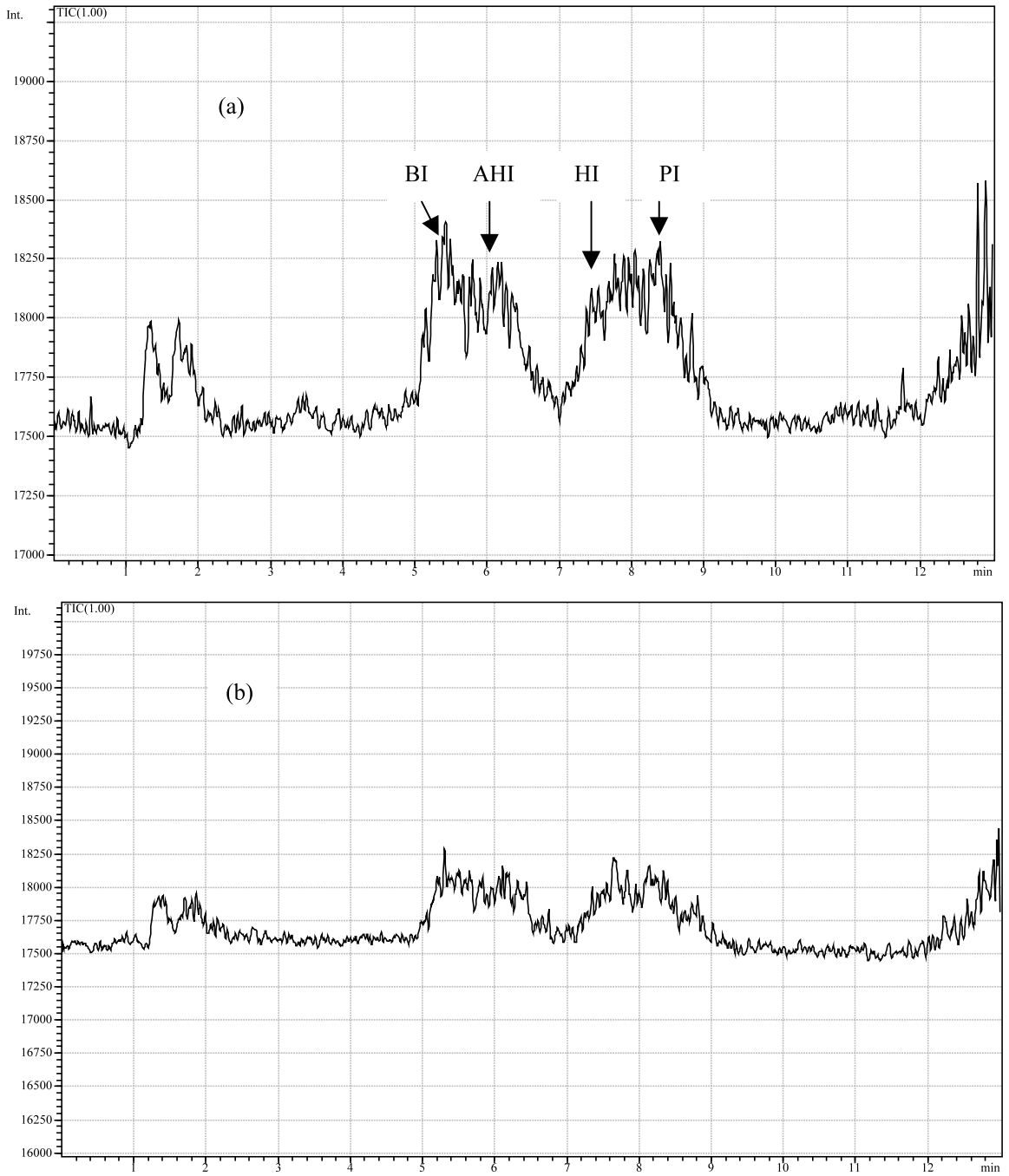

Fig. 8. On-line SPE–RP-LC–UV–MS chromatogram of, (a) 100 nmol/l mix of BI, AHI, HI and PI; (b) diluted human plasma sample spiked with 100 nmol/l mix of BI, AHI, HI and PI after ACN protein precipitation.

sample spiked to 100 nmol/l of the four insulin derivatives can be seen.

4. Conclusions

The developed on-line SPE–RP-LC system could be used to measure insulin in aqueous and spiked plasma samples to concentrations of 50 and 100 nmol/l, respectively, when UV detection was used. LODs of 50 and 100 nmol/l, respectively, were obtained when ESI-MS detection was used in the SIM mode. Next to better LODs, incomplete separated insulin derivatives can be separated using the selective MS detection. Also MS can help to identify different insulin derivatives.

The described on-line SPE–RP-LC system is fully automated. Due to the coupling of two different separation mechanism, ion-exchange and reversed-phase chromatography, a more selective sample clean-up procedure is obtained. No interferences were seen in the separation window in which the four insulin derivatives were determined. Since a second SPE cartridge is used to trap and recondition the sample plug eluted from the first SPE cartridge, a versatile system is obtained in which SPE procedures could be used, which are not directly compatible with the LC separation.

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