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Synthesis and Application of Glibenclamide Imprinted Polymer for Solid Phase Extraction in Serum Samples Using Itaconic Acid as Functional Monomer

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ABSTRACT

Glibenclamide is a second-generation sulfonylurea drugs for treatment of diabetes mellitus. Up to now, a glibenclamide imprinted polymer is not reported for molecular recognition in biological samples. This research is conducted to have Molecular Imprinted Solid Phase Extraction (MISPE) for separation of glibenclamide from serum samples. The results showed that the itaconic acid is the functional monomer that provides the best interaction with the template (glibenclamide) from the computational study using Gaussian 09 software. The MISPE made from itaconic acid monomer at a ratio of 1:6:70 gives the best binding to glibenclamide in methanol pH 4. Serum sample which was spiked with glibenclamide gives recovery more than 80% after pretreatment with MISPE 2 in all concentration ranges. Selectivity test showed that MISPE 2 can be used for selective extraction of glibenclamide from serum samples spiked with other sulfonylurea drugs. This developed MISPE could be further used as extraction method in antidiabetic drugs analysis from biological samples.

Key words: Molecularly imprinted polymer, glibenclamide, itaconic acid, solid phase extraction

INTRODUCTION

Molecular Imprinted Polymer (MIP) is a polymer that is made using molecular imprinting techniques with an affinity for the template molecule, its prepared by the existence of template as a print for the conformation of a complementary binding site of the template (Zheng *et al.*, 2002; Yoshimi *et al.*, 2013). The use of MIP in the Solid Phase Extraction (SPE) has high benefits because it produces selective extraction of analytes and eliminates sample matrices. It is able to produce the receptor binding site-like artificial memory on the shape and position of the functional groups of the template molecule

(Rezaei *et al.*, 2010; Khodadadian and Farhad, 2010). Molecular Imprinted Solid Phase Extraction (MISPE) is able to provide the stationary phase which selectively isolate the specific compound or its structural analog of a complex matrix (Qiao *et al.*, 2006; Lulinski *et al.*, 2014; Pichon, 2007; Yin *et al.*, 2005). The selectivity of the MIP comes from synthetic procedure to prepare the MIP wherein a template molecule is linked by noncovalent or covalent bonding to a monomer with functional groups (Caro *et al.*, 2006). Glibenclamide is a second-generation sulfonylurea drugs for the treatment of noninsulin dependant diabetes mellitus (NIDDM) diabetes type. Glibenclamide is capable to stimulate

the release of insulin from pancreatic beta cells (Binz *et al.*, 2012). As a drug that was used for long term treatment, drug monitoring was needed for glibenclamide. The concentration of glibenclamide was small in biological matrices so preparation of the sample with high accuracy and precision was important in this case. Hence, the development of molecular imprinted polymer for selective extraction of glibenclamide from biological matrices was needed. Up to now, a glibenclamide imprinted polymer for selective extraction of glibenclamide from biological samples never been reported with full study (Hasanah *et al.*, 2014). Thus, this study evaluates the polymer performance for selective recognition of glibenclamide which are made from selection of ten common monomer used in imprinting technology. In addition, this study also aims to determines the best extraction condition for glibenclamide recognition in serum samples with high selectivity.

MATERIALS AND METHODS

Chemicals and apparatus: Itaconic Acid (ITA), ethylene glycol dimethacrylate (EGDMA), 2,2-azoisobutyronitrile (AIBN), tetrafluoroacetic acid (TFA) were provided by Sigma Aldrich. Methanol pro HPLC, chloroform pro analysis, acetonitrile pro HPLC, dimethylformamide (DMF) pro analysis, acetone and acetic acid pro analysis were purchased from JT Baker. Glibenclamide (GC) were provided by Hexpharm Pharmaceuticals Industry. Glipizide (GP) were purchased from Indonesia National Agency of Drug and Food Control. Gliclazide (GL) were provided by Dexa Medica Pharmaceuticals Industry. Blood were provided by Indonesian Red Cross after full examination. ChemDraw and Chem 3D Ultra 8.0.3 software (Cambridgesoft Corporation, USA), Gaussian 09 (Gaussian Inc., Wallingford, CT), Waterbath shaker (Mettler), HPLC (Waters 1525 binary pump HPLC pump with photodiode array 2998 and C18 column sunfire 4, 6, 150 mm), Fourier Transform Infra Red (FTIR) Shimadzu prestige-21, scanning electron microscope (Hitachi TM 3000), centrifugation (Eppendorf centrifuge 5424R).

This study has been carried out from January 2013 until December 2014 at Pharmacochemistry Research Group Bandung Institute of Technology and Research Center Laboratory Universitas Padjadjaran.

Monomer template interaction studies using computational studies: The molecular models of the template molecules, functional monomer and their complexations were drawn using ChemDraw and Chem 3D Ultra 8.0.3 (Cambridgesoft Corporation, USA). The 3D structures were drawn and cartesian coordinates of stable conformers were generated to prepare input file for running Gaussian 09 (Gaussian Inc.,

Table 1: Composition of the produced polymers (MIP and NIP)

Polymer	Template (T)	Monomer (M)	Crosslinker (C)	Ratio T:M:C
MIP 1	GC	ITA	EDGMA	1:6:60
NIP 1	-	ITA	EDGMA	0:6:60
MIP 2	GC	ITA	EDGMA	1:6:70
NIP 2	-	ITA	EDGMA	0:6:70

Wallingford, CT) simulations. The Hartree-Fock level of theory in combination with the 6-31 G(d) basis set was used for geometry optimization to obtain structures with minimum energy. The possible modes of interaction between template molecules and functional monomers at molar ratio 1:1 were sampled by manually docking the functional monomer to each functional group of the template molecule in a systematic manner. The Gibbs free energy gains of the complexes were calculated using Eq. 1:

$$\Delta G = G_{\text{template-monomer complex}} - |G_{\text{template}} + G_{\text{monomer}}| \quad (1)$$

where, ΔG is the change in Gibbs free energy on the formation of template-monomer complex, $G_{\text{template-monomer complex}}$ is the Gibbs free energy of template-monomer complex, G_{template} is the Gibbs free energy of template and G_{monomer} is the Gibbs free energy of monomer molecules.

Synthesis of Molecular Imprinted Polymer (MIP): The Molecular Imprinting Polymer (MIPs) and Non Imprinted Polymers (NIPs) were prepared by bulk polymerization. The polymers were prepared as follows: GC as a template, ITA as functional monomer, followed by cross linker EGDMA and initiator AIBN (0.082 mmol) were dissolved in DMF (4.5 mL) in a thick-walled glass tube. The appropriate homogenous solutions were sonicated for 40 min. Then the mixtures were incubated in the waterbath 60°C for 18 h. The final bulk rigid polymers were ground in a laboratory mortar pestle and wet-sieved with acetone to get particles below 100 μm diameter. The particles were extracted to remove the glibenclamide as a template using Soxhlet apparatus for 24 h in methanol: Acetic acid (9:1) and dried at 55°C for 3 h and stored at room temperature for further experiments. Quantitative removal of the template was ensured by monitoring the amount of template remaining in the extraction solvent by HPLC. The Non Imprinted Polymers (NIP) were prepared in a similar ways as used for the corresponding imprinted polymers except without template molecule during polymerization. Composition of the produced polymers can be seen in Table 1.

Characterization: Infrared (IR) spectra from 4000-400 cm^{-1} were obtained on FTIR Shimadzu prestige-21. Morphology of the MIP and NIP were obtained by using SEM Hitachi TM 3000.

Batch rebinding and isotherm adsorption studies: The rebinding batch-mode experiments were performed in methanol, chloroform, methanol pH 4, acetonitrile pH 4 and acetonitrile. Binding analysis was carried out by incubating 20 mg of polymer in 5 mL volume of the GC solution ($5 \mu\text{g mL}^{-1}$) on a vial and oscillated by a shaker 120 rpm for 3 h at room temperature. Then the mixtures were filtrated and an aliquot of solvent was used to analyze by HPLC. Amount of GC bound to the polymer was calculated by subtracting the amount determined after the experiment from starting amount of GC in standard solution. Isotherm Adsorption Studies were performed by incubating 10 mg of polymer in 1.5 mL volume of GC in several concentration (0.05, 0.1, 0.2, 0.5, 1 and 2 mmolar) on a vial for 24 h. The solution were then analyzed by HPLC. The isotherm adsorption graph were then made with the curve between bound and free GC.

Optimization of Solid Phase Extraction (SPE) system: The 200 mg of MIP and NIP particles were dry packed in 3 mL Chromabond® SPE cartridges using 20 mm porous PTFE frits. This further called MISPE and NI-SPE. Equilibration of the columns, loading and washing were performed using 1 mL aliquots of the corresponding solutions and elution of the retained analytes with different elution solvents. Full vacuum was applied between each step in order to dry the stationary phases. The collected fractions were analytes by HPLC and isocratic elution using a mixture of CH_3CN : TFA 0.01% in water (60:40) with the flow-rate set at 1.2 mL per min.

SPE separation of a mixture of structurally related compounds: After establishing the optimum condition of GC application on the MISPE, a mixture of structurally compounds were used to evaluate the selectivity of the MISPE produced. The compounds were from other sulfonylurea antidiabetic drugs which are GL and GP. Equilibration of the MISPE cartridge with 3 mL of methanol, loading with mixture of GL, GP and GC solution in methanol pH 4 and washing with methanol:water (5:95) were performed and elution of the retained analytes and structurally related compounds with 3×1 mL CH_3CN : Methanol (1:1). The recovery percentage of the elution fraction was then calculated after analysis by using HPLC.

Application of MIP for extraction of glibenclamide from serum samples: Blood serum samples were prepared by centrifugation the collected blood from Indonesian Red Cross 5000 rpm for 5 min at 14°C and careful collection of the clear top layer. The blood serum samples then were spiked with 0.5, 1, 2, 3, 4, 5 and 6 ppm of GC in methanol pH 4. The spiked serum then applied to MISPE and NI-SPE system. The SPE system was conditioned with methanol, washing with water:methanol 95:5 and elute with 3×1 mL CH_3CN :Methanol 1:1. Full vacuum was applied between each step in order to

dry the stationary phase. The elution fraction then analytes by HPLC and isocratic elution using a mixture of acetonitrile: TFA 0.01% in water (60:40) with the flow-rate set at 1.2 mL per min. The recovery percentage of the elution fraction was then calculated.

RESULTS AND DISCUSSION

Computational studies of monomer-template interaction:

In order to optimize the preparation of the MIP, ten monomer were tested for the interaction with template by using Gaussian 09. The result of the studies can be seen in Table 2.

Based on computational studies from Gibbs energy, complex between ITA-GC has a lower Gibbs energy value compare to others. It is mean that this two compounds has good interaction. Negative value of Gibbs energy means this interaction were spontaneous in prepolymerization solution (Piacham *et al.*, 2009). Strong interaction between monomer-template in prepolymerization will lead to good molecular imprinted polymer (Cheong *et al.*, 2013). The interaction between GC and ITA can be seen in Fig. 1.

Table 2: Monomer-template binding energy in prepolymerization mixture by using Gaussian 09

Template-monomer	ΔG (Kcal mol ⁻¹)
GC- ITA	-2.744
GC-Acrylamide	0.378
GC-HEMA	6.121
GC-vinylglycine	2.832
GC-MMA	1.654
GC-methacrylic acid	1.859
GC-methacrylamide	3.509
GC-acrylic acid	5.253
GC-allyl alcohol	5.805
GC-p vinylbenzoic acid	2.97

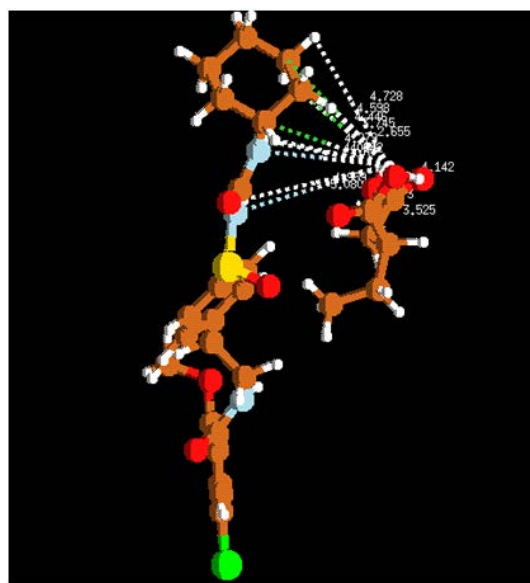


Fig. 1: Interaction of GC-ITA (drawn by Molden® Software)

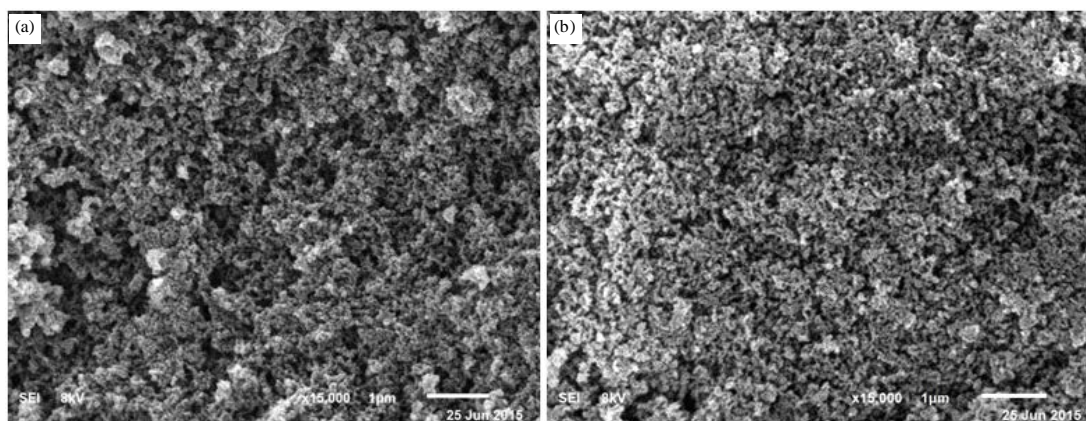


Fig. 2(a-b): SEM Images of (a) MIP 2 and (b) NIP 2

Table 3: Langmuir Fitting Parameters for MIP 2 and NIP 2

Polymers	q (mg g ⁻¹)	R ²	Ka (L mol ⁻¹)
MIP 2	6.24	0.9958	8.00×10 ¹
NIP 2	4.30	0.9994	1.78×10 ²

Characterization: Results of SEM spectrums image of MIP 2 and NIP 2 on Fig. 2 shows the same spectrums, this is because both are made from the same composition only without template on the non-imprinted one. The morphology of the surface of the MIP looks more porous than the NIP, this is due to the extraction process performed on the MIP template resulting in the formation of the bonding that is characterized by a more porous structure (Ji *et al.*, 2014).

From the results of spectrophotometer infra red spectra in Fig. 3a and b, there is a frequency shifting in C = O and -NH which indicates hydrogen bonding between the template and monomer. Because of the hydrogen bonding between template and ITA, there is a reduction in electron density in NH and C = O, which causes a reduction in vibrational frequency before template extraction (Kartasmita *et al.*, 2013).

Batch rebinding and isotherm adsorption studies: The test results from Fig. 4 showed that the binding of MIP made from itaconic acid monomer at a ratio of 1:6:70 (MIP 2) gives 76.37 and 19.93% for non imprinted one in methanol pH 4 against GC solution. ITA has two carboxylate group with pKa 1 = 3.85 and pKa 2 = 5.55 (Gulicovski *et al.*, 2008). At pH 4 one of the carboxylate groups was ionize, this ionization makes ITA interacted with urea groups of GC by hydrogen bonds.

From the Table 3 and Fig. 5, the experimental data were fitted with Langmuir Isotherm adsorption and from the coefficient correlation value, we found out that the data were suitable with Langmuir. Langmuir isotherm adsorption based on the assumption that the adsorption were monolayer and

adsorption site has the same affinity to the template. An adsorption on one site does not affect the other site (Sadeghi and Jahani, 2013).

Optimization of the elution condition on SPE: In order to find out the best condition for elution of GC, a series of solvents were used and the recovery percentage was measured. From Fig. 6, we found out that methanol: ACN 1:1 (3×1 mL) was the best elution solvent. The recovery was reached 99.69% using that solvents. Mixture between methanol and acetonitrile on the elution solvent lead to higher recovery, compare to the solvent alone.

SPE separation of a mixture of structurally related compounds: To find out the selectivity of the MIP 2 in SPE system (MISPE 2), some sulfonylurea drugs were selected. These studies was compared the recoveries percentage of GC, GP and GL through MISPE 2. The recoveries percentage of each compound was determined using MISPE 2 cartridges with three replicates. Percentage recoveries of 91.58±2.19, 79.89±3.99 and 5.16±2.54 were obtained for GC, GP and GL consecutively. GP has relatively higher recovery compare to GL, this is because GP has higher similarity in molecular structure with GC compare to GL. The differences is only in pyridine ring in GP (Pahwa *et al.*, 2010). The interaction between GC and ITA was suggested happened between -NH functional groups from urea and -NH functional groups from amide at GC molecular structure. Molecular structure for all compounds depicted in Fig. 7.

Application of MISPE on serum samples: The performance of MISPE 2 as sorbent was also determined for extraction of GC from blood serum samples in a different concentration. In order to ascertain the performance was based on molecular recognition not from any unspecific binding, the recovery

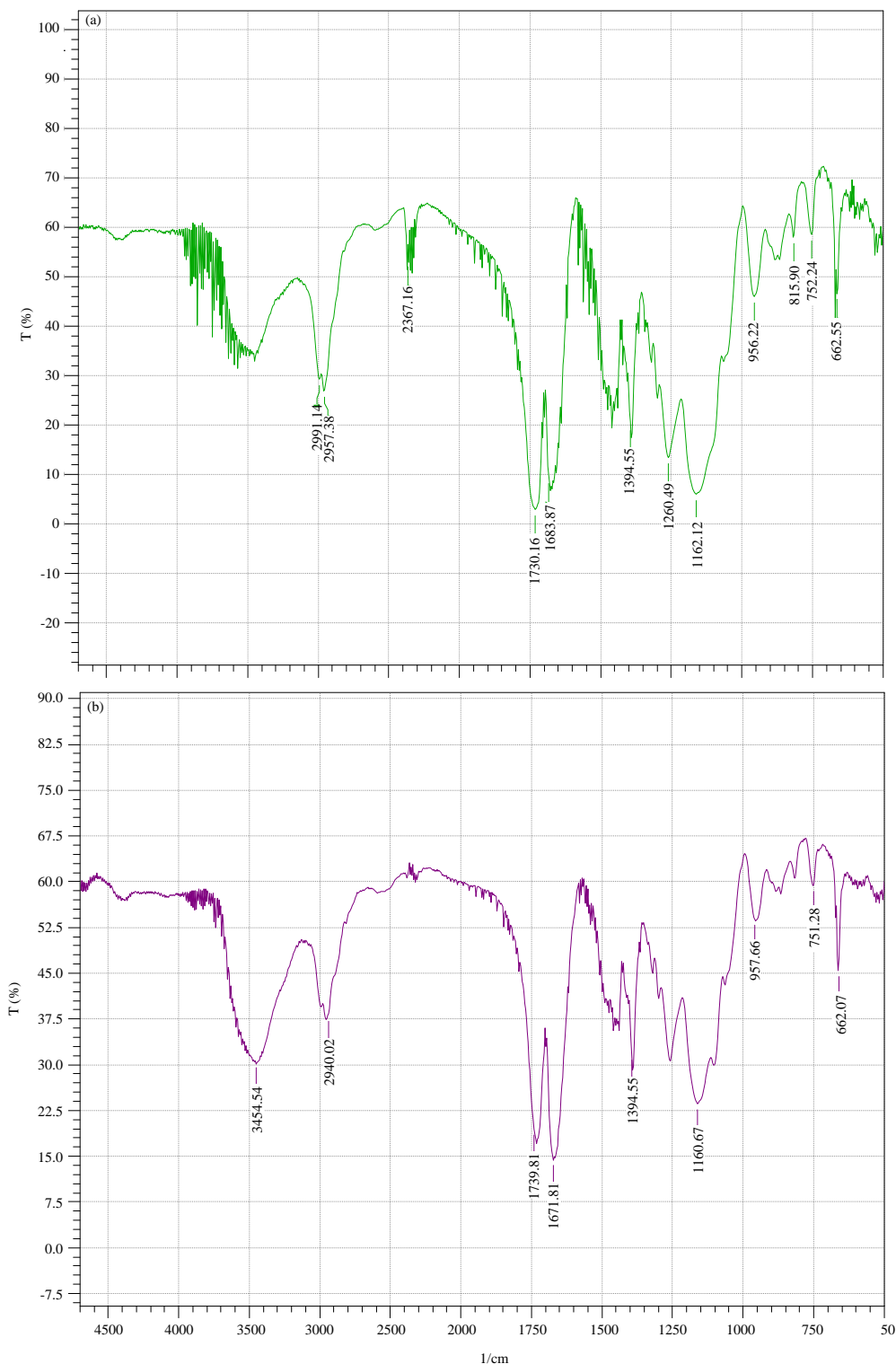


Fig. 3(a-b): FTIR spectra of (a) MIP 2 before template extraction and (b) MIP 2 after template extraction and FTIR spectra of MIP 2 after template extraction

result was compared with NI-SPE 2. Serum sample which was spiked with GC gives recovery 94.22, 92.36, 94.24, 88.2,

89.03, 97.93 and 93.44% for concentration 0.5, 1, 2, 3, 4, 5 and 6 ppm after pretreatment with MISPE 2 (Fig. 8).

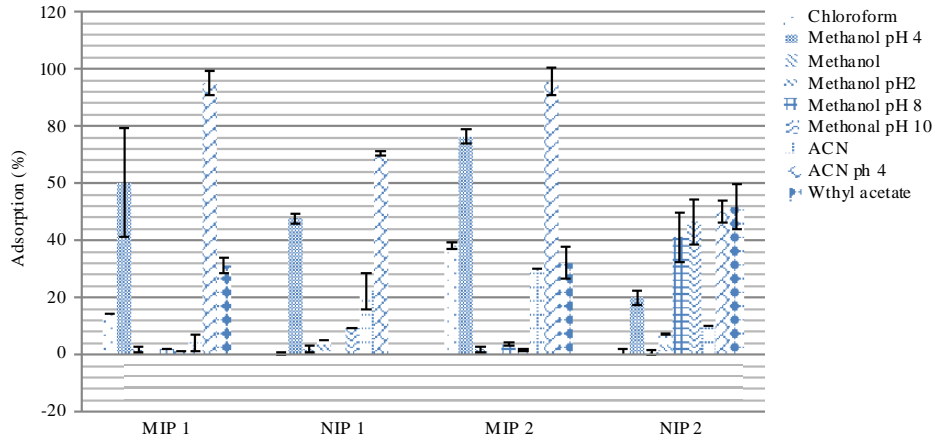


Fig. 4: Binding ability in a various solvents condition

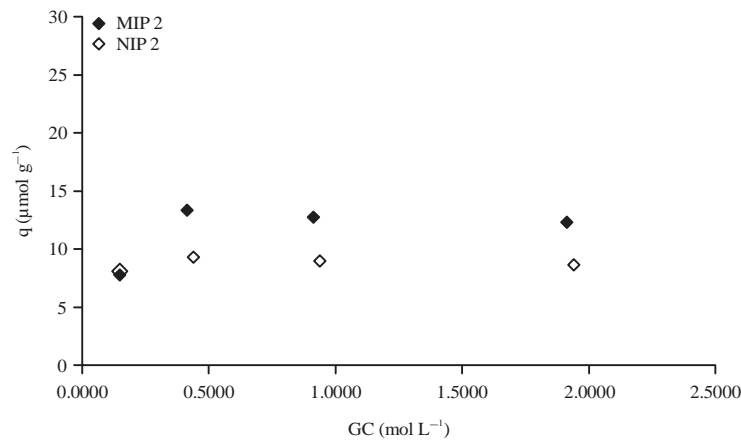


Fig. 5: Isotherm adsorption curve of MIP 2 and NIP 2

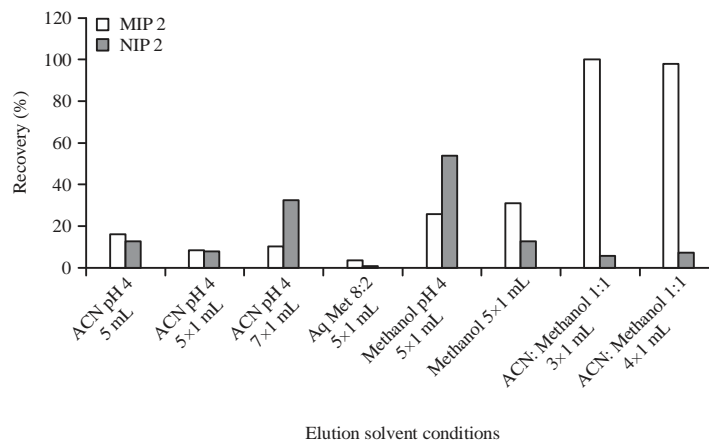


Fig. 6: Recovery percentage of elution condition

From chromatograms of spiked blood serum before and after SPE process (Fig. 9), indicate that MISPE process

produced better peak of GC. The peak of GC is higher and gives good recoveries percentage fitted with the criteria of

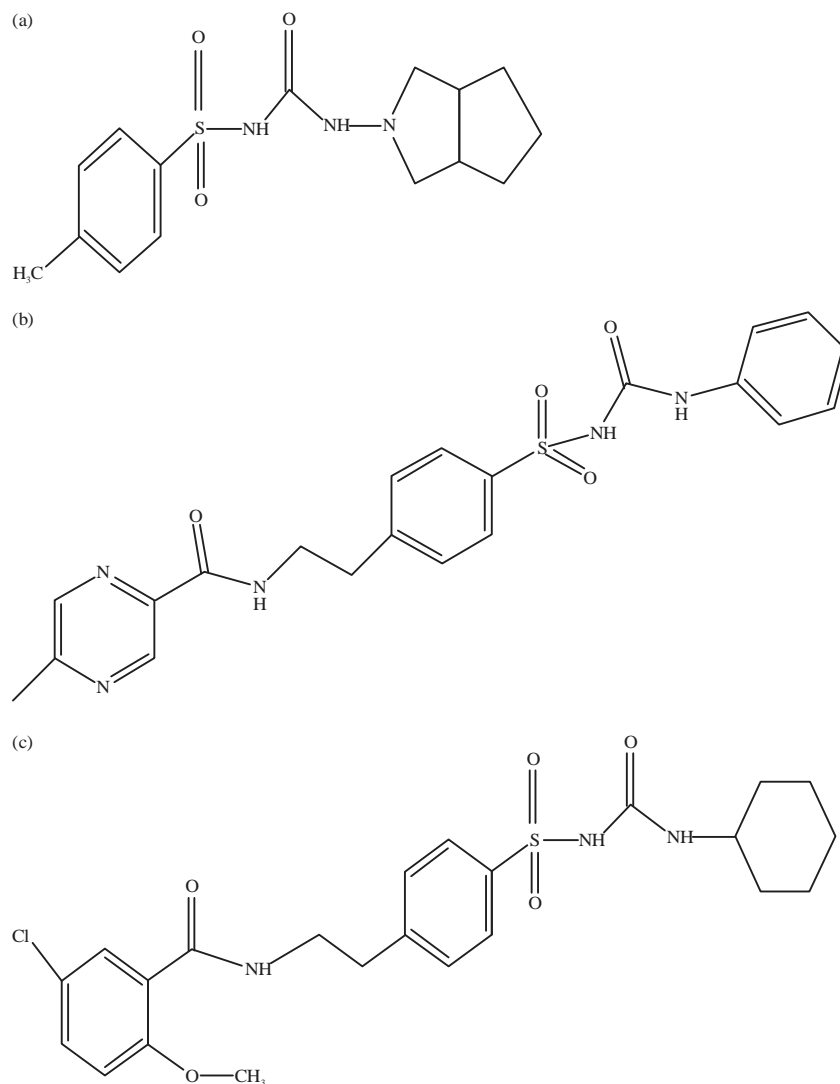


Fig. 7(a-c): Molecular Structure of (a) GL, (b) GP and (c) GC

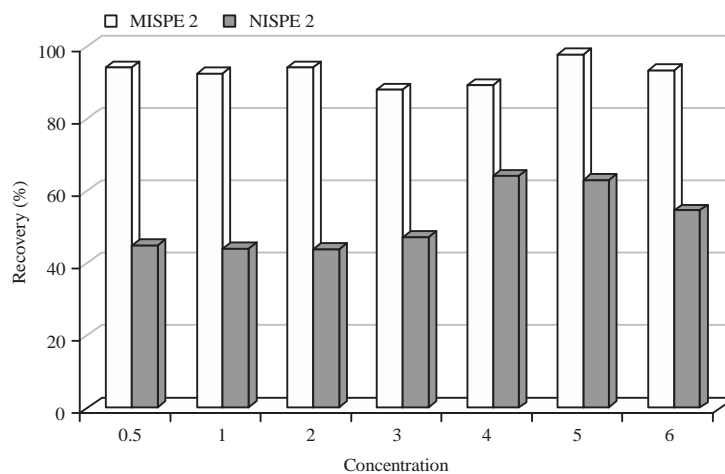


Fig. 8: Recovery percentage of MISPE 2 and NISPE 2

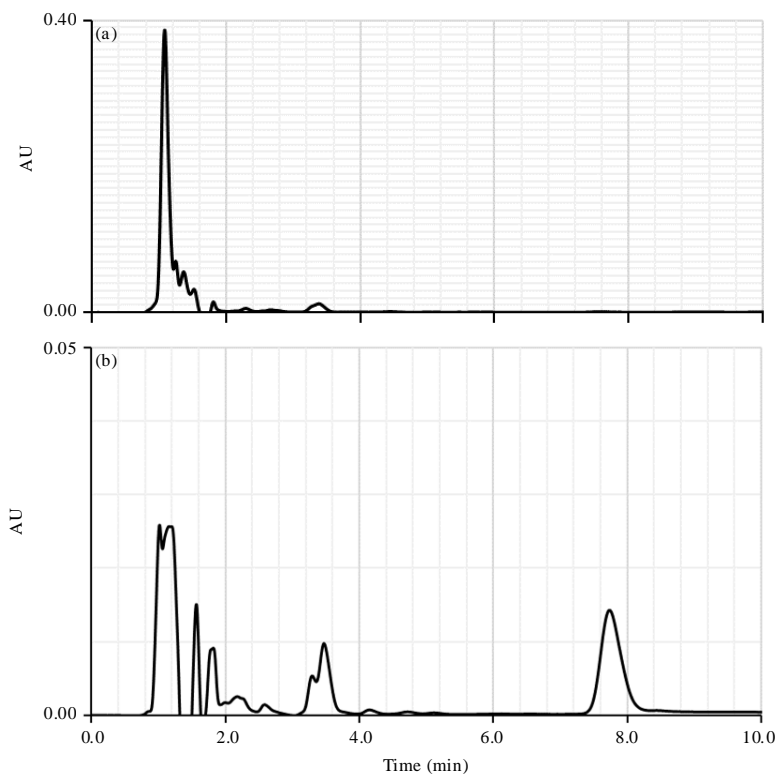


Fig. 9(a-b): Chromatograms of (a) Blood serum spike with GC without MISPE pretreatment and (b) Blood serum spike with GC after MISPE pretreatment

recovery for biological matrices which is more than 80% (DHHS., 2001; Magnusson and Ornemark, 2014; Miura *et al.*, 2011).

CONCLUSION

Glibenclamide molecular imprinted solid phase extraction made from itaconic acid as a monomer in composition 1:6:70 can be used for selective extraction of glibenclamide from serum samples. This results was noteworthy for therapeutic drug monitoring of glibenclamide in diabetic patients.

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