Talanta 85 (2011) 950–957

Contents lists available at ScienceDirect

Talanta

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

# Development of a molecularly imprinted polymer for selective extraction followed by liquid chromatographic determination of sitagliptin in rat plasma and urine

R. Nageswara Rao*, Pawan K. Maurya, Sara Khalid

*HPLC Group, Analytical Chemistry Division, Discovery Laboratory, Indian Institute of Chemical Technology (IICT), Tarnaka, Hyderabad 500607, India*

ARTICLE INFO

*Article history:*
Received 5 February 2011
Received in revised form 30 April 2011
Accepted 3 May 2011
Available online 11 May 2011

*Keywords:*
Zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC)
Molecularly imprinted polymer
Solid-phase extraction
Sitagliptin
Rat plasma

ABSTRACT

A novel water-compatible molecularly imprinted solid-phase extraction (MISPE) combined with zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) method for selective extraction and determination of sitagliptin in rat serum and urine was developed and validated. The effects of progenic solvents, pH, cross linker and amount of monomer were studied to optimize the efficiency and selectivity. The adsorption kinetics and isotherms were measured. The molecularly imprinted polymer (MIP) showed good specific adsorption capacity with an optimum of 180 mg/g at pH 7.5 and selective extraction of sitagliptin from rat plasma and urine. The recovery of sitagliptin from rat urine and plasma was >98%. The limits of detection (LOD) and quantification (LOQ) were 0.03 and 0.10 μg/L respectively. The proposed method overcomes the matrix effects of phospholipids generally encountered while preparation of plasma samples by precipitation of proteins.



## 1. Introduction

Sitagliptin, (2R)-1-(2,4,5-trifluorophenyl)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]butan-2-amine (see Electronic Supplementary Material, Fig. S1), is an orally active, potent and selective inhibitor of dipeptidyl peptidase-IV (DPP-IV), for treatment of type 2 diabetes [1,2]. It also improves glycemic control and regulates the growth of insulin producing β-cells in pancreatic islets [3,4]. Sitagliptin has a bioavailability of 87% and exhibits low reversible binding to plasma proteins [5]. The majority of sitagliptin is excreted unchanged in urine [6,7]. Thus development of analytical methods capable of quantifying sitagliptin at low biologically active concentration is essential not only for understanding its pharmacological mechanism and therapeutic drug monitoring (TDM). Its pharmacokinetic applications require highly sensitive and selective assays with high sample throughput capacity.

In the literature, several analytical methods were found for determination of sitagliptin in biological fluids. However, these methods are not only time consuming, require expensive instrumentation but also affected by matrix effects and have low recoveries. Recently, sitagliptin was quantified in human plasma by liquid–liquid extraction followed by LC-MS/MS [8]. However, the method not only required relatively large amount of plasma (500 μL) but also the average recovery of sitagliptin was less than 68%. The retention of sitagliptin (2.8 min) and internal standard (IS) fluoxetine (2.9 min) on $C_{18}$ column were poor. Turbulent flow online extraction with tandem mass spectrometry was also used for determination of sitagliptin in biological fluids [9,10]. Multidimensional liquid chromatography could provide high resolution, but at the expense of significant material losses and increased costs with each additional chromatographic step. Protein precipitation, liquid–liquid extraction and solid phase extraction (SPE) are the common techniques used for sample preparation of biological materials. Plasma phospholipids are one of the major contributing sources of matrix effects in protein precipitation based sample preparation techniques [11]. SPE is the most popularly used technique for its advantages like time and cost saving application and minimal consumption of organic solvents [12]. However, one of the major drawbacks associated with SPE is the low selectivity. Therefore, it is important to develop techniques for the rapid and selective extraction of sitagliptin from biological matrices.

Molecularly imprinted polymers (MIPs) possess pre-defined specific cavities designed for target molecules. These are stable to extremes of pH, organic solvents and temperature which provides for more flexibility in the development of bioanalytical methods [13–15]. MIPs found a broad range of applications including synthesis and catalysis [16], controlled drug delivery

* Corresponding author. Tel.: +91 40 27193193; fax: +91 40 27160387.
*E-mail address:* rnrao@iict.res.in (R.N. Rao).

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.05.002

systems [17], sensors [18] and separations [19]. Use of MIPs as packing materials in molecularly imprinted solid phase extraction (MISPE) is relatively a new concept in clean-up of biological samples [20,21]. High affinity binding sites are generated by forming covalent/non-covalent pre-association complexes between the template molecules and appropriate functional monomers. The complex is then trapped within highly cross-linked polymeric matrices. Removal of the template molecule creates a polymer cavity with appropriately positioned functional groups [22]. The use of such MIPs as adsorbents may facilitate SPE for development of highly selective and sensitive methods for analysis of low concentrations of drugs in biological matrices. The MIPs for SPE can be used in different modes, (i) conventional, where the MIP was packed into cartridges [23,24] and (ii) batch mode, where the MIP was incubated with the sample [25]. The batch mode using automatic solid phase extractor provides a way towards high throughput sample preparations. Another advantage of MIP-based SPE, relates to the high selectivity of the sorbent and efficient sample clean-up. To the best of our knowledge, there was no MIP based SPE technique reported earlier to determine sitagliptin in biological matrices.

The present manuscript deals with the preparation of a sitagliptin imprinted polymer via non-covalent self-assembly of specific functional monomer units. Its selectivity for sitagliptin compared to commonly co-administered drugs was assessed. A zwitterionic hydrophilic interaction liquid chromatography (HILIC) for the analysis of the extracts was used.

## 2. Experimental

### 2.1. Materials

Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) inhibited with 250 mg/L hydroquinone and 100 mg/L monomethyl ether hydroquinone, respectively and 2,2-azobis isobutyronitrile (AIBN) (Sigma–Aldrich, Banglore, India) of reagent grade were used. EGDMA, MAA were distilled in vacuum prior to use to remove stabilizers. Acetonitrile (ACN), methanol (MeOH), dimethylformamide (DMF), dimethylsulphoxide (DMSO), acetic acid, and ammonium acetate (SD Fine Chemicals, Mumbai, India) were used. Glass-distilled and de-ionized water (Nanopure, Barnsted, USA) was used. Sitagliptin, pioglitazone, simvastatin, metformin, atorvastatin, acetaminophen, caffeine, ibuprofen, pheniramine, dextromethorphan, nicotine, pseudoephedrine, diphenhydramine, acetylsalicylic acid and phenylephrine were obtained from local industries in Hyderabad, India. Drug-free rat plasma and urine were obtained from the Pharmacology Division (Indian Institute of Chemical Technology, Hyderabad) and stored at −20 °C until use after gentle thawing.

### 2.2. Apparatus

An HPLC system consisting of two LC-20AT pumps, an SPD-M20A diode array detector (PDA), a SIL-20AC auto sampler, a DGU-20A3 degasser and a CBM-20A communications bus module (all from Shimadzu, Kyoto, Japan) was used. The chromatographic and the integrated data were recorded using an HP-Vectra (Hewlett Packard, Waldron, Germany) computer system using LC-Solution data acquiring software (Shimadzu, Kyoto, Japan). Scanning Electron Microscope (SEM) (Hitachi S-3000N) and FT-IR spectrometer (Thermo Nicolet Nexus 670 USA) were used for characterization of MIPs and non imprinted polymers (NIPs).

## 3. Methods

### 3.1. Preparation of MIPs and NIPs

The template (sitagliptin), functional monomer (MAA), cross-linking monomer (EGDMA), and initiator (AIBN) were mixed in 25 mL DMSO in a 25 mL thick walled glass tube. The mixture was uniformly dispersed by sonication for 20 min and a stream of nitrogen was passed for one minute. The solution was incubated at 60 °C for 48 h. Fig. 1 depicts the synthesis process of sitagliptin imprinted polymers. A NIP was prepared by the same procedure. The hard polymers thus obtained were crushed. After the polymerization and drying, the polymer particles were washed with 1% acetic acid in MeOH for three times and with distillated water two times. The complete removal of template was followed by HPLC.

### 3.2. Working standards

Primary stock solutions of sitagliptin, pioglitazone, simvastatin, metformin, atorvastatin, acetaminophen, caffeine, ibuprofen, pheniramine, dextromethorphan, nicotine, pseudoephedrine, diphenhydramine, acetylsalicylic acid and phenylephrine were prepared in MeOH (1 mg/mL). Working standards of drugs in mobile phase (ACN: 15 mM ammonium acetate (pH 4.5) 90:10, v/v) were prepared from the primary stocks. All the solutions were stored in dark at 4 °C and brought to room temperature before use.

### 3.3. Rebinding experiments

Batch adsorption experiments were conducted to evaluate the binding affinity of the polymer as reported elsewhere [26]. The general procedure for extraction of sitagliptin is shown in Fig. 2. The free concentration of sitagliptin after the adsorption was monitored by HPLC at 268 nm. Three replicate extractions and measurements were performed for each aqueous solution. The adsorbed sitagliptin was desorbed from the MIP by treatment with 2 mL of MeOH with acetic acid (99:1, v/v) after sonication for 10 min. The sitagliptin concentration in the aqueous phase was determined. Similar procedure was followed for NIP particles.

### 3.4. MISPE conditions for rats' plasma and urine samples

50 mg of MIPs and NIPs were packed into 1 mL empty SPE cartridges and conditioned sequentially with 2 mL MeOH, 1 mL of 0.1% acetic acid and 2 mL of ultra-pure water. After conditioning the MISPE cartridge was thoroughly dried under vacuum. Protein precipitation of plasma and urine solutions were performed by adding 5 mL of plasma/urine sample to a 10 mL volumetric flask and the solutions were diluted up to the mark with 25 mM ammonium acetate (pH 4.5), vortexed for 5 min and were centrifuged for 20 min at 2500 rpm. Filtrates were collected in glass containers and stored at −20 °C until the analysis was performed. 2 mL of the filtered supernatants were loaded on to the cartridges and elution was performed by passing 1 mL of 1% acetic acid solution in MeOH. 10 μL of this elute was injected onto the analytical column for analysis by HPLC. Fig. 3 shows the schematic representation of extraction procedure followed for rats' plasma and urine.

## 4. Results and discussion

### 4.1. Optimization of MIP formulation and progenic solvent

There are different variables viz. amount of monomer, nature of cross-linker and solvent that affect the final characteristics of the obtained materials in terms of capacity, affinity and selectivity for the targeted sitagliptin. An optimized combination of cross-linker

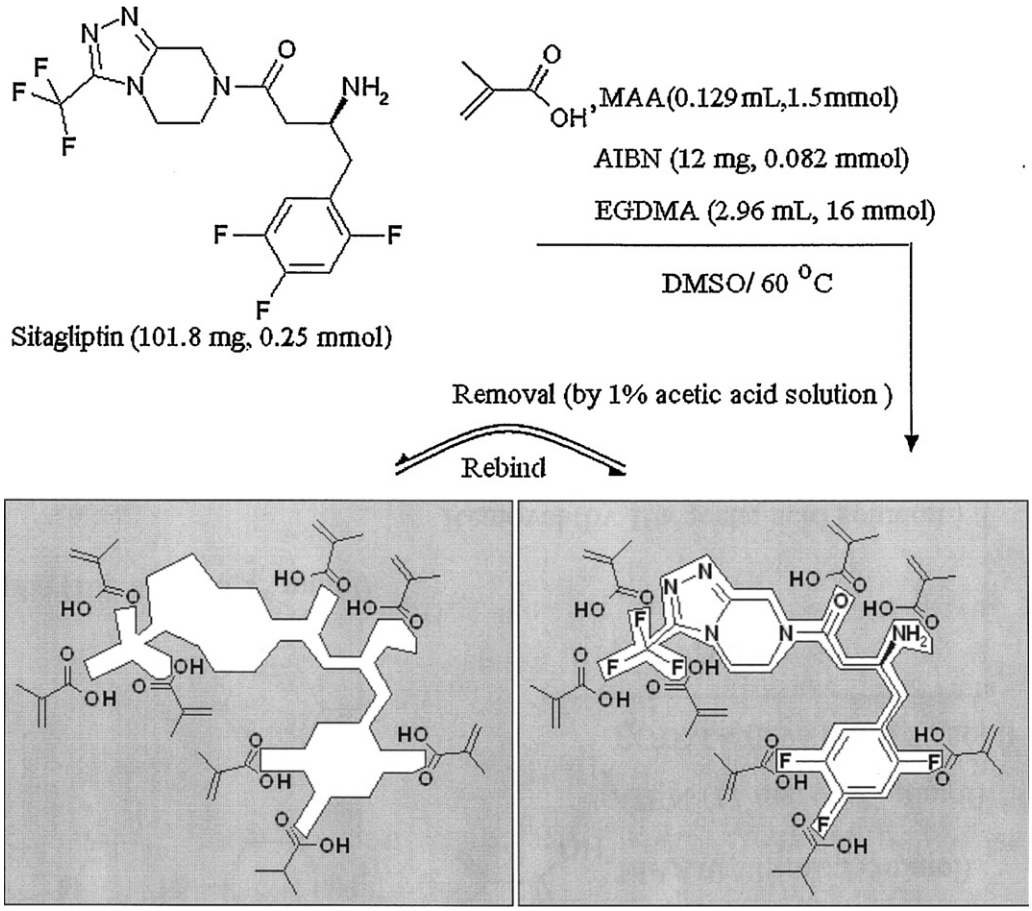


**Fig. 1.** Synthesis process for sitagliptin imprinted polymer.

and functional monomer minimizes non-specific binding. Primary experiments showed that the imprinted polymers prepared in DMSO have better molecular recognition ability than those prepared in MeOH and DMF in aqueous environment (see Electronic Supplementary Material, Fig. S2). Different formulations of MIPs prepared in DMSO with improved molecular recognition capabilities have been used. 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 high non-specific affinity, while low ratios produce fewer complexation due to insufficient functional groups [27]. Four molar ratios of the monomer MAA to the template of 2:1, 4:1, 6:1 and 8:1 were tried experimentally. The optimum ratio of functional monomer to template for the specific rebinding of sitagliptin was found to be 4:1 (Table 1), therefore, a typical 4:1:64 template: monomer: cross-linker molar ratio was used for further studies. Examination of the recoveries of aqueous samples clearly illustrates that the developed MIP is highly water compatible (i.e. applicable in the aqueous environment) giving a high recovery of the analytes (98.94%), with a low contribution due to non-specific adsorption of sitagliptin to the polymer surface (Table 1).

### 4.2. Characterization

FT-IR (KBr) spectrum (see Electronic Supplementary Material, Fig. S3) of NIP, unleached and leached MIPs displayed characteristic peaks, indicating the similarity in the backbone structure of the polymers. The stretching and bending vibrations in the leached MIP materials were shifted to 3446 $cm^{-1}$ and 1456 $cm^{-1}$ compared to 3460 $cm^{-1}$ and 1461 $cm^{-1}$ in the unleached MIP materials due to the hydrogen bonding between the carboxyl group of MAA and

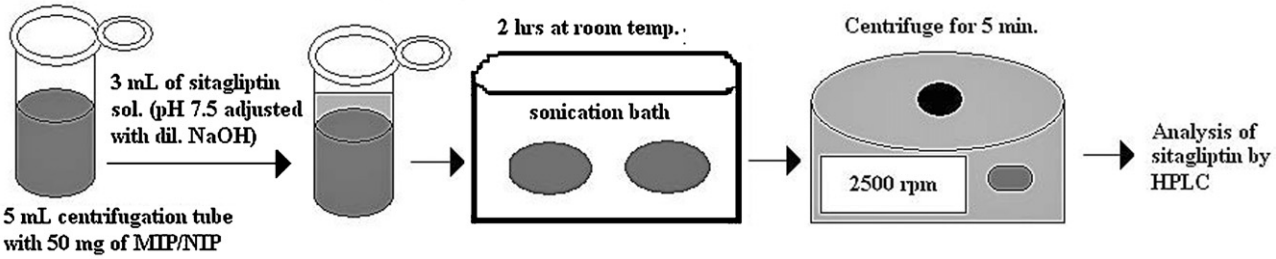


**Fig. 2.** General procedure to check the rebinding affinity of sitagliptin imprinted polymers.

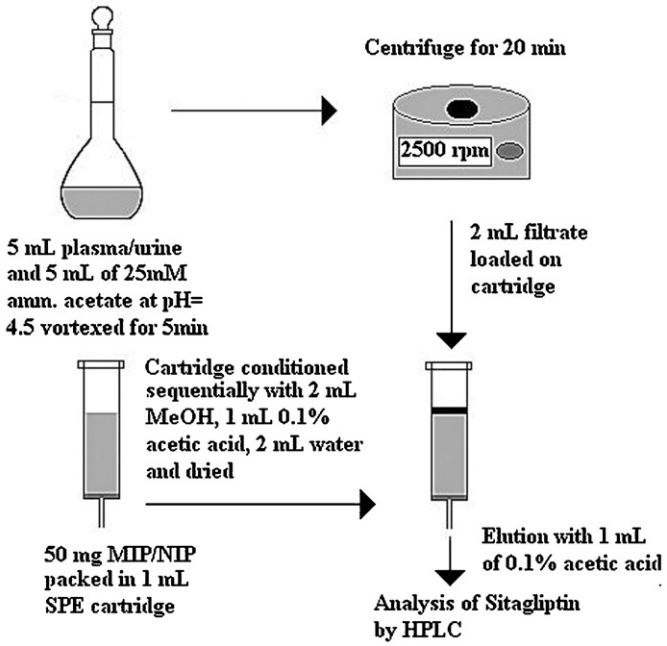


**Fig. 3.** Extraction of sitagliptin from rats' plasma and urine samples using MISPE.

the hydroxyl group of leached MIP materials. Stretching vibration of carbonyl groups (1731–1733 $cm^{-1}$) matched well with those of MIP, as well as NIP. The size of the sieved MIPs and NIPs after crushing was between 10 and 20 μm as determined by SEM (see Electronic Supplementary Material, Fig. S4).

### 4.3. Effect of pH

It was observed that efficient imprint rebinding is possible in aqueous buffer solutions, showing high binding affinity and selectivity as a result of hydrophobic interactions. The effect of pH on the rebinding efficiency of sitagliptin was investigated by varying the solution pH from 4.0 to 8.0 (Fig. 4). Several batch experiments were performed by equilibrating 50 mg of the imprinted particles with 5 mL of solutions containing 100 μg/mL of sitagliptin under the desired range of pH. It was observed that sitagliptin underwent complete rebinding/elution at pH 7.5.

### 4.4. Adsorption kinetics

To investigate the adsorption kinetics of the MIP of sitagliptin, 100 mg MIP was dispersed in 10 mL sitagliptin solution with an initial concentration of 1.0 mg/mL in a 25 mL flask and the sealed flask was placed in a thermostat at 25 °C. 100 μL samples were collected after an hour interval, and concentrations were determined by HPLC. Adsorption quantity was calculated by the following equation:

$$Q_t = \frac{(C_0 - C_t)V}{m}$$

where $Q_t$ (mg/g) is the experimental adsorption quantity at different times, $C_0$ (mg/mL) is the initial concentration of sitagliptin, $C_t$ (mg/mL) is the concentration of sitagliptin at different times, $V$ (mL) is volume of sitagliptin solution, and $m$ (g) is weight of the MIP.

Fig. 5a shows the adsorption kinetic curve of MIP binding sitagliptin. It could be observed from the kinetic curve that the adsorption quantity increased rapidly and about half of the total adsorption quantity was achieved during the first 2–3 h. After this period, adsorption rate slowed down and equilibrium was reached at about 8 h. The explanation for the adsorption process exhibiting two different steps is that the recognition sites on the surfaces of MIP profits the combination with sitagliptin molecules in the first period, and then the mass transfer barrier for sitagliptin into the internal binding sites results in the slow adsorption rate after the surface binding sites getting saturated.

**Table 1**
Compositions and comparisons of the extraction of sitagliptin from sitagliptin standard solution (5 mL, 100 μg/mL) using 50 mg of various polymers as sorbents at pH 7.5, elute 1 mL MeOH with 1% AcOH.

| Polymer | MAA (mmol) | Sitagliptin (mmol) | EGDMA (mmol) | AIBN (mmol) | Extraction (%) (mean ± SD)[a] |
|---|---|---|---|---|---|
| MIP1 | 0.5 | 0.25 | 16 | 0.082 | 64.1 (±3.1) |
| MIP2 | 1.0 | 0.25 | 16 | 0.082 | 98.9 (±1.7) |
| MIP3 | 1.5 | 0.25 | 16 | 0.082 | 71.3 (±2.3) |
| MIP4 | 2.0 | 0.25 | 16 | 0.082 | 53.2 (±2.6) |
| NIP1 | 0.5 | 0.00 | 16 | 0.082 | 24.8 (±1.2) |
| NIP2 | 1.0 | 0.00 | 16 | 0.082 | 22.4 (±2.9) |
| NIP3 | 1.5 | 0.00 | 16 | 0.082 | 25.3 (±2.4) |
| NIP4 | 2.0 | 0.00 | 16 | 0.082 | 31.1 (±2.8) |

[a] Average of five determination.

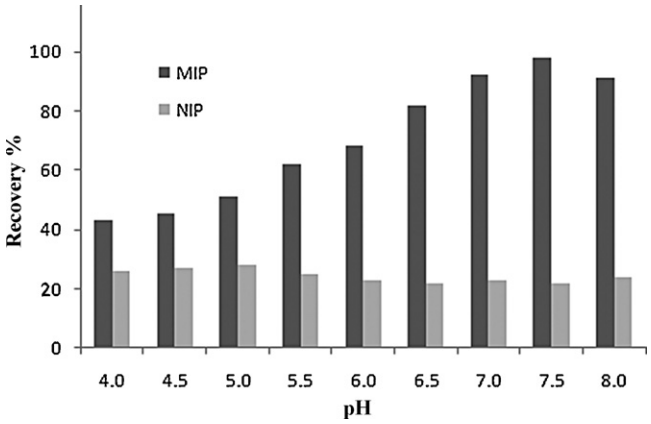


**Fig. 4.** Recoveries obtained using MIP and NIP polymers at different pH.

### 4.5. Measurement of adsorption capacity

The capacity of the sorbent is an important factor that determines how much sorbent is required to remove a specific amount of drug from the solution quantitatively. To measure the adsorption capacity of MIP and NIP absorbents, experiments were performed like rebinding experiments and the isothermal adsorptions are plotted in Fig. 5b. According to the results, the maximum amount of sitagliptin adsorbed by MIP was found to be 180 mg/g at pH 7.5. For higher sitagliptin amounts, a slight increase of retained sitagliptin was observed on MIP capacity curve. As all the accessible specific cavities of the MIP are saturated, the retention of the analyte was only due to non-specific interactions which can be approximately identical to MIP and NIP polymers.

### 4.6. MIP selectivity

To evaluate the specificity of MIP for sitagliptin batch rebinding experiments were performed. For this, 5 mL of initial concentrations of sitagliptin ($C_{i,}$), 100 μg/mL, was extracted by 50 mg of imprinted material at pH of 7.5 on MIP and NIP. Once the system has been equilibrated, the concentration of free sitagliptin ($C_f$) in solution is measured and the mass ($m$) of template adsorbed to the MIP calculated. $K_D$, distribution factor (mL/g) of sitagliptin

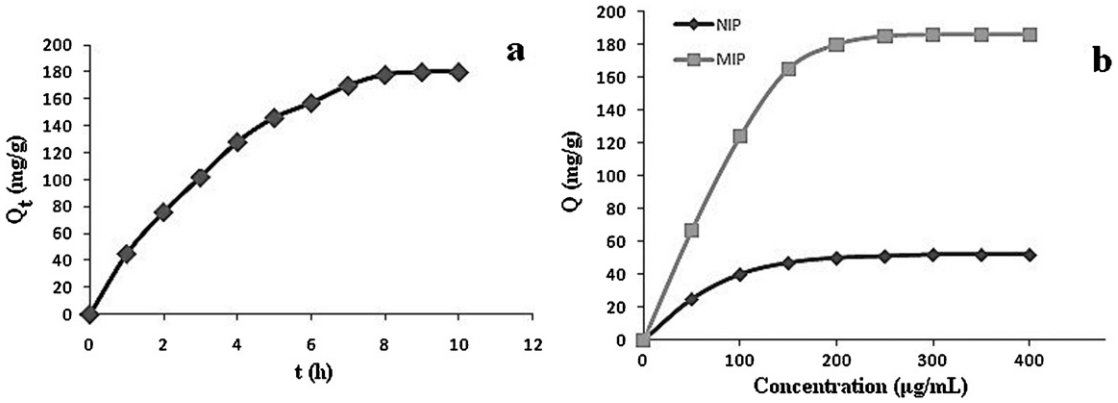


**Fig. 5.** (a) Adsorption kinetic curve of MIP adsorbing sitagliptin (b) Effect of sitagliptin concentration on the retention capacities of MIP and NIP at pH 7.5.

between the MIP particles and aqueous solution was determined by the following equation:

$$K_D = \frac{(C_i - C_f)}{mC_f} V \quad (1)$$

where $V$ is the volume of initial solution.

Selectivity coefficients for sitagliptin ($K^{sel}_{Sitagliptin/j}$) relative to foreign compounds was defined as the ratio of distribution factors ($K_D$) sitagliptin and foreign compounds (j).

$$K^{sel}_{Sitagliptin/j} = \frac{K_D^{Sitagliptin}}{K_D^{j}} \quad (2)$$

The relative selectivity factor $K'$ was also determined by the following equation:

$$K' = \frac{K(MIP)}{K(NIP)} \quad (3)$$

where $K$(MIP) and $K$(NIP) are selectivity coefficient for MIP and NIP, respectively. These parameters are given in Table 2. A representative chromatogram is shown in Fig. 6, which indicates that MIP is highly selective for sitagliptin recognition than NIP, either in urine or plasma samples.

### 4.7. HPLC method development

Sitagliptin is relatively a polar compound having high solubility in water. Preliminary attempts using reversed-phase HPLC were not successful. HILIC has one of the highest degrees of orthogonality to reversed-phase liquid chromatography (RPLC) [28]. Zwitterionic separation materials (polymeric sulfoalkylbetaine in this study) are uniquely characterized by carrying both positive and negative charges on the surface. The electrostatic interactions between those two oppositely charged groups in close proximity at a stoichiometric ratio relatively weaken the interactions of this stationary phase with the charged analytes when compared to normal ionic exchangers such as strong-cation exchange (SCX) [29–32].

The HPLC method was developed using Merck ZIC-HILIC Column (100 mm × 4.6 mm × 5 μm). Mobile phase, ACN and 15 mM ammonium acetate (pH 4.5) 90:10, v/v was filtered through a 0.45 mm polytetrafluoroethylene (PTFE) filter and degassed by helium purging. Analysis was carried out under isocratic conditions using a flow rate of 0.4 mL/min at 25 °C. The chromatograms were recorded at 268 nm using a PDA detector.

**Table 2**
Selectivity of sitagliptin (obtained after the loading of the MIP and NIP cartridges with 5 mL of urine solution spiked with 100 μg/mL sitagliptin, at pH 7.5).

| Analyte | $K_D$ (MIP) (mL/g) | $K_D$ (NIP) (mL/g) | $K^{sel}$ (MIP) | $K^{sel}$ (NIP) | $K'$ |
|---|---|---|---|---|---|
| Sitagliptin | 1598 | 36 | – | – | – |
| Pioglitazone | 89 | 34 | 18.0 | 1.0 | 18.0 |
| Simvastatin | 72 | 35 | 22.2 | 1.0 | 22.2 |
| Metformin | 30 | 28 | 53.3 | 1.3 | 41.0 |
| Atorvastatin | 28 | 25 | 57.1 | 1.4 | 40.8 |
| Acetaminophen | 77 | 30 | 20.8 | 1.2 | 17.3 |
| Caffeine | 67 | 29 | 23.8 | 1.2 | 19.8 |
| Ibuprofen | 48 | 37 | 33.3 | 1.0 | 33.3 |
| Pheniramine | 51 | 29 | 31.3 | 1.2 | 26.1 |
| Dextromethorphan | 52 | 31 | 30.7 | 1.2 | 25.6 |
| Nicotine | 53 | 29 | 30.2 | 1.2 | 25.2 |
| Pseudoephedrine | 92 | 48 | 17.4 | 0.8 | 21.8 |
| Diphenhydramine | 57 | 35 | 28.0 | 1.0 | 28.0 |
| Acetylsalicylic acid | 60 | 42 | 26.6 | 0.8 | 33.2 |
| Phenylephrine | 67 | 31 | 23.8 | 1.2 | 19.8 |

### 4.8. HPLC method validation

#### 4.8.1. Selectivity

No endogenous components extracted from blank plasma were eluted at the retention time of the peak of sitagliptin. The developed method was found to be selective for determination of sitagliptin without interference from the endogenous constituents of plasma and urine. The commonly co-administered drugs pioglitazone, simvastatin, metformin, atorvastatin, acetaminophen, caffeine, ibuprofen, pheniramine, dextromethorphan, nicotine, pseudoephedrine, diphenhydramine, acetylsalicylic acid and phenylephrine were tested for exogenous selectivity. Since, there was no interferencs from these drugs, the developed method was considered to be selective for exogenous as well as endogenous substances.

#### 4.8.2. Calibration curve and linearity

Analysis was based on the external standard method. Calibration standards were prepared fresh on the day of analysis by diluting the appropriate working solutions with mobile phase. The standard calibration curve was constructed using blank plasma and urine samples spiked with sitagliptin at five different concentrations from 0.1, 1, 5, 10 and 100 μg/mL. The data were subjected to statistical analysis using a linear-regression model. The calibration curves were obtained by weighted linear regression (weighing factor $1/x^2$) using Microsoft Excel 2007 software. The suitability of the calibration model was confirmed by back calculating the concentrations of the calibration standards. The developed method was linear over the tested concentrations with correlation coefficient $r^2 = 0.998$ (for plasma) and $r^2 = 0.999$ (for urine), the calibration curve was described by equation $y = 498.22x + 54.50$ (for plasma) and $y = 498.01x + 82.78$ (for urine).

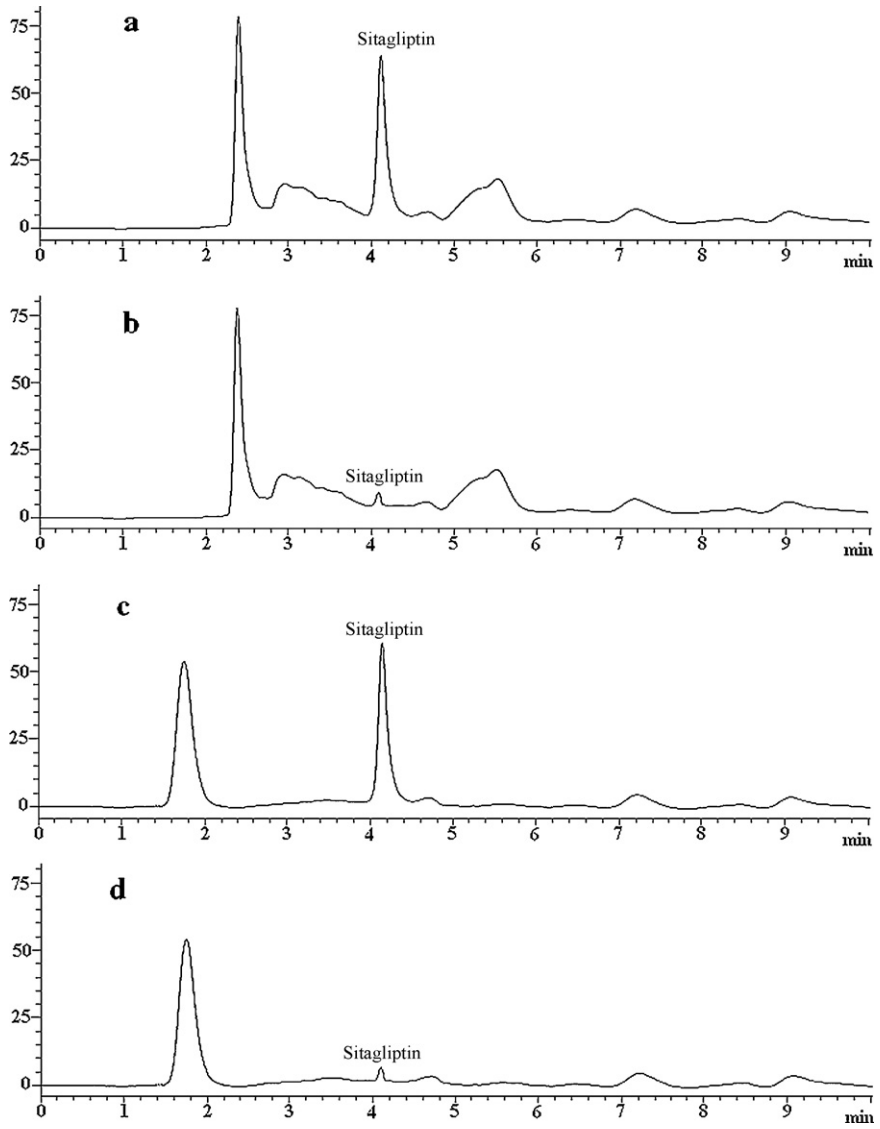


**Fig. 6.** HPLC chromatograms obtained after clean-up of a 50.0 μg/mL solution of sitagliptin in plasma (a) [MIP] and (b) [NIP] and urine (c) [MIP] and (d) [NIP] samples.

*4.8.3. Accuracy and precision*

Two concentrations of sitagliptin viz., 0.1 and 100 μg/mL, in five replicates were used to validate the accuracy and precision of the developed method. The results showed that the intra- and inter-day accuracy (% bias) for the method ranged between −4.0% and 1.0%, respectively (Table 3). The % CV of intra- and inter-day precision was <11.079%. The developed method was thus found to meet generally accepted requirements of accuracy and precision over the studied concentration ranges.

*4.8.4. Limit of detection (LOD) and quantitation (LOQ)*

After sample clean-up, the extracts from spiked plasma were injected into the chromatographic systems. The analysis was carried out at decreasing concentrations to determine the minimal concentration with a signal-to-noise ratio of 3:1. The LOD and LOQ for sitagliptin in urine and plasma were 0.03 and 0.10 μg/L, respectively.

*4.8.5. Matrix effects and recovery*

The matrix-effects were identified by preparing five blank samples. After sample preparation the aliquot was injected, while infusing a standard solution of sitagliptin. Quantification of the matrix-effect was performed by preparing five post-extraction spiked calibration lines from five different batches of plasma and urine, followed by comparing the slopes of these calibration lines with slopes of neat calibration lines. Slope of the neat calibration curve were 0.002045 (for plasma) and 0.002017 (for urine). The results showed that the matrix effect was insignificant in this method and overall recovery of sitagliptin was 98.94% (Table 3).

*4.8.6. Stability*

Sitagliptin in plasma and urine was subjected to three freeze/thaw (−10 to −30 °C to room temperature) cycles. The obtained bias of sitagliptin was >−2.80% of the theoretical value and coefficient of variation (CV) was <4.13%. No significant degradation of the sitagliptin was observed even after 48 h storage period in the autosampler tray with the bias of >−3.59% and CV < 8.29%. In addition, the long-term stability of sitagliptin in quality control samples after 30 days of storage at −10 to −30 °C and room temperature stability for 48 h was also evaluated with the bias of >−3.57% and >−4.00%, CV < 4.71% and <5.69% respectively.

**Table 3**
Intra- and Inter-assay of sitagliptin in rats' urine and plasma by developed MISPE-HPLC protocol[a].

| | | Nominal concentration (in μg/mL) | | | |
|---|---|---|---|---|---|
| | | Plasma | | Urine | |
| | | 0.100 | 100.00 | 0.100 | 100.00 |
| Individual (intra-run) statistics | | | | | |
| Run 1 | Mean concentration (μg/mL) | 0.101 | 99.26 | 0.101 | 98.92 |
| | SD | 0.005 | 1.24 | 0.011 | 1.23 |
| | Precision (% CV) | 4.911 | 1.24 | 11.079 | 1.25 |
| | Accuracy (% bias) | 1.000 | −0.74 | 1.000 | −1.08 |
| Run 2 | Mean concentration (μg/mL) | 0.099 | 99.14 | 0.099 | 98.88 |
| | SD | 0.007 | 0.06 | 0.001 | 0.85 |
| | Precision (% CV) | 7.284 | 1.06 | 1.104 | 0.86 |
| | Accuracy (% bias) | −1.000 | −0.86 | −1.000 | −1.12 |
| Run 3 | Mean concentration (μg/mL) | 0.097 | 98.62 | 0.096 | 99.33 |
| | SD | 0.002 | 1.20 | 0.004 | 0.57 |
| | Precision (% CV) | 2.001 | 1.213 | 4.191 | 0.57 |
| | Accuracy (% bias) | −3.000 | −1.38 | −4.000 | −0.67 |
| Overall (inter-run) statistics | | | | | |
| | Mean concentration (μg/mL) | 0.098 | 99.78 | 0.099 | 99.18 |
| | SD | 0.002 | 0.15 | 0.001 | 0.63 |
| | Precision (% CV) | 1.953 | 0.15 | 1.448 | 0.64 |
| | Accuracy (% bias) | −2.000 | −0.22 | −1.000 | −0.82 |
| Overall recovery = 98.94% | | | | | |

[a] $n = 5$, for each concentration level in each individual run.

*4.8.7. Robustness*

A "Plackett–Burman" design was used to test the robustness of chromatographic separation. Design of experiment (DOE) is a useful tool as it facilitates the investigation of several parameters at the same time while reducing the number of experiments [33,34]. Five factors which are likely to be significant in practical use of the method: ACN content of the mobile phase, molarity and pH of the ammonium acetate buffer, flow-rate and column oven temperature were investigated using three variables with upper and lower limits. The experiments were run randomly with plasma sample spiked with 100 μg/mL sitagliptin. The selected responses were capacity factor ($K'$), resolution ($R_s$), number of theoretical plates ($N$) and tailing factor ($T_f$). The design matrix with the factor settings is shown in Table S1 (see Electronic Supplementary Material). Plotting the scaled and centered coefficient plots (see Electronic Supplementary Material, Fig. S5) for $K'$, $R_s$, $N$ and $T_f$ revealed that different combinations of significant parameters will not drastically affect responses, so that the developed method was considered to be robust.

## 5. Conclusions

For the first time, a simple MISPE-HPLC method based on water compatible MIP for extraction of sitagliptin from wistar rats' plasma and urine samples was developed. The sitagliptin MIP showed higher molecular recognition than NIP. The prepared MIP proved to be very efficient for selective extraction of sitagliptin from plasma and urine samples. It has been demonstrated that by exerting judicious choice and control over polymer composition, the polymerization conditions and the analyte analogues used as templates, it is possible to overcome the practical obstacles that arise frequently during the application of MIPs to the trace analysis of small molecules. The extraction recovery of the analytes from plasma and urine samples was more than 98%. The blockage of MISPE cartridges was observed after loading the rats' plasma and urine samples directly on cartridges; to overcome this problem protein precipitation step was introduced before sample loading.

## Acknowledgements

The authors wish to thank Dr J. S.Yadav, Director, IICT; Dr. M. Vairamani, Head, Analytical Chemistry Division for encouragement and permission to communicate the manuscript for publication. The authors also thank Mr. Sachin B. Agwane, Scientist, Pharmacology Division, Indian Institute of Chemical Technology (Hyderabad, India) for assistance in collection of samples of rats' plasma and urine. Mr. Pawan K. Maurya thanks University Grants Commission (UGC), New Delhi, India for a research fellowship.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.05.002.

## References

[1] B. Gallwitz, Vasc. Health Risk Manag. 3 (2007) 203–210.
[2] G.A. Herman, C. Stevens, K.V. Dyck, A. Bergman, B. Yi, M. Smet, K. Snyder, D. Hilliard, M. Tanen, W. Tanaka, A.Q. Wang, W. Zeng, D. Musson, G. Winchell, M. Davies, S. Ramael, K.M. Gottesdiener, J.A. Wagner, Clin. Pharm. Ther. 78 (2005) 675–688.
[3] T.J. Kieffer, J.F. Habener, Endocr. Rev. 20 (1999) 876–913.
[4] J.J. Meier, M.A. Nauck, Best Pract. Res. Clin. Endocrinol. Metab. 18 (2004) 587–606.
[5] G.A. Herman, P.P. Stein, N.A. Thornberry, J.A. Wagner, Clin. Pharm. Ther. 81 (2007) 761–767.
[6] A.E. Weber, J. Med. Chem. 47 (2004) 4135–4141.
[7] C.F. Deacon, B. Ahren, J.J. Holst, Expert Opin. Invest. Drugs 13 (2004) 1091–1102.
[8] R. Nirogi, V. Kandikere, K. Mudigonda, P. Komarneni, R. Aleti, R. Boggavarapu, Biomed. Chromatogr. 22 (2008) 214–222.
[9] W. Zeng, D.G. Musson, A.L. Fisher, A.Q. Wang, Rapid Commun. Mass Spectrom. 20 (2006) 1169–1175.
[10] W. Zeng, D.G. Musson, A.L. Fisher, L. Chen, M.S. Schwartz, E.J. Woolf, A.Q. Wang, J. Pharm. Biomed. Anal. 46 (2008) 534–542.
[11] W. Zeng, Y. Xu, M. Constanzer, E.J. Woolf, J. Chromatogr. B 878 (2010) 1817–1823.
[12] M.-C. Hennion, J. Chromatogr. A 856 (1999) 3–54.
[13] B. Sellergren (Ed.), Molecularly Imprinted Polymers: Man-Made Mimics of Antibodies and their Applications in Analytical Chemistry (Techniques and Instrumentation in Analytical Chemistry), vol. 23, Elsevier, Amsterdam, 2001.
[14] M. Javanbakht, A.M. Attaran, M.H. Namjumanesh, M.E. Manesh, B.A. Adergani, J. Chromatogr. B 878 (2010) 1700–1706.
[15] C. He, Y. Long, J. Pan, K. Li, F. Liu, J. Biochem. Biophys. Methods 70 (2007) 133–150.
[16] C. Alexander, H.S. Andersson, L.I. Andersson, R.J. Ansell, N. Kirsch, I.A. Nicholls, J. O'Mahony, M.J. Whitcombe, J. Mol. Recognit. 19 (2006) 106–180.
[17] J.Z. Hilt, M.E. Byrne, Adv. Drug Deliv. Rev. 56 (2004) 1599–1620.
[18] F.L. Dickert, P.A. Lieberzeit, O. Hayden, S. Gazda-Miarecka, K. Halikias, K.J. Mann, C. Palfinger, Sensors 3 (2003) 381–392.
[19] C. Baggiani, L. Anfossi, C. Giovannoli, Anal. Chim. Acta 591 (2007) 29–39.

[20] E. Caro, R. Marce, P. Cormack, D. Sherrington, F. Borrul, Anal. Chim. Acta 562 (2006) 145–151.
[21] N. Masque, R.M. Marce, F. Borrull, Trends Anal. Chem. 20 (2001) 477–486.
[22] L.J. Schwarz, B. Danylec, S.J. Harris, R.I. Boysen, M.T.W. Hearn, J. Chromatogr. A 1218 (2011) 2189–2195.
[23] M. Walshe, J. Howarth, M.T. Kelly, R. O'Kennedy, M.R. Smyth, J. Pharm. Biomed. Anal. 16 (1997) 319–325.
[24] A. Zander, P. Findlay, T. Renner, B. Sellergren, A. Swietlow, Anal. Chem. 70 (1998) 3304–3314.
[25] L.I. Andersson, A. Paprica, T. Arvidsson, Chromatographia 46 (1997) 57–62.
[26] Y.H. Zhai, Y.W. Liu, X.J. Chang, S. Chen, X.J. Huang, S.B. Chen, X.P. Huang, Anal. Chim. Acta 593 (2007) 123–128.
[27] A. Rachkov, N. Minour, J. Chromatogr. A 889 (2000) 111–118.
[28] M. Gilar, P. Olivova, A.E. Daly, J.C. Gebler, Anal. Chem. 77 (2005) 6426–6434.
[29] S.D. Palma, P.J. Boersema, A.J.R. Heck, S. Mohammed, Anal. Chem. 83 (2011) 3440–3447.
[30] W. Dormeyer, S. Mohammed, B. Breukelen, J. Krijgsveld, A.J. Heck, J. Proteome Res. 6 (2007) 4634–4645.
[31] P. Hemstrom, K. Irgum, J. Sep. Sci. 29 (2006) 1784–1821.
[32] P. Jandera, J. Sep. Sci. 31 (2008) 1421–1437.
[33] R.N. Rao, P.K. Maurya, M. Ramesh, R. Srinivas, S.B. Agwane, Biomed. Chromatogr. 24 (2010) 1356–1364.
[34] R.N. Rao, P.K. Maurya, D.D. Shinde, S. Khalid, J. Pharm. Biomed. Anal. 55 (2011) 282–287.