CHAPTER III

MOLECULARLY IMPRINTED SOLID-PHASE EXTRACTION FOR NEVIRAPINE

3.1 Introduction

Nowadays, various analytical methods such as HPLC, LC-MS, GC and immunoassay have been developed and used for monitoring the level of NVP in biological samples, such as urine or human plasma. These techniques generally require sample clean-up or sample pre-treatment procedure before analysis. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) have been used to simplify and improve the efficiency of sample preparation. Nevertheless, the growth of SPE is more than LLE because of the advantages of SPE over LLE in which the technique require less organic solvents, easy to automate and safe time.

The commercial SPE sorbents such as C_8, C_{18}, an anion or cation-exchanger were commonly used. Although those SPE sorbents offer advantages such as easy to prepare, high load ability and high recovery, but they are expensive and utilize a wider range of extraction. Therefore, the SPE protocol that have selectivity to target analyte and less expensive is needed. Nowadays, molecularly imprinted polymers (MIPs), a highly rigid polymeric materials containing specific binding site to the analyte, are a new choice to be used as SPE sorbent. From their recognition properties, these materials have been applied as selective sorbent for molecularly imprinted solid-phase extraction (MISPE). The first MIPs used for SPE was reported

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by B. Sellergren in 1994 for sample pre-treatment of pentamide, the AIDS-related pneumonia [52]. Since that study, MISPE has been developed for the wide range of biological, pharmaceutical, food and environmental samples.

The aim of this study is to apply MIPs as a solid sorbent in solid phase extraction (SPE) for NVP sample pre-treatment and pre-concentration. In the previous experiments, it was found that P(NAM) showed a good performance in binding with NVP in aqueous media. Therefore, MISPE using P(NAM) as a solid sorbent was developed. After washing and eluting condition were optimized, the suitable MISPE condition was then applied with real plasma samples.

3.2 Experimental

3.2.1 Chemicals

Nevirapine (NVP), $C_{15}H_{14}N_4O$, Government Pharmaceutical Organization (GPO) Thailand Plasma sample, Research Institute for Health and Sciences, Chiang Mai University, Thailand

Nicotinamide (NAM), $C_6H_6N_2O$, assay 98.5%, BDH, England

Benzamide (BZM), $C_7H_{16}NO$, assay 98.5%, Aldrich, U.S.A.

Methacrylic acid (MAA), $C_4H_6O_2$, assay 98%, Fluka, Switzerland

Benzophenone (BzP), $C_{13}H_{10}O$, assay 98.5%, Aldrich, U.S.A.

Trimethylopropane trimethacrylate (TRIM), $C_{18}H_{26}O_6$, Aldrich, U.S.A.

Benzoyl peroxide, $C_{14}H_{10}O_4$, Janssen Chemica, Belgium

Potassum hydrogen phosphate, K_2HPO_4, Fluka, Switzerland

Potassium dihydrogen phosphate, KH_2PO_4, Fluka, Switzerland

Methanol, CH_3OH, HPLC grade, Fisher, England

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Acetonitrile, CH_3CN, HPLC grade, Fisher, England

Triethylamine, $C_6H_{15}N$, Fluka, Switzerland

Acetic acid glacial, $C_2H_4O_2$, Carlo erba, Italy

Formic acid, CH_2O, BDH, England

Tween 20, BDH, England

Dimethylsulfoxide, C_2H_6SO, Fluka, Switzerland

Methanol, CH_4O, Fluka, Switzerland

3.2.2 Instrumental

UV-Vis spectrophotometer (Perkin Elmer, Lambda25), U.S.A.

Centrifuge (BECKMAN COULTER, Allergra), U.S.A.

High performance liquid chromatography (HPLC) (Aligent), U.S.A.

C_{18}column 4.0×250 mm, 5µm (Hewlett-Packard) Germany

3.2.3 General procedure for MISPE

Twenty-five milligrams of the obtained P(NAM) was packed dry into SPE cartridge and fitted with plate. Before used, the cartridge was conditioned by washing with twice of 1 ml MeOH followed by twice of 1 ml phosphate buffer 0.01 M pH 7 containing 0.05% Tween 20. Extraction experiments consist of loading the MISPE cartridge with 1 ml of NVP in loading solution. Then washed and eluted the packing with five times of 1 ml of washing and eluting solvent. All the solution after each step was collected, evaporated to removed solvent and the volume of sample were

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adjusted to 0.5ml using acetonitrile. All samples were then analyzed by HPLC using UV detector. The schematic representation of MISPE method is shown in Figure 3.1.

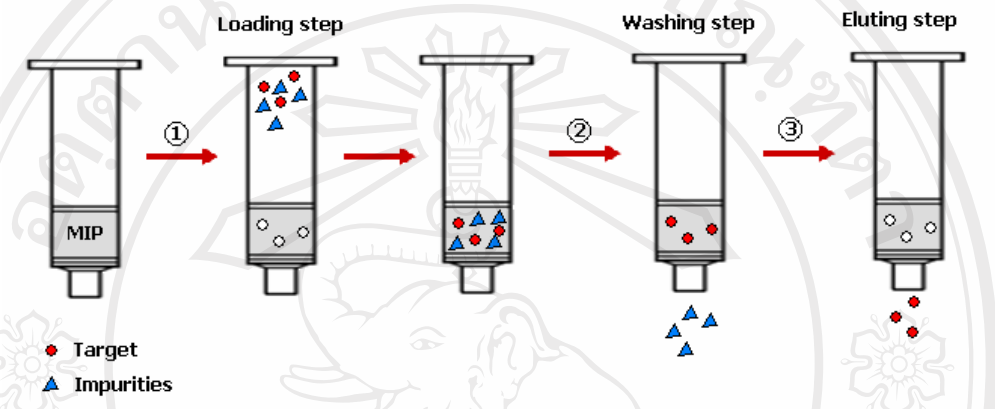

Figure 3.1 MISPE method (1) loading step (2) washing step (3) eluting step.

3.2.4 Optimization of the extraction conditions

3.2.4.1 Optimization of the washing condition

To optimization the washing condition, the MISPE process were done as described above. The MISPE cartridge was loaded by 1 ml of 0.2 mM NVP in phosphate buffer pH 7 containing 0.05% Tween 20 then washed the packing with five times of 1 ml of washing solution. The solution after each step was collected and then analyzed by HPLC. The washing condition in this experiment is shown in Table 3.1.

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Table 3.1 The washing conditions

Condition	Washing solution
a	0.01 M phosphate buffer pH 4 with 0.05% Tween 20
b	0.01 M phosphate buffer pH 7 with 0.05% Tween 20
c	0.01 M phosphate buffer pH 10.5 with 0.05% Tween 20
d	5% MeOH in (b)
e	10% MeOH in (b)
f	20% MeOH in (b)
g	5% acetonitrile in (b)
h	10% acetonitrile in (b)
i	20% acetonitrile in (b)

The percentages of the retained of NVP after washing step were calculated by equation:

$$R_{af.wash} = \left(\frac{A_{initial} - A_{af.load} - A_{af.wash}}{A_{initial} - A_{af.load}} \right) \times 100 \ ;$$

$R_{af.wash}$ is the percentage of the retained NVP after washing step

$A_{initial}$ is the area of 0.2 mM NVP in phosphate buffer pH 7 containing 0.05% Tween 20 before MISPE process.

$A_{af.load}$ is the area of 0.02 mM NVP in phosphate buffer pH 7 containing 0.05% Tween 20 after loading step.

$A_{af.wash}$ is the area of 0.02 mM NVP in phosphate buffer pH 7 containing 0.05% Tween 20 after washing step.

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3.2.4.2 Optimization of the eluting condition

The eluting conditions were investigated through the MISPE process as previously described. The MISPE cartridge was loaded with 1 ml of 0.2 mM NVP in phosphate buffer pH 7 containing 0.05% Tween 20. Then the packing was washed with five times of 1 ml of appropriate washing solution obtained from the previous experiment. After that, the packing was eluted with the eluting solvent and the final solution was collected to HPLC analysis. The eluting conditions in this experiment are shown in Table 3.2.

Table 3.2 The eluting conditions

Condition	Eluting solvent
a	Acetonitrile
b	1% TEA in acetonitrile
c	1% formic acid in acetonitrile
d	1% acetic acid in acetonitrile

The percentage recoveries of NVP were calculated following equation;

$$\%\,\mathrm{Recovery} = \frac{Amount_{af.elute}}{Amount_{initial}} \times 100$$

where $amount_{af.elute}$ is gram of NVP that was eluted from MISPE.

$amount_{initial}$ is gram of NVP before added to MISPE.

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3.2.5 MISPE with the real plasma sample

Plasma samples were spiked with NVP in various concentrations. The amount of NVP in samples was analyzed by MISPE using the optimized washing and eluting condition, followed by HPLC. The %recovery of NVP after extraction was calculated as previously described.

3.2.6 Effect of NAM in MISPE process

To investigate the effect of template molecule on the recovery of the NVP analyte, real plasma sample was spiked with mixed solution of NAM and NVP in ratio of 1:1, 6:1 and 12:1 of NAM: NVP. All samples were pre-treated with MISPE and analyzed by HPLC.

3.2.7 Method validation

The developed MISPE method for NVP analysis was validated using plasma sample standard. The linearity of standard calibration curve for NVP was determined by spiking blank plasma with known amount of analyte in the range of 0.5-100 μg/ml and a linear regression coefficient (R^2) was observed. The accuracy was done the MISPE process in 10 times of constant 10 μg/ml spiked NVP in plasma sample. The relative accuracy (%RA) of this method was calculated by the equation:

$$\%RA = \frac{x_i}{\bar{x}} \times 100$$

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where x_i is the gram of NVP that was eluted from MISPE and $\bar{x}$ is the average of gram of NVP that was eluted from MISPE

The precision of this method was determined and evaluated at three analyte concentrations of 1, 10 and 100 µg/ml., and the relative accuracy and the relative standard deviation (%RSD) of this method were investigated by following this equation:

$$\%RSD = \frac{SD}{\bar{x}} \times 100$$

where $\bar{x}$ is the average of gram of NVP that was eluted from MISPE.

3.2.8 Plasma sample preparation

Aliquots of 0.5 ml of blank human plasma were spiked with NVP (0.1 ml) and cold acetonitrile (0.1 ml) for protein precipitation to yield of NVP in spiked plasma sample concentrations corresponding to 0.2 mM. These mixtures were vortex-mixed for 1 min followed by centrifiguration at 8000 rpm for 5 min. Aliquots of 500µl of the samples were diluted to 1 ml with addition of water and applied on MISPE cartridge.

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3.2.9 Chromatographic conditions

HPLC analysis was performed using Agilant technologies (USA) HPLC system employing a model 1100 quaternary gradient pump and variable-wavelength detector. The injection volume was 20 µl throughout the study. Separation was carried out on 4.0×250 mm, 5µm Hewlett-Packard (Germany) C_{18} column. The gradient mobile phases consist of 15mM of phosphate buffer pH7.2 as solvent A and

pure acetonitrile as solvent B. The gradient system and the wavelength for analysis as shown in Table 3.3 in was 10-90% solvent B, 10 min for analysis of NVP and NAM, and with a typical flow rate of 1.0 ml/min.

Table 3.3 Chromatographic conditions for analysis.

Substrate	Gradient system	λ(nm)
NVP	10-90% solvent A, 10 min	281
Mixed of NVP and NAM	10-90% solvent A, 10 min	254

3.3 Results and discussions

3.3.1 Optimization of the washing condition

Generally, SPE was usually done by the three-step procedure via, loading, washing and eluting. NVP (0.2 mM) was prepared in 0.01M phosphate buffer pH7 containing 0.05% Tween 20, which represents the pH of human plasma. In the washing step, washing solution should effectively extract unwanted impurity in sample matrices without removing the target analyte. Therefore, in this study, the effect of pH and organic solvent in washing solution were investigated.

The washing conditions were firstly optimized by varying pH of the phosphate buffer from 4 to 10.5. Figure 3.3 showed the percentage of NVP retained after washing step. The results showed that high percentage of NVP was retained in MISPE column in pH range investigated (97.42±0.115, 95.77±0.299 and 98.68±0.219 for phosphate buffer pH 4, 7 and 10.5, respectively). Therefore, pH of washing

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solution has almost no effect on the removal of NVP. For the ease of operation, phosphate buffer pH 7 was selected for further study.

To study the effect of organic solvent in washing solution, increasing amount of methanol and acetonitrile (5-20%) in phosphate buffer pH7 were used to wash MISPE column after loading with NVP. Figure 3.2 showed that the washing solution containing 5-10% of organic solvent did not interfere with the retention of NVP, whereas, at 20% of organic solvent, most of NVP was removed from MISPE column. In order to prevent the leak out of NVP and avoid the use of organic solvent, washing condition (b) was selected for the washing step.

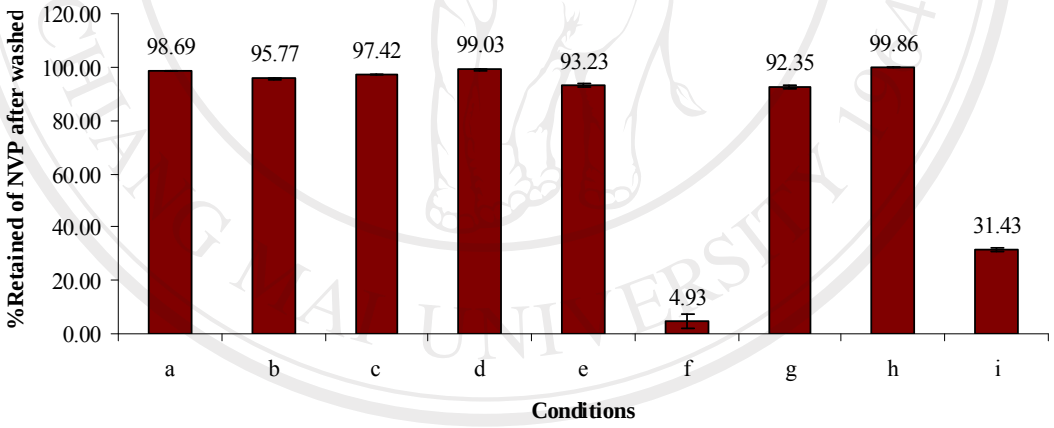

Figure 3.2 The percentage of retained NVP after washing step in various washing conditions; (a) 0.01 M phosphate buffer pH 4 containing 0.05% Tween 20, (b) 0.01 M phosphate buffer pH 7 containing 0.05% Tween 20, (c) 0.01 M phosphate buffer pH 10.5 containing 0.05% Tween 20, (d) 5% MeOH in (b), (e) 10% MeOH in (b), (f) 20% MeOH in (b), (g) 5% acetonitrile in (b), (h) 10% acetonitrile in (b) and (i) 20% acetonitrile in (b).

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3.3.2 Optimization of the eluting condition

To obtained high percent NVP recovery, the eluting conditions were optimized. NVP sample in 0.01 M phosphate buffer pH 7 containing 0.05% Tween 20 was loaded in MISPE column and washed with the washing condition (b). NVP was then eluted with neutral, basic and acidic eluent using acetonitrile, 1% TEA in acetonitrile, 1% formic acid in acetonitrile and 1% acetic acid in acetonitrile, respectively. Acetonitrile was selected to be used as eluting solvent for the ease of sample preparation for further HPLC analysis. Figure 3.3 showed the percentage of recovery NVP after eluting step. The percentage recoveries of NVP were 98.57±8.85, 116.13±11.07, 84.79±9.10, 106.13±2.81 and 101.12±1.61 for condition (a) to (d), respectively. From the results, it can be seen that the similarly high %recoveries were observed in all condition. When sample was eluted with acidic solvent (condition (c) and (d)), higher recovery of NVP were obtained than eluting with neutral solvent (condition (a)). Meanwhile under basic condition (condition (b)), lowest NVP recovery was observed. This observation is corresponding well with the basic property of NVP molecule. Since P(NAM) has acidic functional monomer and NVP is the basic molecule, when binding occurred in aqueous media at neutral pH, the binding interaction should be ionic or hydrogen bonding. Therefore, in acidic media, NVP can be easily eluted off, because the binding sites in NVP were protonated and caused the disruption of interaction between the polymer and NVP. However, under basic condition, those binding sites in the polymer were deprotonated causing an enhancement in template-polymer binding interaction via ionic bond. Nevertheless, recovery of NVP did not affected much with pH therefore pure acetonitrile was selected to be used as the eluting solvent.

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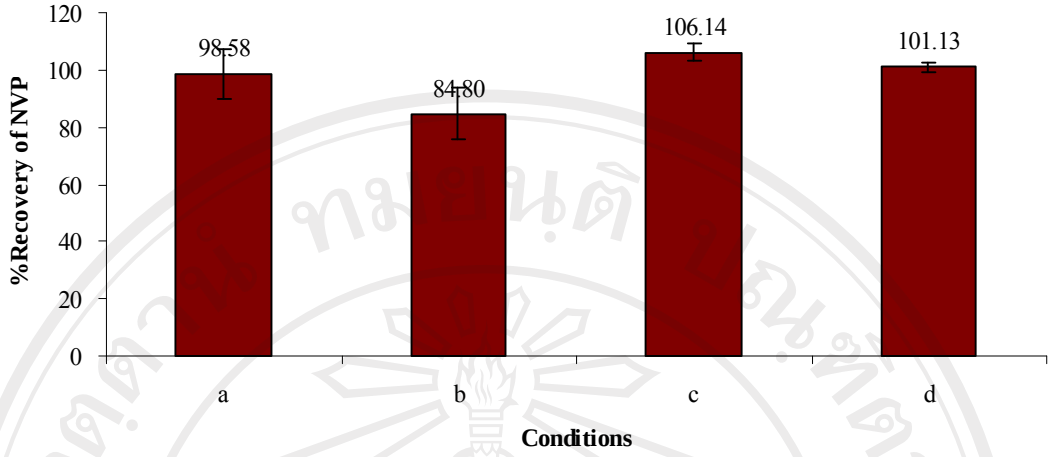

Figure 3.3 The percentage of recovery NVP after eluting step in various eluting conditions; (a) acetonitrile, (b) 1% TEA in acetonitrile, (c) 1% formic acid in acetonitrile and (d) 1% acetic acid in acetonitrile.

3.3.3 MISPE with real plasma sample

After MISPE conditions were optimized, the selected protocol was applied with the real plasma samples to study the effect of sample matrix on extraction ability. SPE cartridge was loaded with NVP spiked in plasma sample at concentration range 0.5-100 μg/ml, washed with 0.01 M phosphate buffer pH 7 with 0.05% Tween 20 and eluted with pure acetonitrile. Figure 3.4 shows HPLC chromatogram of the solution obtained after each MISPE process. From the results, the developed MISPE protocol can efficiently remove impurities from the plasma matrix. After pre-concentration, NVP can be obtained in high percentage recovery.

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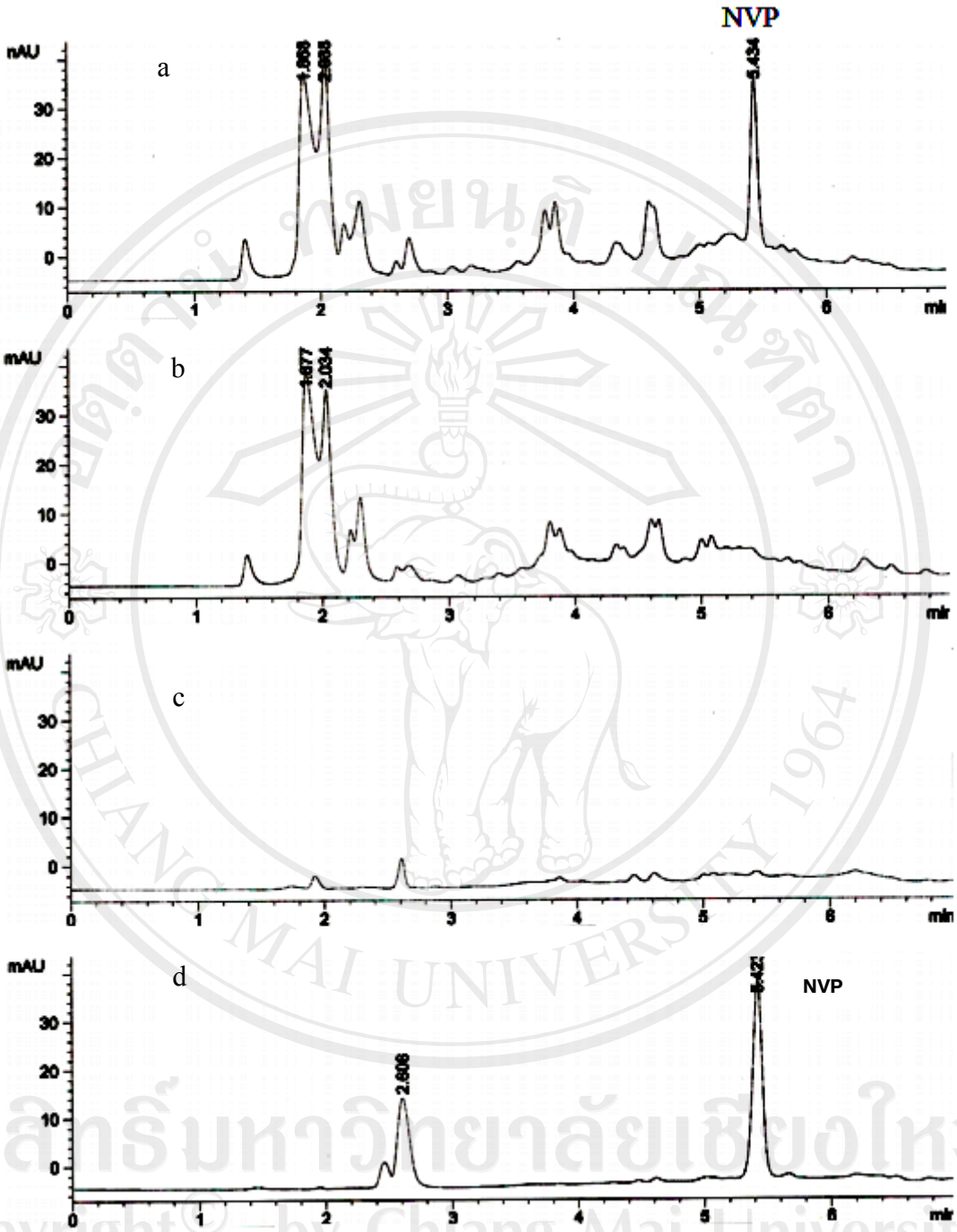

Figure 3.4 HPLC chromatogram of plasma sample monitored at λ 281 nm ; (a) before MISPE process, (b) after loaded, (c) after washed and (d) after eluted.

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3.3.4 Effect of NAM on the recovery of NVP

To study the effect of NAM on the pre-treatment ability of the developed MISPE for NVP, percentage recovery of samples containing NVP and NAM were examined. In this study, fixed amount of NVP (25 μg/ml) were mixed with increasing amount of NAM (25-300 μg/ml). These mixtures were then loaded into MISPE column using previously optimized condition. The eluted solution was then analyzed by HPLC. Effect of NAM for recovery of NVP was shown in Figure 3.5. The percentage recoveries of NVP were 97.29±4.49, 98.47±1.91 and 89.67±1.26 when the amount of NAM increased from 25-300 μg/ml. It can be seen that, from the results, the recovery of NVP was slightly decreased when increasing the NAM amount, suggesting that the excess amount of NAM can promote the competitive binding between NVP and NAM in MIP solid sorbent.

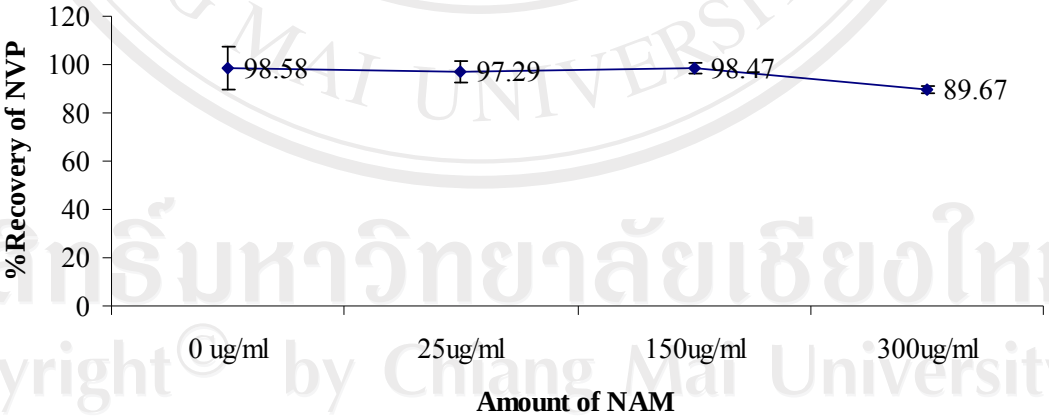

Figure 3.5 Effect of NAM for recovery of NVP.

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3.3.5 Method validation

To validated MISPE protocol, linearity, accuracy and precision were determined. The linearity of standard calibration curve for was determined by spiking blank plasma sample with NVP to obtained the concentration in range of 0.5-100 μg/ml. that created a linear regression coefficient (R^2) observed was 0.995. To determine the accuracy of the method, MISPE of NVP in plasma sample at concentration of 10 μg/ml were performed repeatedly for a ten times. The recovery of 97.60±1.39% was obtained with relative accuracy was calculated as 100.00 (Appendix A). The precision of this method was determined by evaluation of NVP extracted from spiked plasma sample of NVP concentration of 1, 10 and 100 μg/ml. The relative standard deviations were 2.48, 10.81 and 3.46%, respectively.

From the results, the developed MISPE method and protocol can effectively be used to clean-up and pre-concentration of NVP from plasma samples with good accuracy and precision.

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