Research Article

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Microsphere molecularly imprinted solid-phase extraction for diazepam analysis using itaconic acid as a monomer in propanol

<https://doi.org/10.1515/chem-2021-0050>

received February 14, 2021; accepted April 13, 2021

Abstract: Diazepam (DZP) is a benzodiazepine drug used as an anti-drug and sedative. It is often misused to induce or create euphoria in combination with other drugs (high or fly sensation) or administered alone. So far, screening for DZP abuse with sensitive analytical methods is needed, as its small concentrations make it difficult to detect. Increased sensitivity of the analytical method can be obtained by using a preparation method that selectively separates the analyte from the sample matrix. Molecularly imprinted polymer (MIP) is one of the preparation solutions with good selectivity, specificity, and sensitivity. MIP was made from DZP as a template, itaconic acid, and ethylene glycol dimethacrylate in a composition of 1:4:20. MIP was made by precipitation polymerisation to obtain microsphere polymer type. MIP had a binding capacity value of 0.0557 mg/g and followed the Freundlich isotherm. Application of the microsphere MIP on spiked blood serum resulted in a recovery of $105.63 \pm 1.0\%$ for MIP compared to $21.28 \pm 0.4\%$ for non-imprinted polymer, with the imprinting factor value reaching 4.96. Hence, MIP DZP with itaconic acid as a

functional monomer and propanol as a porogen, fabricated by the precipitation polymerisation method, is a promising sorbent for DZP extraction in biological fluids.

Keywords: diazepam, itaconic acid, precipitation polymerisation, molecularly imprinted solid-phase extraction, solid-phase extraction, propanol

1 Introduction

Diazepam (DZP) is an anticonvulsant drug used to treat epilepsy and is also used for sedative effects in sleeping pills. DZP is one of the benzodiazepines, providing positive allosteric modulation of the gamma-aminobutyric acid (GABA) receptor, which makes more GABA molecules to bind to the receptor. This modulation will make the GABA receptor (channel ligand) undergo repolarisation and create a relaxing effect. These receptors are found in the central nervous system and explain the mechanism of the sedative effect of DZP to reduce stress and induce drowsiness [1]. In Indonesia, DZP is included as one of the psychotropic drugs, and its prescription is controlled under government law and restrictions. To get DZP, someone needs to visit a doctor and get a prescription. However, DZP is widely abused by those who have become addicted and dependent, to seek the sedative and euphoria effect. It can lead to emergencies, such as overdoses, and end up with comas and fatalities. This behaviour is also carried out by a combination of DZP together with opiates. Detection and monitoring of DZP levels in patients who are undergoing treatment, or who have finished treatment prescribed by doctors, as well as people with symptoms of addiction and dependence of DZP, need to be conducted [2].

Analysis of DZP levels from users or patients poses a new challenge on extraction in biological fluids with a complexity that makes sample preparation necessary, especially due to its low plasma level after oral and

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intramuscular administration. Plasma level of DZP is in the range of 70–400 ng/mL after oral administration and 43–300 ng/mL after intramuscular injection [3]. A large variety of compounds from biological fluids will also have a significant effect on DZP analysis and pureness. Therefore, the novel method of molecularly imprinted polymer (MIP) is an advanced solution for sample preparation because of the high selectivity, sensitivity, and specificity of the molecular template. The ability of each MIP is determined by its components, its manufacturing method, and also its analytical parameters compared with non-imprinted polymer (NIP) [4]. NIP is a polymer molecule synthesised by the same procedure as MIP synthesis, but without the use of templates in the polymerisation process [5]. The components used are templates (analytes or compounds to be analysed), functional monomers, cross-linkers, initiators, and porogens (solvents), which are synthesised using the bulk and precipitation method [6]. The type of porogen is an important choice because the interaction of each component will depend on the dissolving media, i.e. the solvent itself [4].

DZP-imprinted polymers as an MI-SPE sorbent for the separation of DZP in serum have been successfully synthesised using the bulk polymerisation method [7]. However, the bulk method has drawbacks, such as the irregular size and shape of particles, a decrease in the MIP loading capacity, and some interaction sites being destroyed because of the grinding process [8]. To overcome these drawbacks of bulk polymerisation, precipitation polymerisation can be chosen to obviate the grinding and sieving processes. Precipitation polymerisation produces a uniform size and shape of the MIP microspheres [9]. Moreover, MIP that was synthesised by precipitation polymerisation had a higher binding capacity and selectivity than MIP that was synthesised using the bulk polymerisation [10]. Precipitation polymerisation is an easy, simple, and commonly used strategy in producing microsphere MIP and does not need any surfactant in the making [11]. In precipitation polymerisation, the morphology of the polymer is not significantly affected by the presence or absence of a template [12,13].

In this article, MIP microspheres as solid-phase extraction (SPE) sorbents for the selective extraction of DZP in serum samples were synthesised by precipitation polymerisation using itaconic acid as a functional monomer, EDGMA as a cross-linker, benzoyl peroxide as an initiator, and propanol as a porogen. Then, the binding affinity of the MIP microsphere was determined using the adsorption capability test, adsorption capacity test, and selectivity test. The physical characterisation of the MIP microsphere was carried out using scanning electron microscopy (SEM), Fourier-transform infrared (FTIR), and Brunauer–

Emmett–Teller (BET). The MIP microsphere was evaluated as an SPE sorbent for selective extraction of DZP in the spiked blood serum.

2 Materials and methods

2.1 Instruments

Agitator (IKA®HS 260 basic), mesh sieve 60, FTIR (IR Prestige-21; Shimadzu), oven (Memmert), centrifugator (Yenaco dan Hettich), UV-Vis spectrophotometer (Specord 200; Analytik Jena), ultrasonicator (NEY 19H), water bath (Memmert), and SPE vacuum manifold 12 ports (Phenomenex) were used. Reagents used were as follows: itaconic acid (Sigma Aldrich), acetonitrile (Fisher Scientific), benzoyl peroxide (Merck), DZP (TCI), ethylene glycol dimethacrylate (EGDMA) (Sigma Aldrich), ethanol (Merck), potassium bromide (KBr; Merck), chloroform (Merck), methanol (Merck), and alprazolam (TCI). All solvents used are of HPLC grade.

Ethical approval: The conducted research is not related to either human or animal use.

2.2 Measurement of association constants between itaconic acid as a monomer and DZP as a template using UV-Vis spectrophotometer

For the spectrophotometry measurements using a UV-Vis spectrophotometer, 2 mL of 8.54×10^{-4} mg/L DZP solution was dissolved in propanol. Then, itaconic acid 1.30 mg/L was added to the DZP solution in increments. The addition was made from 10 μ L and rising in multiples (20, 30, 40 μ L, and so on) until the addition no longer changed the absorbance of the previous measurement. Each addition of itaconic acid was measured and the absorbance recorded. Based on the results obtained, the association constants can be determined from the Benesi–Hildebrand equation graph [14].

2.3 Synthesis of microsphere MIP and NIP via precipitation polymerisation

A total of 285 mg of DZP (1 mmol) was dissolved in 350 mL of propanol inside a bottle and sonicated for 5 min. Then,

520 mg itaconic acid (4 mmol), 3.775 mL (1.05×10^6 mg/L) EGDMA (20 mmol), and 250 mg benzoyl peroxide were added and sonicated for 5 min. The closed bottle was then sealed with parafilm and heated in an oven for 1 h at 70°C. After that, the bottle was transferred to a water bath shaker at 70°C for 18 h. The polymer from the precipitation method was then separated using centrifugation and rinsed with 20 mL of methanol and water mixture, and dried again in the oven for 18 h at 70°C. The same procedure was carried out for making NIP but without the addition of the DZP template [15].

2.4 Template extraction from MIP

The template was removed by the ultrasonic extraction method for over 3 h using 50 mL of ethanol, then filtered using a filter paper, and the filtrate was separated. The extracted MIP was then rinsed with a 50 mL mixture of ethanol and acetic acid (1:1), followed by drying in an oven over a period of 18 h at a temperature of 55°C [16]. To ensure the DZP template was completely extracted, monitoring was done through 20 mg of MIP added to 5 mL of ethanol, and then the absorbance was measured using a UV spectrophotometer [7].

2.5 Evaluation of MIP and NIP adsorption capacity

Solutions of DZP were made in a variety of concentrations (2.5, 5, 7.5, and 10 mg/L). A total of 5 mL of DZP solution from each concentration was put into vials containing 20 mg of MIP sorbent and agitated for 3 h. The mixture was decanted, and the filtrate absorbance was measured using a UV spectrophotometer. This evaluation was also carried out on NIP with the same treatment [17].

2.6 Physical characterisation of sorbents with SEM, FTIR, and BET

A total of 2 mg of MIP sorbent was crushed together with 200 mg of KBr and then printed into pellets. The MIP sorbent infrared spectrum was observed using the FTIR instrument. The transmission was measured at wavenumbers between 4,000 and 400 cm^{-1} . The determination of the MIP sorbent functional group was carried

out before and after the extraction. The same method was also carried out for the NIP sorbent. Morphological observations of MIP and NIP sorbents were carried out with SEM, whilst BET was used to observe the surface area of the MIP sorbents [17].

2.7 Application of molecularly imprinted solid-phase extraction (MI-SPE) and non-imprinted solid-phase extraction (NI-SPE) on spiked blood serum

Applications of the fabricated MIP and NIP were carried out on spiked blood serum with standard DZP solution without other substances and DZP solution mixed with other compounds, namely, hydromorphone (HDF) and codeine (COD). Each 200 mg MIP and NIP was put into a 3 mL SPE cartridge [6]. SPE process was done using Phenomenex SPE vacuum manifold 12 ports. SPE optimisation was performed with various solvents to obtain optimal conditions during the conditioning, washing, and eluting steps. The optimal condition was determined from the highest percent recovery obtained through analysis by HPLC. The optimal conditions were then used to extract the blood serum samples that had been spiked with 2 mg/L DZP alone and the mixtures of DZP with other substances. In order to make sure the treatment using MIP is efficient, blank serum treated with protein precipitation using acetonitrile $3 \times$ (3 times) serum volume and centrifuged at 5,000 rpm for 15 min was injected. All analyses of MI-SPE and NI-SPE were done by validated HPLC condition with coefficient of correlation for linearity (r) 0.9912, accuracy $101.12 \pm 2.55\%$, %RSD for intermediate precision 1.1% using acetonitrile:water 60:40 as the mobile phase with 1 mL/min flow rate.

3 Results

3.1 Determination of constants from the monomer–template association using a UV visible spectrophotometer

Determination of the association constant (K_A) with UV-Vis spectrophotometry was aimed to see how strong the interaction was between itaconic acid monomers and DZP [18]. The maximum wavelength of DZP was obtained at 233 nm. DZP solution concentration of 8.54×10^{-4} mg/L

in propanol was measured using the titration method by adding itaconic acid 1.02 mg/L little by little. Measurable additions were made and changes in absorbance with each addition of itaconic acid were recorded and plotted on a graph of $1/[\text{Itaconic acid}] [\text{M}]^{-1}$ vs $1/\Delta\text{absorbance}$ (Figure 1). The ratio between monomers and templates contributes to the observations of the interaction between the two components. The ratio between the itaconic acid and DZP used was arranged in such a manner through optimisation stages and according to the ideal ratio (ro) ≥ 100 [14,15].

The KA value was determined using the calculation of the Benesi–Hildebrand formula with the KA value obtained as $381.9 \text{ M}^{-1} \pm 0.4$. KA values have no restrictions and can vary between very large (more than $10,000 \text{ M}^{-1}$) and very small values (less than 1 M^{-1}). According to Wang and Yu, the KA value of weak interactions between molecules is less than 25 M^{-1} , and the interaction between strong molecules has a value of more than 100 M^{-1} [18,19]. Thus, the interaction formed between DZP and itaconic acid was strong.

3.2 Synthesis of MIP and NIP by precipitation polymerisation method

Synthesis of MIP and NIP following the free radical polymerisation reaction was initiated by the thermal decomposition of BPO as an initiator after the mixture was put into the oven [15]. BPO is active in the form of free radicals and initiates polymerisation by giving free electron pairs to carbon monomer double bonds that will then form carbon radicals. The process continues in sequence until it forms a long polymer chain [20]. Illustration of

Figure 1: Correlation between $1/[\text{Itaconic acid}] [\text{M}]^{-1}$ and $1/\Delta\text{absorbance}$.

DZP MIP synthesis and the possible interactions between functional monomers and templates can be seen in Figure 2.

3.3 Template extraction from MIP

Extraction was repeated ten times. The extraction monitoring was carried out using a UV spectrophotometer by observing the spectrum formed from the MIP solution filtrate. A spectrum like the DZP spectrum with the same wavelength indicates incomplete MIP extraction. This was the basis for a repeated extraction such that the spectrum of the MIP filtrate did not indicate the presence of DZP. The measurement results showed that the template was completely extracted from the sorbent.

3.4 MIP and NIP-SPE adsorption capacity evaluation results

The adsorption capacity evaluation aimed to determine the bond affinity or interaction of MIP with DZP. The MIP binding site distribution can be determined using the Freundlich isotherm or Langmuir isotherm equations to determine the affinity, capacity measurement, and homogeneity index of the polymer adsorption site. MIP binding or adsorption sites can be homogeneous or heterogeneous. If an MIP follows the Langmuir isotherm, the distribution is homogeneous, while the distribution becomes heterogeneous if it follows some of the provisions of the Freundlich isotherm [21]. MIP follows the Langmuir isotherms if the Langmuir (k) constant is positive [22]. For the Freundlich isotherm, MIP with a homogeneous binding site distribution is indicated by a homogeneity index (m) close to one. The higher the value of m , the more heterogeneous the distribution [23]. The results of the isotherms are given in Table 1. Based on Table 1, MIP follow Freundlich isotherm according to r^2 value and smaller error showing its heterogenous adsorption site.

3.5 Physical characterisation of sorbents with SEM, FTIR, and BET

Characterisation using FTIR was performed on MIP before extraction, MIP after extraction, and NIP, to see the differences in the results (Table 2). The measurement results

Figure 2: The synthesis scheme of MIP DZP with itaconic acid as a monomer.

confirmed that the polymerisation process was complete, characterised by the presence of branched chains in the form of a tertiary butyl group. This is a result of an alkene group or a double bond of itaconic acid and EGDMA that undergoes a radical polymerisation process so that it turns into a single bond. A peak in the wavenumber region $1,600\text{ cm}^{-1}$ indicated the presence of $\text{C}=\text{C}$ stretching from the double C bond. The bond can originate from double C in the aromatic ring structure belonging to DZP, the remnants of monomers, and EGDMA that are not completely polymerised, or the aromatic rings from the BPO side reactions [24]. The tertiary butyl group branched chain shows the twin absorption peaks of $\text{C}-\text{H}$ bending at wavenumbers $1,370$ and $1,385\text{--}1,400\text{ cm}^{-1}$. $\text{C}-\text{O}$ stretching in the $1,200\text{ cm}^{-1}$ wavenumber region with a peak that was broader than $\text{C}=\text{O}$ stretching at $1,700\text{ cm}^{-1}$ was characteristic for the $\text{C}-\text{C}(=\text{O})-\text{C}$ or ester group of EGDMA. Broad or wide absorption peaks were seen at $2,500\text{--}3,300\text{ cm}^{-1}$

wavenumbers. The broad peak was a sign that $\text{O}-\text{H}$ is in a state of interacting intermolecularly or intramolecularly with other $\text{O}-\text{H}$. This interaction can occur between carboxylic acid groups such that the broad peak was the absorption of carboxylic acid and itaconic acid [24].

Measurement with SEM was used to observe the surface characteristics of MIP and NIP polymers microscopically. The SEM results are shown in Figure 3a for MIP and Figure 3b for NIP. The pore size in NIP was relatively larger and not homogeneous compared to MIP. It is believed that this phenomenon resulted in the adsorption capacity with the Freundlich or Langmuir isotherm approach having lower homogeneity values compared to MIP.

The particle size of the MIP sorbent was more homogeneous compared to the NIP. The larger particle size results in a lower adsorption capacity of the polymer.

Table 1: The value of isotherm Freundlich and Langmuir parameters on MIP precipitation polymerisation

Polymer		MIP	NIP
Isotherm Freundlich	r^2	1.000	0.1253
	Intercept	-1.2538	-0.754
	Slope	0.8039	-0.2603
	Homogeneity	1.2439	-3.8417
	Affinity (mg/g)	0.0557	0.1762
	Standard error	0.27	0.31
Isotherm Langmuir	r^2	0.9854	0.3389
	Intercept	18.7370	9.3029
	Slope	1.0311	9.2159
	Capacity (mg/g)	0.96984	0.10851
	Constant	0.0550	0.9906
	Standard error	2.90	3.28

Table 2: The FTIR result of MIP and NIP by precipitation polymerisation method

Wavenumber (cm^{-1})		Functional group	
MIP		NIP	
Before extraction	After extraction		
3551.98	3592.48	3585.73	$\text{O}-\text{H}$ stretching
2986.82	2955.96	2955.90	$\text{C}-\text{H}$ stretching
2359.95	—	—	$\text{N}-\text{H}$ stretching
1730.18	1728.25	1735.96	$\text{C}=\text{O}$ stretching
1456.28	1455.32	1458.21	$\text{C}-\text{H}$ bending
1387.81	1389.74	1388.77	$\text{C}-\text{H}$ bending
1258.57	1259.54	1259.50	$\text{C}-\text{O}$ stretching
1159.24	1161.17	1161.17	$\text{C}-\text{O}$ stretching
957.67	960.57	960.57	$=\text{C}-\text{H}$ wagging
881.48	880.52	880.52	$=\text{C}-\text{H}$ wagging

Figure 3: The morphology of MIP of precipitation using scanning electron microscope magnification of 5,000×: (a) MIP and (b) NIP.

This was consistent with the results of the adsorption capacity with the Langmuir isotherm approach in that the affinity of the MIP by precipitation method was lower compared to its NIP [25].

Based on the BET results shown in Table 3, MIP has a larger surface area than NIP and a little bit larger total pore volume. The larger surface area indicates the effect of the imprinting process. The presence of the molecular template during the synthesis of MIP created the surface of pores or imprinted cavities [22–24]. Therefore, the MIP has more accessibility and specific interaction with the analyte or adsorbate [29]. This BET result corresponds to an adsorption of MIP that is larger than NIP. According to the International Union of Pure and Applied Chemistry classification, MIP and NIP have a mesoporous form as the pore diameter size is in the range of 2–50 nm or 20–500 Å [26,27].

3.6 Application of MI-SPE and NI-SPE on spiked blood serum

3.6.1 SPE optimisation

SPE optimisation is done by varying the amount of loading volume, the type of washing and eluting solvents, and by varying the total eluting volume. Before

SPE optimisation, batch adsorption test was done to choose the solvent for loading condition. According to batch result (Figure 4), methanol was used in SPE optimisation further as a loading solvent.

Based on the optimisation results of a standard solution of DZP, the best recovery percentage was achieved when the sorbent conditioning used was water:acetonitrile (9:1) 1 mL, loading with 0.5 mL solution, washing with 0.5 mL water, and eluting was done with methanol:acetic acid (9:1) 6 × 1 mL. The recovery percentage reached $88.65 \pm 3.29\%$. The result is shown in Figure 5. In SPE extraction, the type of washing solvent has an important role. It removes the matrix component in the sorbent matrix and reduces non-specific interaction at the binding site to ensure a selective extraction before the elution step. Therefore, the interaction between the analytes and sorbent's binding sites may be maximally retained [28,29]. The result showed that when the washing condition used toluene, the recovery was higher than the other solvents. This result indicates that toluene, the non-polar solvent, has disrupted the interaction between the functional monomer itaconic acid and DZP. However, when 0.5 mL of water is used as a washing solvent, the

Table 3: BET result of MIP and NIP

Polymer	Surface area (m ² /g)	Pore volume total (cc/g)	Average pore radius (nm)
MIP	10.131	0.0238	4.701
NIP	5.976	0.0213	7.137

Figure 4: Batch adsorption test result.

Figure 5: SPE optimisation result according to different conditioning solvents, washing, and eluting conditions based on solvent type and volume of the solvents used.

recovery was lower than the other solvents, indicating the interaction is not disrupted by the polar solvent. Therefore, the possibility of the interaction of DZP and a functional monomer is a hydrophobic interaction.

3.6.2 Application of SPE sorbent on spiked blood serum

Blood serum was spiked with 2 mg/L DZP alone, and SPE was done according to the optimisation result (Figure 6). Recovery percentages of the DZP on blood serum reached $105.63 \pm 1.0\%$ for MIP and $21.28 \pm 0.4\%$ for NIP (Figure 5). The result was better than the previous result [7] that reached $95.31 \pm 1.1\%$ for MIP and $60.83 \pm 0.3\%$ for NIP. This result correlated with the imprinting factor (IF) value that reached 4.96, much better than the previous result of 1.56 [7], and this shows that the sorbent with the smaller pore diameter has better binding. When the blood was spiked with other compounds (HDF and COD) together with DZP (DZP mix as shown in Figure 5), the recovery was still in the same range, which is $105.82 \pm 1.1\%$ for DZP, without any interference results from the presence of other compounds. IF is a parameter that describes how well the quality of imprinted sites is owned by MIP [25]. IF

was obtained from the ratio between the MIP distribution coefficient with NIPs. A good IF is indicated by a greater value of KD MIP than KD NIP, and therefore, a good IF is indicated by a KD value of >1 . The recovery of MIP and NIP has different result in blood compared to

Figure 6: Recoveries of DZP spiked blood serum with and without another compound. DZP: serum spiked with DZP alone without other compounds. DZP mix: serum spiked with DZP, HDF, and COD and measured the DZP recoveries. HDF mix: serum spiked with DZP, HDF, and COD and measured the HDF recoveries. COD mix: serum spiked with DZP, HDF, and COD and measured the COD recoveries.

Figure 7: The chromatogram of blank serum (a), serum spiked with DZP without MIP treatment (b), and with MIP treatment (c).

optimisation using standard solution, and it is assumed that this has happened due to changes in the surface of the polymer. This phenomenon still needs an observation (for what really happened inside the polymer) to arrive at a conclusion.

Figure 7 shows the chromatogram of serum spiked with DZP without MIP treatment (Figure 7b) and with MIP treatment (Figure 7c). The result shows that when the spike DZP in the serum sample was directly injected (without MIP treatment) in HPLC, many interferences can be seen, and a peak of DZP is not detected in Figure 7(a). However, the peak of DZP of about 6.880 min is detected and there is less interference when the spiked sample is treated with MIP. Therefore, the result indicates that MIP is an effective enrichment for DZP [30–35]. This study still has limitations, as we could not analyse nordiazepam as a metabolite of DZP for selectivity test based on Indonesian law on psychotropic drug.

4 Conclusion

The MIP of DZP with itaconic acid as the functional monomer in propanol, using the precipitation polymerisation method, had better analytical performances than previous results, with a recovery of $105.63 \pm 1.0\%$ for MIP and $21.28 \pm 0.4\%$ for NIP. The sorbent has an IF value of 4.96. The physical characterisation showed that the MIP synthesis undergoes a complete polymerisation. From the study, it is observed that MIP DZP using itaconic acid as a monomer in propanol, synthesised by the precipitation polymerisation method, is a promising sorbent to extract DZP in the biological fluid.

Acknowledgements: The authors thank the Ministry of Research and Higher Education of the Republic of Indonesia for funding this research through Fundamental Research Grant 2020.

Funding information: This research was funded by the Ministry of Research and Higher Education of the Republic of Indonesia, grant number 1827/UN6.3.1/LT/2020, and the APC was funded by Universitas Padjadjaran.

Author contributions: Conceptualisation – A. N. H. and R. P.; methodology – A. N. H. and R. P.; formal analysis – G. J. M. and M. M.; investigation – M. M. and F. A. S.; writing – original draft preparation – F. A. S.; writing – review and editing – A. N. H. and I. S.; project administration – G. J. P.; funding acquisition – A. N. H. All authors have read and agreed to the published version of the manuscript.

Conflict of interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Data availability statement: All data generated or analysed during this study are included in this published article.

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