Talanta 121 (2014) 30–36

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Molecularly imprinted polymer cartridges coupled on-line with high performance liquid chromatography for simple and rapid analysis of human insulin in plasma and pharmaceutical formulations

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ARTICLE INFO

Article history:
Received 6 August 2013
Received in revised form
2 December 2013
Accepted 4 December 2013
Available online 31 December 2013

Keywords:
Molecularly imprinted polymer
Insulin
Human plasma
Pharmaceutical formulations
Automated
Sample clean-up

ABSTRACT

In this paper, a novel method is described for automated determination of human insulin in biological fluids using principle of sequential injection on a molecularly imprinted solid-phase extraction (MISPE) cartridge as a sample clean-up technique combined with high performance liquid chromatography (HPLC). The water-compatible molecularly imprinted polymers (MIPs) were prepared using methacrylic acid as a functional monomer, ethylene glycol dimethacrylate as a cross-linker, chloroform as a porogen and insulin as a template molecule. The imprinted polymers were then employed as the solid-phase extraction sorbent for on-line extraction of insulin from human plasma samples. To achieve the best condition, influential parameters on the extraction efficiency were thoroughly investigated. Rapid and simple analysis of the hormone was successfully accomplished through the good selectivity of the prepared sorbent coupled with HPLC. Limits of detection (LOD) and quantification (LOQ) of 0.2 ng mL^{-1}, 0.7 ng mL^{-1}, and 0.03 ng mL^{-1}, 0.1 ng mL^{-1} were obtained in plasma and urine respectively. The obtained data exhibited the great recoveries for extraction of insulin from human plasma and pharmaceutical samples, higher than 87%.

1. Introduction

Insulin is the most important regulatory hormone that is central to regulating carbohydrate and fat metabolism in the body. It plays a vital role in the control of glucose homeostasis, consisting of 51 amino acids shared between two intramolecular chains and with a molecular weight of about 5800. Insulin is not a stable entity. However, enables molecules in its vicinity to be modified by chemical reactions. So, during storage, use of pharmaceuticals preparation and during inappropriate sample preparations, insulin is degraded by hydrolytic reactions or is transformed by formation of intermolecular covalent bonds with other insulin molecules, leading to higher molecular weight transformation products.

The insulin has been separated and quantified using different methods such as capillary electrophoresis [1], HPLC method [2,3], LC/MS analysis [4], immunoassays [5], electrochemical method [6], amperometry [7], surface plasmon resonance [8] and mass-sensitive biosensors [9,10]. Solid-phase extraction (SPE), nowadays, is a well-founded technique and has been used for the preconcentration and clean up of numerous different kinds of compounds in a variety of matrices before the chromatographic separation [11,12]. However, it is considered as an appropriate sample preparation technique, but the selectivity of the SPE sorbents has still remained as a challenging demand sought after by many research to enhance the selectivity of the method particularly in complex matrices. Hence, molecularly imprinted polymers (MIP) are currently being investigated by many researchers [13,14]. They are composed of immune sorbents (ISs) in which its affinity and selectivity stem from antigen–antibody interactions. Therefore, a selective extraction of the target analyte and of similarly structured compounds could be easily achieved [15,16]. One of the interesting configurations of the SPE sorbents could be considered as on-line combination with HPLC if the applied solid support is pressure resistant [17–21]. Despite their important interest as a selective sample pretreatment sorbents, the development of ISs is time consuming and relatively expensive. These drawbacks have contributed to the development of molecularly imprinted polymers (MIPs). MIPs are synthetic materials possessing specific cavities specially designed for the recognition of an analyte of interest. Their synthesis procedures for SPE application are mainly based on strong noncovalent interactions (such as hydrogen bonds or ionic interactions) between a target molecule (template) with functional monomers, followed by polymerization in the

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http://dx.doi.org/10.1016/j.talanta.2013.12.011

presence of a cross-linker, usually in a non-protic and weakly polar solvent. Once the template is removed, selective molecular recognition sites, often described as three-dimensional shapes in the polymer, are available for the selective rebinding of the target molecule and some structurally related compounds. The first application was carried out by the group of Sellergren in 1994 for the extraction of pentamidine present at low concentration level in urine [22]. Nowadays, MIPs have been largely applied to the extraction of target analytes from various complex matrices [23,24]. Concerning the pharmaceutical field, many examples deal with the extraction of a biomolecule by MIP from tablets [25–27], plasma [28–33] or from urine [34,35] samples. Most of these applications were based on off-line procedures. A very few applications were carried out in on-line mode [33,34,36–38] while trends in analytical chemistry are for high throughput approaches that require to minimize the time spent performing analysis. Therefore, MIP cartridges coupled on-line with high performance liquid chromatography (HPLC) can allow a straightforward and fast pretreatment due to their specific recognition properties. We applied MIPs as artificial receptors for off-line solid-phase extraction of bromhexine [31], metoclopramide [32], verapamil [39], and tramadol [40] in biological fluids. Recently, the applicability of an on-line solid phase extraction method using molecularly imprinted monolithic column was developed for the assay of tramadol in urine and plasma samples [41].

In this work a novel automated method has been developed for the selective extraction and further chromatographic determination of insulin based MIPs. It was intended to be used as the selective SPE cartridge sorbents for efficient sample clean-up of the analyte from complex biological matrices. The new SPE cartridge sorbents allows the sensitive, simple and inexpensive extraction and determination of the target drug in human plasma and urine samples while various pharmaceutical compounds found in the biological fluid were not well retained on the MIP support. To the best of our knowledge, it is rarely reported that the on-line MIP–SPE coupled to HPLC system is employed to monitor insulin.

2. Experiment section

2.1. Reagents

Prior to use methacrylic acid (MAA) from Merck (Darmstadt, Germany), it was distilled in vacuum in order to remove the stabilizers. Ethylene glycol dimethacrylate (EGDMA) and 2, 2′-azobis isobutyronitrile (AIBN) as the initiator of polymerization from Sigma-Aldrich (Steinheim, Germany) were used without any purification. All solvents used in chromatography analyses were HPLC grade and purchased from Merck. Pure insulin was supplied by Sigma (Munich, Germany). All solutions were made with deionized water purified using a Milli-Q Plus System (ELGA Corp., Purelab UHQ) and dissolved in water to obtain concentrations of 10, 50, 100, 150 and 200 μg mL^{-1}. It was also some insulin formulations which were purchased from post-market and analyzed as real samples: Insulin regular human (Lansulin® R, 100 IU 1 ml, Vial, Exir pharmaceuticals), Insulin Isophane (NPH)-(Lansulin® N, 1000 IU 1 mL^{-1}, Vial, Darou Pakhsh Pharmaceuticals) and Insulin Aspart (NovoRapid®, 100 IU 1 mL^{-1}, Pen, Nove Nordisk, Australia) were obtained as pharmaceutical preparations. All experiments and validation steps were performed utilizing fresh freeze plasma obtaining from healthy volunteers in Tehran University Hospital (Tehran, Iran) and kept frozen until use after gentle thawing. All standard solutions were freshly prepared daily and stored at 2–8 °C. Aliquots of standard stock solution of insulin were dispensed into 10 mL volumetric flasks and the flasks made up to volume with the mobile phase to give final concentrations range of 0.2–250 ng mL^{-1}.

2.2. Apparatus

An Alliance HPLC instrument from Waters Company used to separate and analyze insulin in biological samples. The chromatographic system composed of a multisolvent gradient Water pumps, a Water 2996 photodiode array detector and an online degasser. Chromatographic separation was achieved on an ACE C18, 5 μm, 4.6 mm × 250 mm column. Regarding the mobile phase, a degassed mixture of methanol:phosphate buffer (60:40) was prepared and delivered via P_A pump in the first stage of a gradient elution process. This is for eluting of our analyte from SPE cartridge. In the second stage, a prefiltered mixture of 60 volume of 1 mM sodium sulfate and 0.2% triethylamine in water (pH = 3.2 by *o*-phosphoric acid) and 40 volume of acetonitrile were prepared and delivered via P_B and P_C pumps at flow rate of 1.0 mL min^{-1}. This time programmed moving up the insulin through the SPE cartridge and analytical column is indicated in the Table 1. All of the analyses were carried out at an operation wavelength of 214 nm and HPLC data were acquired and processed using a PC and Millennium 2010 chromatogram manager software (Version 2.1 Waters). The mean retention time of insulin was 4.45 min. Re-equilibration of the column was accomplished within 30 min.

Scanning electron microscopy (SEM, PhilipsXL30 scanning microscope, Philips, the Netherlands) was employed to determine the shape and surface morphology of the produced polymer particles.

Table 1
The optimized sequence for the performance of the automated SPE–HPLC system.

Pump type duration (min)	P_1	P_2	P_3	P_4	P_5	P_6	P_A	P_B	P_C	Pump action
SPE phase										
0–1	Del.	Asp.	Asp.	Asp.	Asp.	Asp.	Off	Off	Off	Conditioning with methanol
1–2	Asp.	Del.	Asp.	Asp.	Asp.	Asp.	Off	Off	Off	Conditioning with ultra-pure water
2–3	Asp.	Asp.	Del.	Asp.	Asp.	Asp.	Off	Off	Off	Conditioning with ammonium phosphate
3–8	Asp.	Asp.	Asp.	Del.	Asp.	Asp.	Off	Off	Off	Loading insulin into the MIP cartridge
8–9	Asp.	Asp.	Asp.	Asp.	Del.	Asp.	Off	Off	Off	Washing with 0.1 M HCl
9–10	Asp.	Del.	Asp.	Asp.	Asp.	Asp.	Off	Off	Off	Washing with ultra-pure water
HPLC phase										
10–13	Asp.	Asp.	Asp.	Asp.	Asp.	Asp.	On	Off	Off	Eluting insulin from SPE cartridge with methanol:phosphate buffer 100%→0%
12–25	Asp.	Asp.	Asp.	Asp.	Asp.	Asp.	Off	On	On	Eluting insulin through HPLC column with 1 mmol sodium sulfate and 0.2% triethylamine in water:acetonitrile (60:40) 0%→100%
25–30	Asp.	Asp.	Asp.	Asp.	Asp.	Del.	Off	Off	Off	Regeneration of SPE cartridge with dichloromethane

Del. = Deliver, and Asp. = Aspirate.

2.3. MIP and NIP preparation with precipitation polymerization

For the preparation of the human insulin imprinted polymer, the template molecule (insulin) (2 mL, 0.01 mmol), the functional monomer (MAA), the cross-linking monomer (EGDMA) (2.6 mL, 14 mmol), and the initiator (AIBN) (0.057 mg, 0.164 mmol) were added to 20 mL of chloroform. The mixture was sonicated for 30 min to remove the oxygen. Then the solution was placed in a water bath at 60 °C for 18 h.

The prepared polymer particles were collected by centrifugation (Sigma 3K30, Germany) at 10,000 rpm for 15 min. The template of unleached imprinted polymers were removed by batch-mode solvent extraction with 40 mL of methanol containing 10% acetic acid (v/v) five times for 1 h, until no template could be detected from the washing solvent by spectrometric measurement (at λ=214 nm) (Scinco S-3100, Korea). The polymer particles were then rinsed with deionized water and acetone (1:1), and finally the resulting leached imprinted polymers were dried in oven (Memmert INB 400, Germany) at 60 °C overnight. Apart from the MIP, Non-imprinted polymers (NIPs) were synthesized at the same conditions as MIP but in the presence of the template.

2.4. Operation of the of SPE–HPLC system

Exactly 120 mg of the optimized MIP polymer was packed in a polypropylene cartridge, which was incorporated in a flow system prior to the HPLC analytical instrumentation. The principle of sequential injection was utilized for a rapid automated and efficient SPE procedure on the MIP. Samples, buffers and washing solvents were introduced to the extraction cartridge via six Waters 515 peristaltic pumps (P1–P6 in Fig. 1). The sequential injection manifold comprised of a micro-electrically actuated 10-port Valco valve. Firstly, the cartridge was conditioned via P1–P3 pumps with 1 mL methanol, 1 mL ultra-pure water and 1 mL 25 mM ammonium phosphate, adjusted to pH 3.0. Extraction experiments included loading the cartridge with 5 mL of sample containing 100 ng mL^{-1} insulin at a flow rate of 1.0 mL min^{-1} with P4 pump. After loading, cartridge was washed with 1 mL HCl 0.01 M and 1 mL ultra-pure water with P5 and P6 pumps, respectively. The elution phase was performed by steady changes of the mobile phase composition during the chromatographic run. This was performed by passing 3 mL methanol:phosphate buffer (60:40) via P_A peristaltic pump. After that, each eluted compound was passed through analytical HPLC column by an isocratic elution of mobile phases of sodium sulfate:triethylamine buffer:acetonitrile (60:40) via P_B and P_C peristaltic pumps.

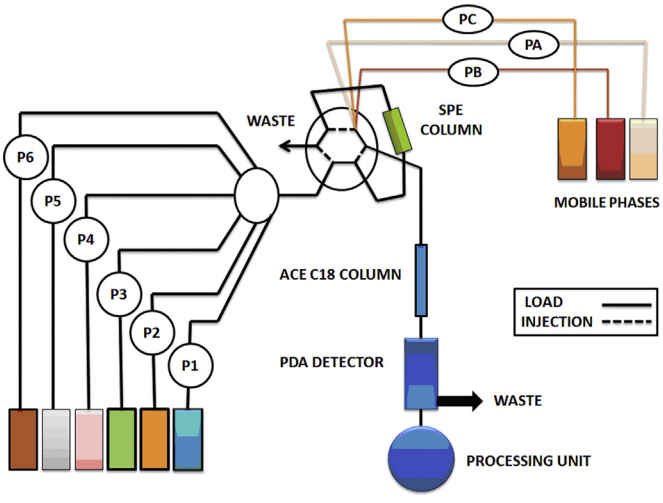

Fig. 1. System configuration for on-line sample purification using MISPE prior to detection in a HPLC system.

2.5. Batch rebinding experiments

Batch adsorption experiments were used to evaluate the binding affinity of the imprinted polymer as reported before [44]. Polymer beads were suspended in aqueous solutions containing insulin solutions of various concentrations. The mixtures were thermo stated at 25 °C for 8 h under continuous stirring followed by filtration by a paper filter (flow rate=50 mL min^{-1} by applied vacuum). The free concentration of insulin after the adsorption was recorded by HPLC-UV at 214 nm. Three replicate extractions and measurements were performed for each aqueous solution.

2.6. Extraction procedure for human plasma and pharmaceutical samples

Drug-free human plasma was obtained from the Iranian blood transfusion service (Tehran, Iran) and stored at −4 °C until use after gentle thawing. Due to possibility of protein-bonding for insulin and reducing the recoveries in solid-phase extraction processes, it is necessary to have some treatments with plasma before extraction with MIP particles. So, the plasma samples were diluted with 25 mM phosphate buffer (pH 7.6), then were centrifuged 20 min at 8000 rpm to remove excess of proteins. Then the supernatant was filtered through a cellulose acetate filter (0.20 μm pore size, Advantec MFS Inc., CA, USA). The filtrate was collected in glass containers and stored at −20 °C until the analysis was performed. Pharmaceutical insulin formulations were also diluted with 25 mM phosphate buffer (pH 7.6), and were centrifuged for 10 min at 4000 rpm and filtered through a cellulose acetate filter. Two milliliters of the filtered supernatant were collected to be directly percolated through the MIP or the NIP cartridges.

3. Results and discussion

3.1. Optimal MIP formulation and porogenic solvent

There are several variables, such as amount of monomer or nature of cross-linker and solvent that affects the final characteristics of the obtained materials in terms of capacity, affinity and selectivity for the target analyte. Thus, by achieving an optimum combination of cross-linker and functional monomer, non-specific binding should be able to be minimized. Primary experiments revealed that the imprinted polymers prepared in chloroform are good recognition ability than methanol and acetonitrile (Fig. 2).

Duo to insulin slightly soluble in chloroform, different volume of the porogen (5–30 mL) in the precipitation polymerization was used. On the other hand the volume of the porogen is very important for specific polymers synthesis.

In this regard, different formulations have been implemented to obtain the MIPs with improved molecular recognition capabilities. Generally, proper molar ratios of functional monomer to template are very important to enhance specific affinity of polymers and number of MIPs recognition sites. High ratios of functional monomer to template result in a high non-specific affinity, while low ratios produce fewer complexation due to insufficient functional groups [39]. For doing so, nine molar ratios of the monomer MAA to the template of 2:1, 4:1, 6:1, 8:1, 10:1, 13:1, 15:1, 20:1 and 22:1 were completely investigated. The optimum ratio of functional monomer to template for the specific rebinding of insulin was 20:1 (Table 2), which had the best specific affinity and the highest recovery of 89%, while the recovery of 25% was

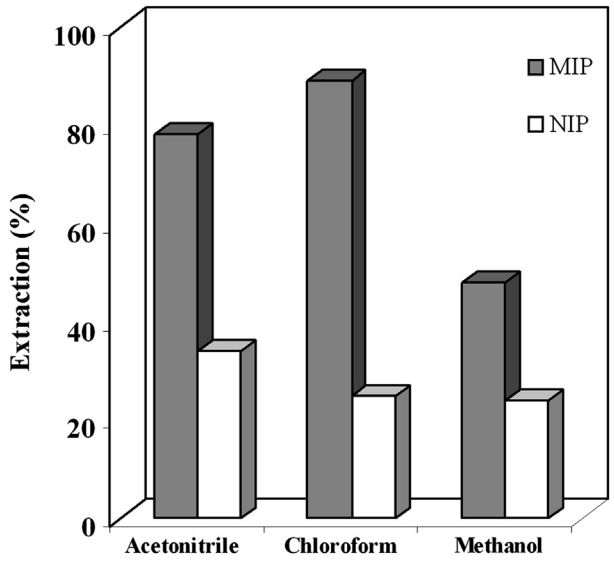

Fig. 2. Effect of solvent on the recognition property of the polymers.

Table 2
Compositions and comparisons of the extraction of insulin from insulin standard solution (5 mL, 50 ng mL^{-1}) using 50 mg of various polymers as sorbents at pH 7.6, elute: 5 mL methanol and acetic acid (10:1, v/v).

MIP	MAA (mmol)	Insulin (mmol)	EGDMA (mmol)	AIBN (mmol)	Extraction (%) (mean ± SD)[a]
MIP1	0.54	0.27	14	0.056	29 (±2.0)
MIP2	1.08	0.27	14	0.056	31 (±2.3)
MIP3	1.62	0.27	14	0.056	35 (±2.0)
MIP4	2.16	0.27	14	0.056	36(±2.2)
MIP5	2.7	0.27	14	0.056	48 (±2.7)
MIP6	3.51	0.27	14	0.056	58 (±2.6)
MIP7	4.05	0.27	14	0.056	68 (±3.0)
MIP8	5.4	0.27	14	0.056	89 (±3.1)
MIP9	5.94	0.27	14	0.056	69 (±2.8)
NIP1	0.54	–	14	0.056	25 (±2.3)
NIP2	1.08	–	14	0.056	23 (±2.1)
NIP3	1.62	–	14	0.056	24 (±2.2)
NIP4	2.16	–	14	0.056	23 (±1.6)
NIP5	2.7	–	14	0.056	24 (±2.2)
NIP6	3.51	–	14	0.056	26 (±2.3)
NIP7	4.05	–	14	0.056	24 (±2.1)
NIP8	5.4	–	14	0.056	25 (±2.2)
NIP9	5.94	–	14	0.056	26 (±2.5)

[a] Average of three determinations.

obtained for the prepared NIPs. The specific adsorption recoveries of insulin at molar ratio of 20:1 was 64%, while at 2:1, 4:1, 6:1, 8:1, 10:1, 13:1, 15:1 and 22:1 were 4%, 8%, 11%, 12%, 24%, 32%, 44% and 43%, respectively. Therefore, a typical 1:20:52 template:monomer: cross-linker molar ratio was selected for further studies.

3.2. Morphologic analysis

Fig. 3 shows the SEM images of MIP and NIP particles. The microspheres were obtained by appropriate amount of template/functional monomer/cross-linker agent (molar ratio of 1:20:52) and gentle mixing during the precipitation polymerization.

3.3. Equilibrium batch rebinding

Template binding by imprinted polymer is result of specific (by recognition sites) and non-specific binding, while it is only result of non-specific binding for non-imprinted polymer. In order to evaluate the binding properties of the polymers, batch wise guest-binding experiments were performed in the buffer solutions of the template. It is found that the incubation time of the polymer with insulin and the amounts of MIP taken greatly affected the rebinding results. The amount of analyte adsorbed by a sorbent is dependent on the distribution constant between sorbent and solution, thickness of an adsorbing phase and diffusion coefficient of analytes. MIP is an equilibrium based method in which the extraction efficiency is expected to increase with time until the equilibrium is reached. The results showed, the extraction efficiency of analytes with MIPs improved almost linearly as the sample loading time increased. Anyway, a loading time of 1 h was selected as for most analytes the equilibrium time was almost achievable.

3.4. Effect of pH

It has been demonstrated that efficient imprint rebinding is possible in aqueous buffer solutions, showing high binding affinity and selectivity as a result of hydrophobic interactions. The influence of pH on insulin has been considered in several works [42–47]. In some of them acidic and in others neutral pHs were reported as a good point for insulin analysis. In this work, the effect of pH on the rebinding efficiency of insulin was investigated by varying the solution pH from 2.0 to 10.0 (Fig. 4). Several batch experiments were performed by equilibrating 50 mg of the imprinted particles with 20 mL of solutions containing 50 ng mL^{-1} of insulin under the desired range of pH. It was observed that insulin underwent complete rebinding/elution at pH 7.6. The protonation of the amine groups of insulin and de-porotonation of carboxyl groups of the polymer at lower and higher pHs might be the main reason of the obtained data, respectively.

3.5. The sample flow rate through the MIP–SPE cartridge

It is known that the required time to obtain extraction equilibrium is proportional to the length of MIP–SPE cartridge, the analyte distribution constant and the volume of coating, while it is inversely proportional to the extraction flow rate. Generally, the flow rate is optimized by keeping the total sample solution volume and the concentration constant. Thus for the MIP–SPE cartridge applied here, the extraction flow rate was initially optimized to obtain high extraction efficiency while still offering a reasonable analyzing time. In doing so, a range of flow rates from 0.2 to 2 mL min^{-1} were considered to be investigated. The results demonstrate the higher extraction efficiencies achieved at flow rates above 1 mL min^{-1} due to improved mass transfer during more extraction cycles. Hence a flow rate of 1 mL min^{-1} has been chosen as the optimum value.

3.6. Choice of loading, washing and eluent solution

Commonly, synthetic polymers have binding ability with both specific and nonspecific interactions. The specific interactions may mainly originate from the imprinting procedure, creating selective recognition sites for the template. The non-specific interactions were assessed by measuring the binding of the non-imprinted polymer. At first, cartridges were conditioned with 1 mL methanol, 1 mL of ultra-pure water and 1 mL of 25 mM ammonium phosphate at pH 3.0. In the following water samples were loaded onto the cartridges at a flow rate of 1 mL min^{-1} and water washing procedure, it was an assessment of obtaining maximum recovery of the analytes using a variety mixtures including; ultra-pure water, hydrochloric acid 0.01 M, acetonitrile, acetone, tetrahydrofuran, dimethyl formamide (DMF), acetonitrile–acetone mixture, dichloromethane (DCM) and acetonitrile–methanol mixture. In order to investigate usefulness of washing stage, 5 mL of 50 μg L^{-1}

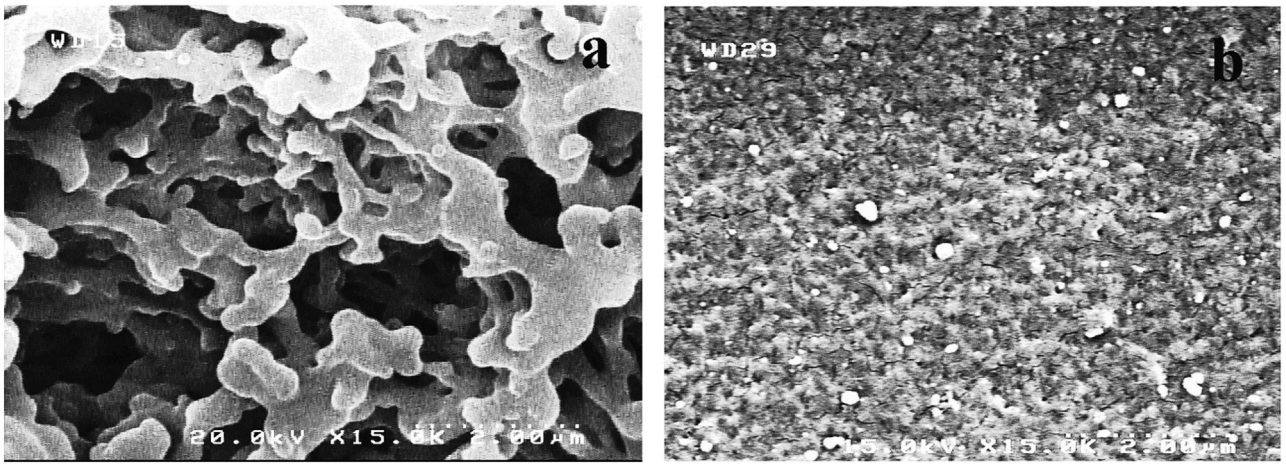

Fig. 3. Scanning electron micrographs of leached MIP (a) and NIP (b) particles.

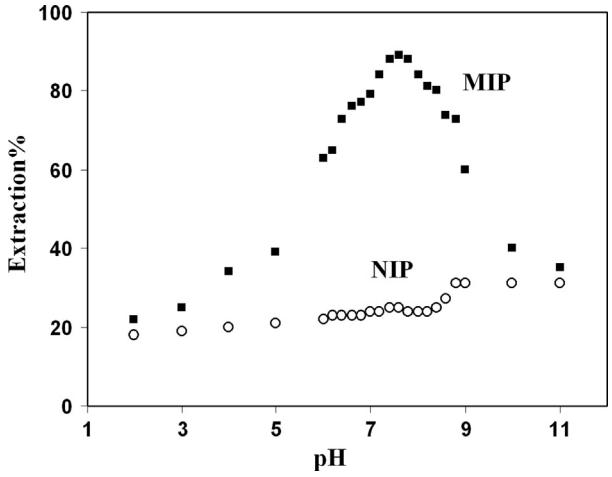

Fig. 4. Effect of pH on extraction of MIP and NIP particles in batch experiments.

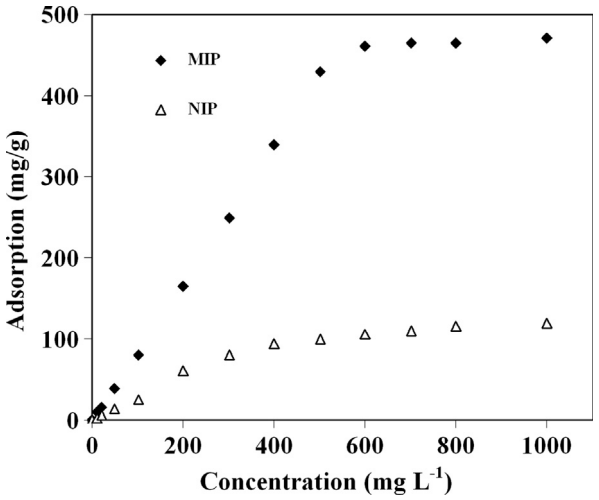

Fig. 5. Effect of insulin concentrations on the retention capacities of MIP and NIP particles at pH 7.6.

insulin aqueous solution (pH 7.6) was loaded on the MIP and NIP cartridges individually, followed by desorption with the washing solvent. The results indicate that washing with 1 mL of hydrochloric acid (0.01 M) and 1 mL of ultra-pure water was the most excellent protocol. The results showed there is a recovery of insulin in NIP cartridge decreasing up to 24%, while the recovery of insulin by the MIP cartridges did not reduce (87%).

It is significant, that, washing via hydrochloric acid is essential to remove ionic species (e.g. metal ions) from complex matrices such as human plasma and urine. Note that after washing stage, we need to dry the SPE cartridge thoroughly by vacuum application. As for the recovery of strongly bounded insulin, the polymeric sorbents were eluted with 3×1 mL of methanol and acetic acid (10:1, v/v). Moreover, increasing of the sample volume up to 50 mL just had a light effect on the extraction of insulin.

3.7. Adsorption capacity

The capacity of sorbent is an important factor determining how much sorbent is required to remove a specific amount of drug from the solution quantitatively. In considering the measurement of the adsorption capacity of MIP and NIP absorbents, the absorbents (50 mg) were added into 50 mL insulin solutions at concentrations of 10–1000 mg L^{-1}, and the suspensions were mechanically shaken for 1 h at room temperature, followed by centrifugally removing of the absorbents. The remained insulin in the supernatant was measured by HPLC. The isothermal adsorptions are plotted in Fig. 5. According to these results, the maximum amount of insulin absorbed by MIP was found to be 460 mg g^{-1} at pH 7.6. Regarding higher insulin amounts, a slight increase of retained insulin was observed on MIP capacity curve. Since all the accessible specific cavities of the MIP are saturated, the retention of the analyte is only due to nonspecific interactions which can be approximately identical for MIP and NIP polymers.

3.8. Calibration curve, precision and calculation of LOD and LOQ

As regards calibration curve of samples, drug-free plasma and urine samples spiked with insulin at the concentration range from 0.07 to 250 ng mL^{-1} and 0.1 to 270 ng mL^{-1}. The area under curves of insulin versus its concentration was linear and a correlation coefficient of 0.999 and 0.9992 across the concentration range was obtained following linear regression analysis, respectively. Typically, the regression equation for the calibration curves was found to be $y=679.02x+571.9$ ($n=4$) and $y=73.21x+12.921$ ($n=4$) for plasma and urine samples, respectively, where x is insulin concentration per ng mL^{-1}. The LOQ and LOD were determined based on a signal-to-noise ratios and were based on analytical responses of 10 and three times the background noise. The LOQ for plasma and urine was found to be 0.7 ng mL^{-1} and 0.1 ng mL^{-1} with a resultant %R.S.D. of

3.35 and 2.8 ($n=4$), respectively. The LOD was found to be 0.20 ng mL^{-1} and 0.03 ng mL^{-1}, respectively.

The reproducibility and repeatability of the method were evaluated from run-to-run MISPE experiments (10 ng mL^{-1} standard solution, $n=7$) and different batch experiments (fore batches) and RSDs of 3.4% and 4.6% for the extraction amounts of insulin were obtained, respectively.

The intra- and inter-day precision and accuracy of method were assessed by analyzing in plasma sample spiked with human insulin at three different concentration levels over the calibration range tested, (in replicates 5) of 10, 70 and 200 ng mL^{-1}, respectively. All samples were prepared either at the same day or at five consecutive days. The inter-day precision was less than 4.8% and the inter-day precision was lower than 3.3% in plasma sample, respectively.

3.9. Pharmaceutical formulations analysis

The applicability of extraction method to demonstrate the potential of MIP for the selective sample clean-up and on-line determination of the drug in real samples was examined by extraction and determination of insulin in pharmaceutical formulations and human plasma and urine samples. The plasma and urine samples were prepared according to Section 2.6. The results for the automated method display the procedure can elute interferences from complex matrices avoiding contaminating HPLC column. The chromatograms obtained for urine and plasma samples were compared in Figs. 6 and 7. HPLC chromatogram obtained for blank plasma sample for comparison was shown in Fig. 8. As can be noticed in Figs. 6 and 7, the method permitted cleaner extracts to obtain and interfering peaks arising from the complex biological matrices to be suppressed. Results from the HPLC analyses showed that the MIP extraction of insulin for plasma and urine samples are linear in the ranges 0.07–250 ng mL^{-1} and 0.1–270 ng mL^{-1} with good precision (4.8% and 3.2% for 50 ng mL^{-1}) and recoveries. The extraction yields of the selected compounds with the MIP and NIP are shown in Table 3. The results showed that the recoveries for plasma and urine samples were between 87–89% and 86–88%, respectively (Table 3).

Typical chromatograms presented in Figs. 6 and 7 reveal that the MIP can be used for the sample clean-up and when novel MIP sorbent was used, a board peak in chromatogram was omitted.

For investigation of usefulness of the automated procedure for pharmaceutical insulin formulations, various formulations such as Insulin regular human (Lansulin® R, Vial), Insulin Isophane (NPH)-(Lansulin® N, Vial) and Insulin Aspart (NovoRapid®, Pen) were selected. Solutions of all compounds were prepared individually with the concentration of 50 ng mL^{-1}. Elution solvent for the cartridges was methanol and acetic acid (10:1, v/v). The extraction yields of the selected pharmaceutical formulations with the automated MIP–HPLC was obtained and compared with the

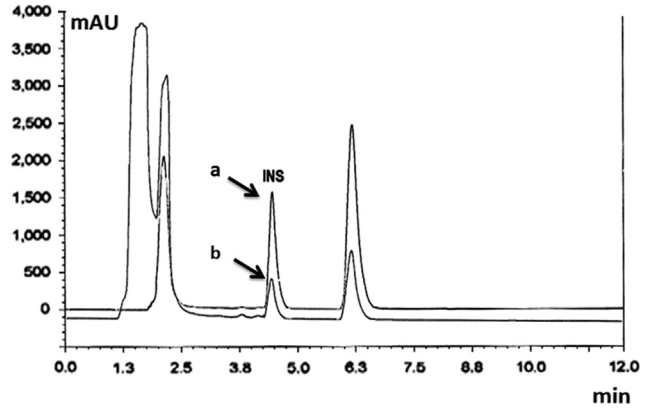

Fig. 6. HPLC chromatograms obtained after percolation of 1.0 mL urine sample spiked with 50.0 ng mL^{-1} of insulin with a clean-up step comprising (a) the imprinted polymer cartridge and (b) the non-imprinted polymer cartridge, monitored at 214 nm. Conditions: column ACE 5 μm, C18 4.6 mm × 250 mm at 40 °C, eluent methanol:phosphate buffer (0.01 mol L^{-1}, pH 5.8) (60:40, v/v) at flow rate of 1.0 mL min^{-1}.

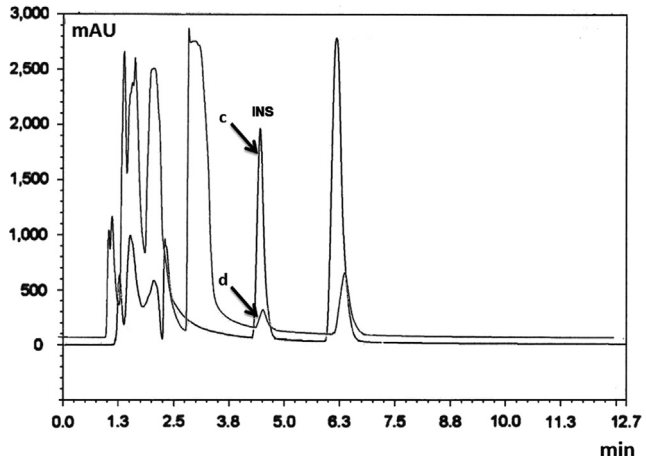

Fig. 7. HPLC chromatograms obtained after percolation of 1.0 mL plasma sample spiked with 50.0 ng mL^{-1} of insulin with a clean-up step comprising (c) the imprinted polymer cartridge, and (d) the non-imprinted polymer cartridge, monitored at 214 nm. Conditions: column ACE 5 μm, C18 4.6 mm × 250 mm at 40 °C, eluent methanol:phosphate buffer (0.01 mol L^{-1}, pH 5.8) (60:40, v/v) at flow rate of 1.0 mL min^{-1}.

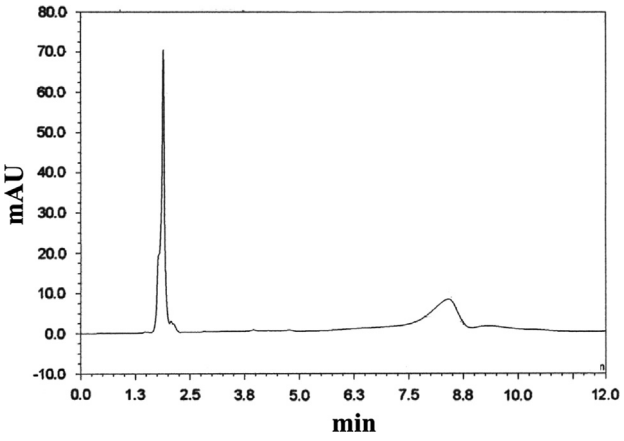

Fig. 8. HPLC chromatogram obtained for blank plasma with a clean-up step comprising the imprinted polymer cartridge monitored at 214 nm. Other conditions are similar to Fig. 7.

Table 3
Assay of insulin in human plasma and urine by means of the automated SPE–HPLC protocol.

Sample	Spiked value (ng/mL)	Proposed SPE–HPLC protocol (Recovery% ± SD)[a]	
		MIP	NIP
Human plasma	1.0	87 ± 3.2	24.1 ± 1.7
	25	89 ± 3.3	25.0 ± 1.4
	50	88 ± 3.9	24.9 ± 1.3
Human urine	1.0	86 ± 3.2	26 ± 2.6
	20	88 ± 3.3	26 ± 2.4
	100	87 ± 3.0	25 ± 2.8

[a] Average of three determinations.

Table 4
Insulin assay in pharmaceutical formulations

Insulin pharmaceutical name/form	Labeled value (mg/Vial or Pen)	Proposed online SPE –HPLC		Reference LC method [35]	
		Observed value (mg/Vial or Pen)	Recovery ± RSD (%)(n=4)	Observed value (mg/Vial or Pen)	Recovery ± RSD (%)(n=3)
Lansulin® R, Vial	100	87	87 ± 3.1	93	93 ± 3.5
Lansulin® N, Vial	1000	880	88 ± 3.4	915	91.5 ± 3.8
NovoRapid®, Pen	100	90	90 ± 3.8	92	92 ± 3.5

conventional method [48] (Table 4). This table shows that the novel SPE–HPLC method can be used for automated determination of insulin in pharmaceutics with very high recovery and accuracy.

4. Conclusions

In this paper, for the first time, insulin molecularly imprinted polymers were synthesized and used for preparation of MIP cartridges coupled on-line with high performance liquid chromatography. The automated MISPE–HPLC method has been developed for simple and rapid analysis of insulin in human plasma and urine samples. This efficient method allowed cleaner extraction to be obtained and interfering peaks arising from the complicated biological samples to be suppressed. The method was applied to the trace insulin determination at three levels, and the recoveries for the spiked human plasma and urine samples were higher than 87%. It can be concluded that the technique has great potential for developing selective extraction and on-line determination method for other compounds.

Acknowledgments

Financial support provided by the Amirkabir University of Technology (Tehran, Iran) is acknowledged.

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