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The Spectrophotometric Determination of Nystatin in Real Samples Using Solid Phase Extraction Based on Sodium Dodecyl Sulphate-Coated Magnetite Nanoparticles

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The purpose of this study was to separate and determine Nystatin in water, urine and plasma samples using a method based on sodium dodecyl sulfate (SDS) coated magnetic nanoparticles (nano-magnets Fe_3O_4) along with spectrophotometry which has been developed for to this end. Due to their excellent adsorption capacity and high chemical stability, nanoparticles can be modified by surfactants. The extraction efficiency of Nystatin can be affected by different factors such as the amount of adsorbent, pH value, eluent type and its volume, extraction time, and ionic strength; these factors have been further studied and optimized. The method was successfully employed for extracting Nystatin from water, urine and plasma samples under optimized condition. The linear response of the method was over ranges of 1-20, 1-18 and 1-15 mg l^{-1} and the coefficients of determination were 0.991, 0.994 and 0.991; these desirable coefficients were achieved for of water, urine and plasma samples, respectively. In addition, it was tried to investigate the relative recovery in different water, urine and plasma matrices and subsequently the values of 99%, 98% and 102% were obtained. It could be concluded that the method employed here was conveniently fast, linear, efficient and economical for extracting Nystatin in water and biological samples.

Keywords: Nystatin, Magnetic solid-phase extraction, Sodium dodecyl sulfate, Magnetic nanoparticle

INTRODUCTION

Nystatin, according to Dorland's pocket medical dictionary (28th ed.2009), is an antifungal agent. Nystatin is produced by the growth of Steptomyces noursei and is used for treating infections caused by Candida albicans or other Candida species. It is an antibiotic and a macro-cyclic lactone as well. This feature is quite effective against various pathogenic as well as non-pathogenic yeasts and fungi [1,2]. Since evaluation of the stability of the drug is of importance, a number of methods have been developed to measure the concentration of Nystatin in plasma, saliva, urine, blood and tissues [3-5]. However, it is difficult to determine the amount of Nystatin in biological fluids because it is solved very slowly in solvents and the available methods for its quantification are relatively expensive [6]. Since the use of microbiological methods has its own limitations, they cannot be employed when it is essential to monitor the degradation of a drug product through stability studies (see for example [6]). Various methods have been used for the analysis of Nystatin in recent years including spectrophotometric [1,7], liquid chromatography [8], high performance liquid chromatography (HPLC) [2,8-9], and liquid chromatography with mass spectrometry (LC-MS) [10]. In 1973, Robinson *et al.* introduced the magnetic carrier technology [11]. Due to its unique and physical properties such as a significantly higher surface area-to-volume ratio and a short diffusion route, the synthesis of micro (or nano) magnetic nanoparticles (Fe_3O_4) have attracted great attention. These properties lead to a higher extraction capacity, rapid dynamics of extraction and its higher extraction efficiencies [12-13]. In addition, its isolation from the sample solutions can be done easily by an external

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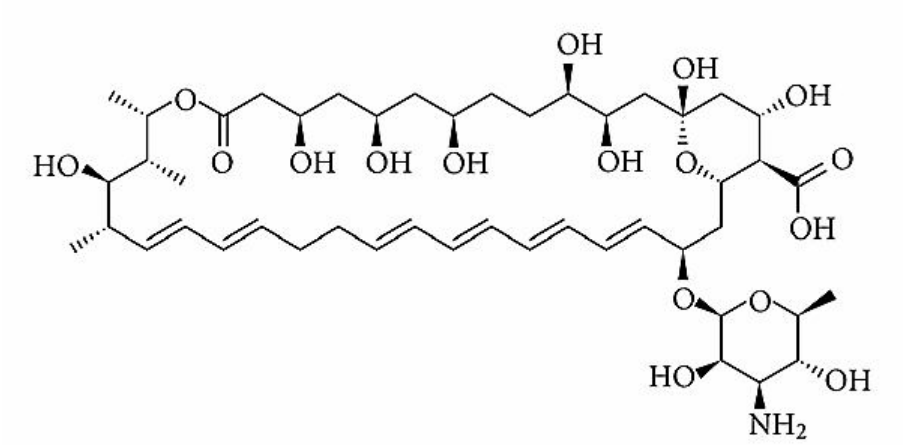


Fig. 1. Molecular structure of Nystatin.

magnetic field without any need for additional centrifugation or filtration of the sample following extraction in comparison with the non-magnetic adsorbent; thus, magnetic nanoparticles are used as sorbents for analytes in aqueous solutions [14]. Analysis methods based on the solid-phase extraction (SPE), which is also called the magnetic solid-phase extraction (MSPE) (For a detailed explanation, see [15-19]) has drawn great attention recently as well. Due to the upper contact between the adsorbent and the analyte, MSPE technique has an excellent adsorption efficiency and rapid separation from the matrix. However, magnetic nanoparticles have some inherent limitations because they tend to aggregate and cannot adsorb analytes effectively due to their surface simplicity. Thus, their properties improve by modifications with different functional groups. Moddification improves their extraction selectivityand extend their application. Some researchers have reported SPE methods based on surfactant-coated Fe_3O_4 NPs [20-22]. Surfactants such as sodium dodecyl sulfate (SDS) are formed by mixing hemimicelles, admicelles and coating with the adsorption of ionic on the Fe_3O_4 NPs [24]. In addition, SPE-CMN (the solid-phase extraction method on coated magnetic nanoparticle) has several benefits, for example, easy preparation, good dispersion, high surface area, high chemical stability, and faster high extraction. Moreover, they are applicable for the pre-concentration and extraction of different analytes such as drugs and dyes [22-28]. Therefore, the extraction time becomes very short for the enrichment process, which is the main advantage of SPE-CMN. In order to extract Nystatin in various matrices, a simple and efficient method was developed based on SPE-CMN in the present study. It was also tried to study and optimize the influence of different experimental parameters on the efficiency of extraction.

EXPERIMENTAL PROCEDURE

Chemicals and Apparatus

Without applying any purification, all the reagents of the analytical grade were used. Figure 1 shows the molecular structure of Nystatin. By dissolving an appropriate amount of Nystatin in methanol purchased from Parsdarou Company (Tehran, Iran), the stock standard solution of Nystatin 1000 mg l^{-1} was prepared. It was then stored at 4 °C. In order to synthesize magnetic nanoparticles (Fe_3O_4), $FeCl_3.6H_2O$, $FeCl_2.4H_2O$, hydrochloric acid, sodium hydroxide and ammonia were used. Methanol, sodium dodecyl sulfate (SDS) were purchased from Merck Chemical Company (Darmstadt, Germany). Deionized water from a Milli-Q system (Millipore, USA) was used to prepare all solutions. The spectrophotometric measurement was performed with the Perkin Elmer UV-Vis spectrophotometer using 1.0 cm quartz cells (λ = 306 nm). In addition, the Metrohm meter (827 pH/mV model) was used to measure pH. The scanning by electron microscopy (SEM) was used for studying the morphological features of the magnetic nanoparticles (Fe_3O_4), and surfactant-coated Fe_3O_4 NPs.

Preparation of Fe_3O_4NPs

By applying the co-precipitation method found in the literature [21], magnetic nanoparticles (Fe_3O_4) were prepared. It is briefly explained here; firstly, 25 ml of deionized water was degassed with nitrogen gas under the N_2 gas and under vigorous stirring conditions. Then, this deionized water was used to dissolve 5.2 g of $FeCl_3 \cdot 6H_2O$, 2 g of $FeCl_2 \cdot 4H_2O$, and 0.85 ml concentrated hydrogen chloride (12 M). This process was performed with nitrogen gas at 85 to 90 ℃. An ammonia aqueous solution was added to the solution immediately after the dissolve process was over. This addition was in the form of drops under intense stirring conditions and under nitrogen gas protection in order to precipitate and produce magnetic nanoparticles. Once the reaction was observed, a magnetic field was used to separate the precipitation from the reaction medium, then the precipitation was completely washed five times using deionizer water.

Coating Fe_3O_4 Nanoparticles with SDS

Using the method described in the literature [26], the coating of nanoparticles with SDS was performed. By adding 2.0 ml of SDS solution (5%, m/v) into a beaker containing 0.1 g of nanoparticles Fe_3O_4 nanoparticles were modified. Using a stirrer, the solution was stirred for 90.0 s, then, the beaker was placed on the magnet. Finally, the modified nanoparticles were washed four times with double distilled water.

Procedure for SPE-CMN Extraction

A tube containing 10 ml of buffer solution (pH = 5.0) was used to mix 50 µl of 1000 mg l^{-1} Nystatin and 20 mg of SDS-coated Fe_3O_4 nanoparticles, then, the mixture was shaken for 5.0 min to ensure that the extraction process was performed completely. Using a powerful Nd-Fe-B magnet (5 cm × 4 cm × 2 cm, 2.1T) at the bottom of the tube, the adsorbent SDS-coated Fe_3O_4 nanoparticles were isolated from the solution afterwards. The aqueous phase was easily decanted by simply inverting the sorbent tube. Nystatin adsorbed on the SDS-coated Fe_3O_4 nanoparticles was desorbed with 2 ml of methanol NaOH (60:40), and the vortex was mixed for two min. Then, the sorbent was isolated from the solution by positioning the magnet outside of the tube. UV-Vis was used to determine the concentration of the solution.

Real Sample Preparation

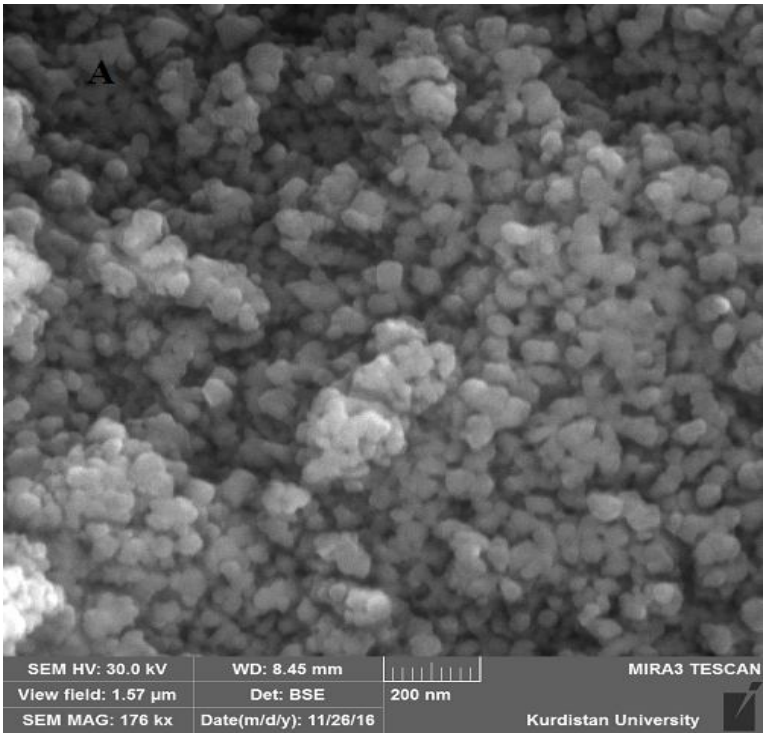
In order to extract Nystatin from urine and plasma samples, the SPE-CMN method developed in the present study was employed. Fresh tap water sample was collected from our laboratory (Khorramabad, Iran) and human urine samples were obtained from healthy female participants. Blood Transfusion Organization (Khorramabad, Iran) supplied the spiked tablet and plasma samples. Using distilled water, the specific urine and plasma samples were diluted tenfold in order to reduce the matrix effect. An extra preparation step was additionally conducted in order to remove protein from plasma in plasma samples; this was done by adding 75 ml of perchloric acid (0.5 M) to 50 ml of the diluted sample, followed by the centrifugation of the mixture to isolate the precipitated proteins. Also, to remove matrix effect in urine samples, the pH of urine samples was adjusted to 11 and centrifuged for 20 min until white lipidic solid was sedimented in the bottom of tube.

As recommended by the literature [26], the extraction was performed on the clear supernatant solution based on the SPE-CMN procedure following the protein isolation.

RESULTS AND DISCUSSION

Characterization of the Adsorbent

SEM instrument was used to characterize the synthesized Fe_3O_4 NPs. The magnetized images for Fe_3O_4 NPs (A) and SDS-coated Fe_3O_4 NPs (B) are shown respectively in Fig. 2 as well. It could be concluded that these synthesized nanoparticles are smaller than measured size in SEM. Because agglomeration of many ultrafine particles has been proven on the Fe_3O_4 surface morphology analysis by analyzing SEM images [13]. Figure 3b shows the IR spectra of pure Fe_3O_4NPs (a) and SDS-coated Fe_3O_4 NPs exposed well to the modified Fe_3O_4 NPs surface by SDS. The peak at ~3392 and ~590 cm^{-1} could be attributed to the stretching vibrations of -OH which in turn is assigned to the OH groups of the Fe_3O_4NPs surface and the Fe-O band vibration of Fe_3O_4, respectively. Moreover, the new absorption peaks observed at almost 1218 cm^{-1} could be attributed to S=O groups of SDS, and peaks at ~2917 and ~2848 cm^{-1} are assigned to stretching mode of the aliphatic



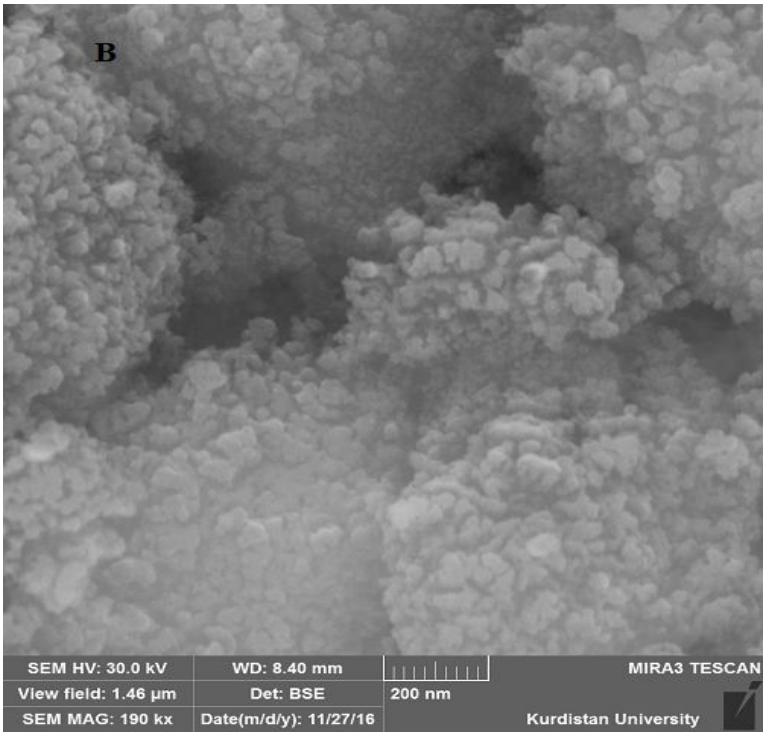


Fig. 2. The SEM images of Fe_3O_4 NPs (A) and SDS-coated Fe_3O_4 NPs (B).

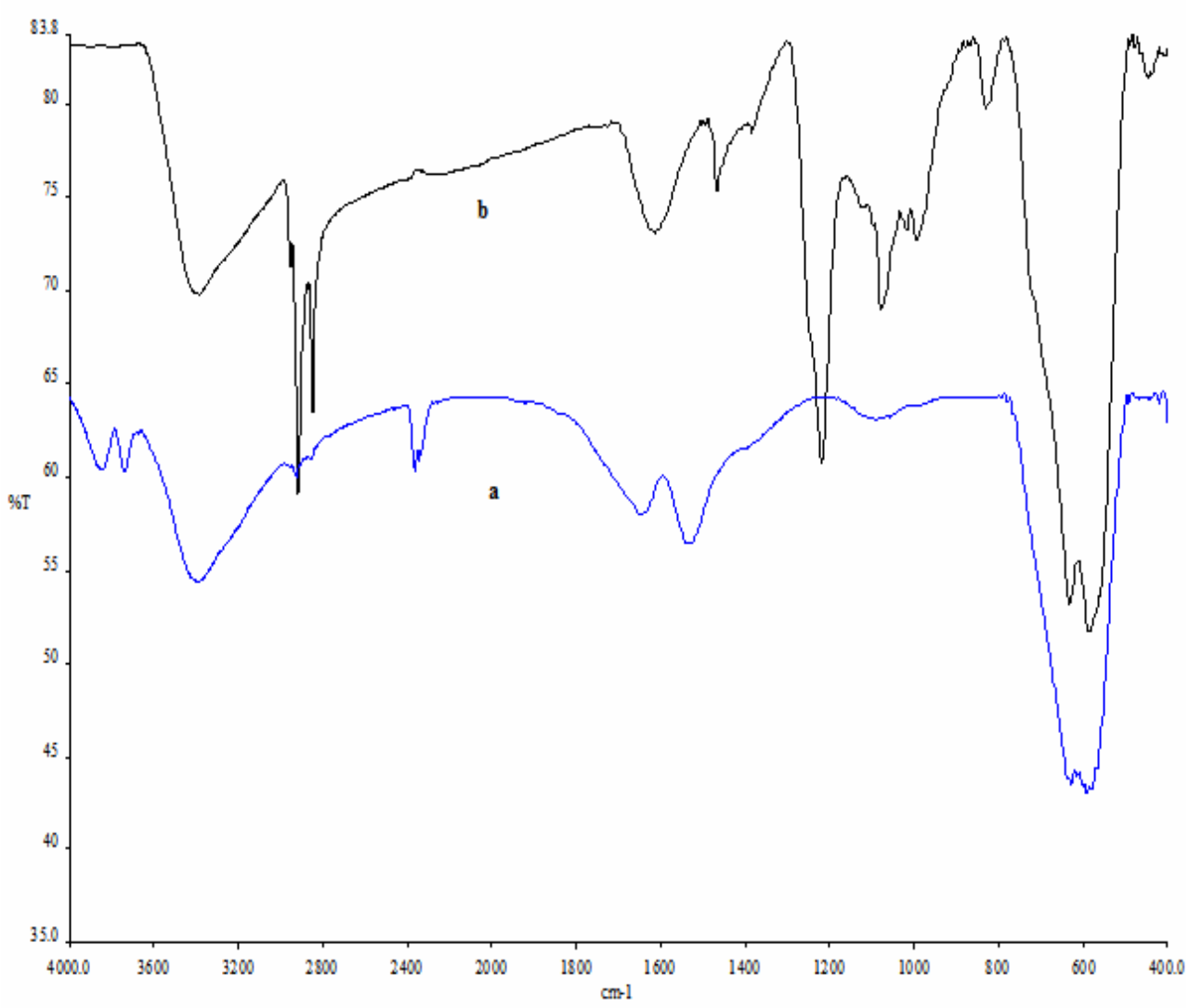


Fig. 3. FT-IR spectra of Fe_3O_4 NPs (a) and SDS-coated Fe_3O_4 NPs (b).

C-H groups of SDS in the final product (as shown in Fig. 3).

Effects of pH

It is believed that the adsorption behavior of the system of mixed-hemimicelles is affected by pH due to the change of the charge density on the MNPs surface [27]. In order to evaluate the adsorption performance of the drug as a function of pH samples, the SDS (anionic surfactant) was used in this study. Several studies have investigated the pH effects on the adsorption of drug at SDS-coated Fe_3O_4 nanoparticles in the pH range of 2-8. For Fe_3O_4 NPs, PZC (the point of zero charge) has an approximately pH of 6.5 [29 & 30]. The surface of Fe_3O_4 NPs is negatively charged when the pH of the solution is above its PZC. However, the surface of Fe_3O_4 NPs is weakly positively charged at the acidic solution (pH < 6.5). Next, the surfactant molecules of sodium dodecyl sulfate are adsorbed gradually on the positively charged Fe_3O_4 NPs. This was done through the electrostatic attraction which led to the formation of a self-assembled monolayer. Additional SDS molecules could form a second layer following the coating of the MNPs

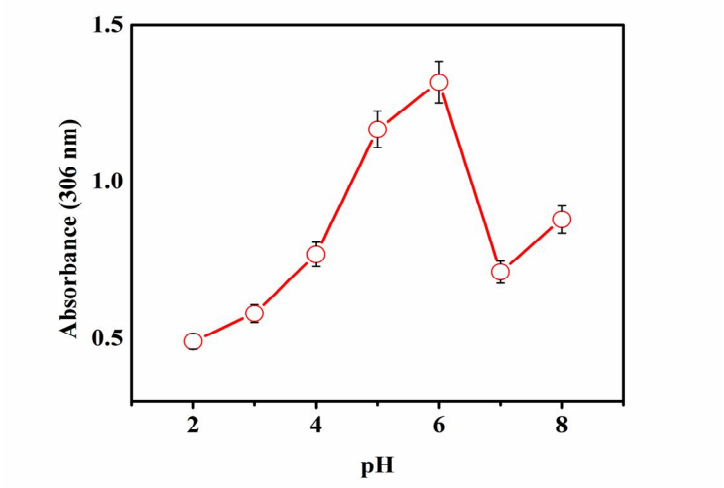


Fig. 4. Effect of pH on extraction efficiency. Sample volume: 10 ml, sample concentration: 5 mg l^{-1}, adsorbent amount: 20 mg, extraction time: 5 min, eluent:2 ml methanol: NaOH (60:40, v/v), desorption time: 2 min.

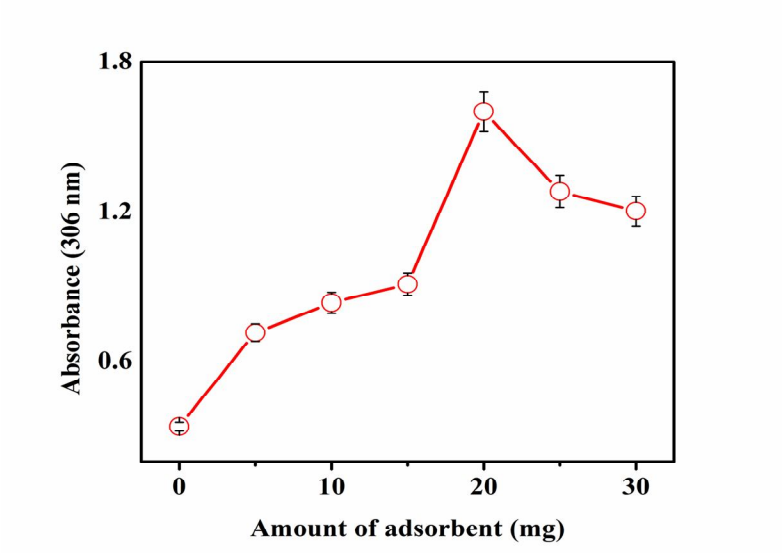


Fig. 5. Effect of the adsorbent amount. Sample volume: 10 ml, sample pH = 5, sample concentration: 5 mg l^{-1}, extraction time: 5 min, eluent: 2 ml methanol: NaOH (60:40, v/v), desorption time: 2 min.

surfaces with a monolayer of SDS. This formation was due to hydrophobic attraction between their organic tails [18]. The combination of these two regions is called the mixed hemimicelles, which would be a suitable medium for extracting the organic compounds [22]. Moreover, it was found that pure Fe_3O_4 NPs are oxidized; that is why, they easily lose their magnetite when pH is below 4. As Fig. 4 shows, the maximum adsorption increases gradually as pH increases up to 5, and then it decreases. Thus, the optimum pH for the subsequent experiments was set at 5.0.

Effects of SDS-coated Fe_3O_4 Nanoparticles

The adsorption and extraction of Nystatin is also influenced by the amount of adsorbent. In comparison with

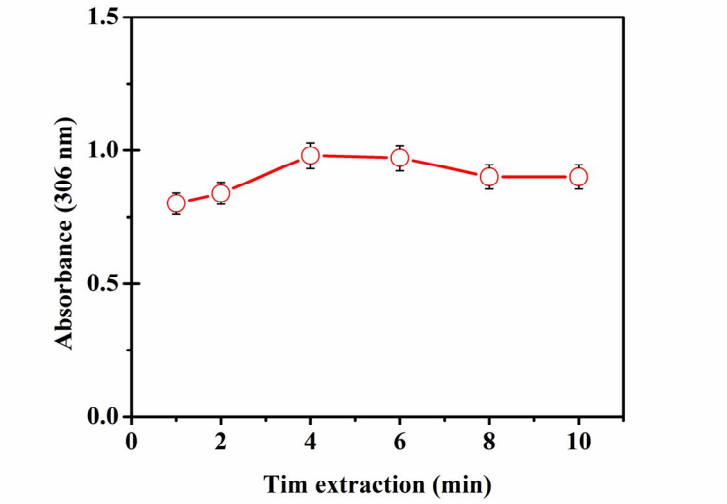


Fig. 6. Effect of the extraction time. Sample volume: 10 ml, sample pH = 5, sample concentration: 5 mg l^{-1}, adsorbent amount: 20 mg, eluent: 2 ml methanol: NaOH (60:40, v/v), desorption time: 2 min.

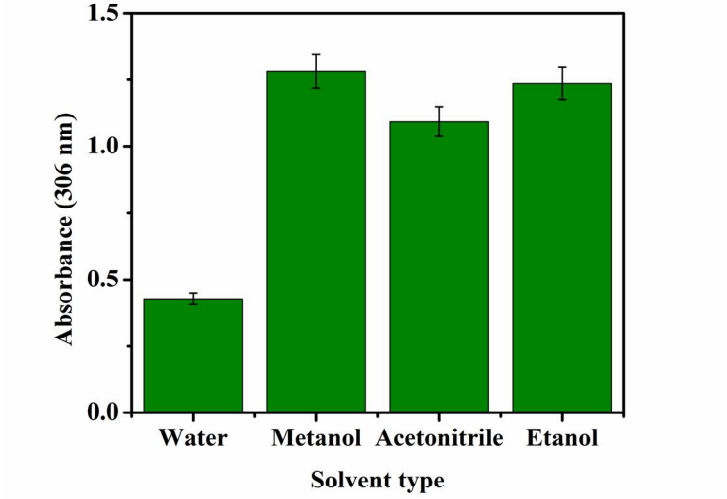


Fig. 7. Effect of the eluent type on the extraction efficiency. Sample volume: 10 ml, sample pH = 5, extraction time: 5 min, sample concentration: 5 mg l^{-1}, adsorbent amount: 20 mg, desorption time: 2 min.

other adsorbents, micron-size particle sorbents (such as carbon active), Fe_3O_4 nanoparticles sorbents have a significantly higher surface area-to-volume ratio. Thus, smaller amounts of Fe_3O_4 nanoparticles could yield better results [21]. The effects of the amounts of SDS-coated Fe_3O_4 nanoparticles were investigated by applying varying amount of the adsorbent from 5 to 30 mg in this study. As shown in Fig. 5, by increasing the amount of adsorbent, the amount of Nystatin adsorption increases remarkably up to 20 mg.

The adsorption of Nystatin decreases gradually when the adsorbent amount is above 20 mg. It could be concluded that when the amount of nanoparticles increases, due to decreased surface area, decreased adsorption and

extraction efficiency, the dispersion will not occur properly, that is why, the optimum amount of the adsorbent in the next studies was set at 20 mg.

Effects of the Ionic Strength

By applying varying amount of sodium chloride within the range of 0 and 8% w/v, the effects of salt concentration on the extraction was investigated in this study. Results showed that when the salt concentration is increased the adsorption capacity of SDS-coated Fe_3O_4 nanoparticles decreases. This can be attributed to the exchange reactions in the solution during the adsorption process. In other words, Nystatin adsorption is expected (Based on Le Chatelier's principle) to decrease when the amount of salt concentration cation (Na^+) is increased. Consequently, all the subsequent experiments were conducted in the absence of salt. Clearly, one can expect lower extractions in the biological samples than those in the aqueous samples since salts are present in the former samples [17,18].

Effects of ExtractionT and Desorption

An experiment was also done to investigate the effects of the extraction time on the adsorption of Nystatin in the range of 1-10 min (Fig. 6). Results showed that the adsorption of Nystatin increased up to 5 min and then remained constant. Since the diffusion routes in SDS-coated Fe_3O_4 nanoparticles are short, adsorption process takes place rapidly and the equilibrium between the sample solution and the adsorbent surface is achieved in a shorter contact time. Thus, the optimal extraction time for analytes in the subsequent experiments was set at 5 min. Moreover, the effects of desorption time was investigated in the range of 1-10 min. No significant change occurred in the desorption efficiencies after 2 min. Consequently, the optimal desorption time for analytes in the subsequent experiments was set at 2 min.

Effects of Eluent Type and Volume

Using the organic solvents, the present study examined the effect of the removal of the species adsorbed on the surface of nanoparticles that had been modified by surfactants. For this purpose, the desorption of Nystatin from SDS-coated Fe_3O_4 nanoparticles was performed by various organic solvents including ethanol, methanol, acetonitrile, water, and 0.01 M NaOH (60:40 v/v). As shown in Fig. 7, the maximum desorption is related to methanol. Moreover, based on the reported results [17,22, 24], as pH increased, more disruption occurred because of the reduced charge density on the Fe_3O_4 nanoparticles surface above the isoelctric pH (pH = 6.5). One can examine the effects of eluent amounts on the recovery of analyte absorption simply by varying the amounts in the range of 1-5 ml. As Fig. 8 shows, the maximum signal was obtained when the eluent volume was less than 2 ml. A decrease in the concentration of analyte resulting from the increased eluent can be considered to trigger this phenomenon. Thus, the optimum volume for further experiments was set at 2 ml.

Analytical Results

The precision and accuracy of the method can be evaluated through separate calibrations in water, urine, and plasma samples, in one hand. On the other hand, it can be evaluated through quantitative parameters such as the linear range, the coefficient of correlation, the detection limit, and the quantification limits (See Table 1). By diluting the stock solution of Nystatin with methanol, a series of practical and standard solutions were prepared. For water, urine, and plasma samples, the calibration graphs were linearized in the ranges of 1-20, 1-18 and 1-15 mg l^{-1}, respectively, under the optimized conditions. LODs were 0.63, 0.76 and 0.85 mg l^{-1} for water, urine, and plasma samples, respectively. LOQs were 2.12, 2.29 and 2.58 mg l^{-1} for water, urine, and plasma samples, respectively, as well. Based on the standard deviation of the response (SD) and the slope of the calibration curve (slop = m) at approximated level the LOD, LODs and LOQs were calculated using the following formula: LOD = 3.3 (SD/m) and LOQ = 10 (SD/m). The standard deviation of y-intercept of regression lines could be used to determine the standard deviation of the response. As shown in Table 1, the proposed method is capable of good linearity, low detection, and quantification limits. Five replicate experiments with 10 ml of the standard solution were used to verify the precision of the analytical method; each experiment contained 5 mg l^{-1} Nystatin. Moreover, by determining the relative error, the accuracy of the method was evaluated. Spiked samples were used to determine the relative error. The spiked urine and plasma samples were

Table 1. Merits of the Proposed Method in Urine and Plasma Matrices for Extraction and Determination of Nystatin

Matrix	LOD ($mg\ l^{-1}$)	LOQ ($mg\ l^{-1}$)	Linear range ($mg\ l^{-1}$)	R^2
Water	0.63	1.9	1-20	0.991
Urine	0.76	2.28	1-15	0.994
Plasma	0.85	2.56	1-12	0.991

Table 2. Results of the Analyzed Real Samples Using the Proposed Method

Matrix	Added ($mg\ l^{-1}$)	Founded ($mg\ l^{-1}$)	RSD%	Recovery (%)
Water	5	4.95 ± 0.2	2.93	99
Urine	5	4.9 ± 0.5	6.3	98
plasma	5	5.1 ± 0.4	7.2	102

Table 3. Comparison of the Results of the Proposed Method with Reports for Determination of Nystatin

Method	Recovery (%)	RSD (%)	LOD ($mg\ l^{-1}$)	Ref.
HPLC	92	0.82	0.01	[9]
HPLC	98.24-100	0.24	-	[6]
	88	5.6	0.78	[2]
HPLC UV	98-102	2.93	0.63	This work

*HPLC: High Performance Liquid Chromatographic.

extracted and analyzed using the proposed method under optimum conditions to evaluate the applicability of the newly developed extraction system for analysis of Nystatin in real samples. Since Nystatin was not detected in the real samples, 5 $mg\ l^{-1}$ of Nystatin was added into the real samples, and both the extraction and determination were conducted based on the above-mentioned procedure. Table 2 shows the results of 10 replicate analyses of each real sample (obtained from the proposed method). These results are in agreement with the spiking amounts.

CONCLUSIONS

Based on the SDS-coated Fe_3O_4 nanoparticles, an SPE method was developed for both the pre-concentration and the determination of Nystatin urine and plasma samples in this research. In the proposed technique, the SPE was coupled with the SDS-coated Fe_3O_4 nanoparticles process. Compared to previous methods of SPE, the SDS-coated Fe_3O_4 nanoparticles method has more advantages such as the simplicity of performance, high rate of separation, high selectivity, and high extraction efficiency due to the fact that the nanoparticle sorbents have a higher surface capacity. In addition, the nanoparticle sorbents are easily applied for extraction by using less nanoparticle sorbents than micron-size particle sorbents; that is why, the new proposed method has a dramatic analytical potential for the pre-concentration in large-volume of real samples. The adsorbed analyte was easily desorbed with the basic methanol, thus, the proposed method was successfully applied to determine the target Nystatin in water, urine and plasma samples. Besides, comparison with other methods for determining Nystatin, Table 1 has revealed that this method is either equally applicable or has significant advantages.

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