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A novel mixed hemimicelles dispersive micro-solid phase extraction using ionic liquid functionalized magnetic graphene oxide/polypyrrole for extraction and pre-concentration of methotrexate from urine samples followed by the spectrophotometric method

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ABSTRACT

Methotrexate (MTX) is an anticancer drug that is widely used in a variety of cancers including primary central nervous system lymphoma. It is also administrated in the treatment of some autoimmune diseases. A simple, accurate, sensitive, and precise mixed hemimicelles dispersive micro-solid phase extraction was proposed for MTX quantification in human urine samples. MTX was quantified by spectrophotometer after dispersive micro-solid phase extraction using ionic liquid functionalized magnetic graphene oxide/polypyrrole. Interactions of adsorbent and MTX were modeled by molecular docking and the interaction energy was predicted to be −8.35 kcal/mol. A larger absolute value of binding energy represents larger adsorption strength, indicating that graphene oxide nanosheets could perform higher adsorption strength toward MTX. The concentrations of MTX were proportional to analytical response in amounts ranging from 10 to 1000 ng/mL with a good correlation (R^2 = 0.99). Inter- and intra-day precisions and accuracies were within the acceptable limit according to FDA guideline (15% for biological determination). The recoveries were ranging from 89 to 93% and the method was specific for routine analysis of MTX. This protocol was applied to the urine of two patients under MTX therapy received an intravenous administration of 1 mg/kg/dose of MTX with acute lymphoblastic leukemia. The accuracy of the method was confirmed by HPLC measurements.

1. Introduction

Methotrexate (MTX), or (2*S*)-2-[[4-[(2,4-diaminopteridin-6-yl) methyl-methylamino] benzoyl] amino] pentanedioic acid (Fig. 1) is a chemotherapeutic drug belonging to the class of antifolates. MTX is widely used to treat a brosd range of cancers including breast cancer, lung cancer, leukemia, lymphoma, and osteosarcoma and also is used in the treatment of some autoimmune diseases. MTX is applied to treat cancer by inhibiting cellular enzymes and preventing cell replication. It is generally administered orally and after absorption, most of the drug is excreted unchanged in urine. Approximately 10% of the orally-administrated MTX is metabolized due to the rapid first-pass effect; the rest remains unchanged for the systemic circulation. The biological half-life of MTX is almost 7 to 10 h.

The analysis of MTX for biomedical disciplines covers a broad range of aims such as therapeutic drug monitoring (TDM), pharmacokinetic study to analyze drug bioavailability, bioequivalence tests to evaluate the effect of formulation parameters, toxicology, and forensic sciences [1,2]. High doses of MTX used in cancer therapy have serious toxic effects. High circulating levels of MTX can cause severe myelosuppression. To minimize toxicity levels and in order to have a safe, and effective use of MTX, continuous monitoring of drug levels in the body is essential for optimizing the therapeutic dosing [3–5]. MTX is a strongly UV–Vis absorbing substance, due to the presence of the heteroaromatic chromophore. The most frequent detection system employed for the determination of MTX is UV–Vis [6]. To overcome the difficulty of poor sensitivity in UV detectors, pre-concentration methods have been recommended; the most important step in the determination of an analyte is sample preparation, and 80% of the analysis time is allocated to it [7–10]. Several methods have been reported to

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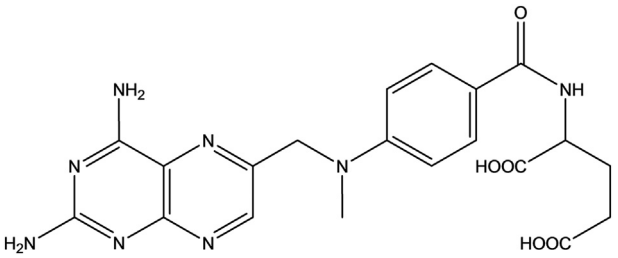

Fig. 1. Chemical structure of MTX.

determine the MTX in various biological matrices using high-performance liquid chromatography (HPLC) [3,4,6,11–13], electrochemical impedance spectroscopy and cyclic voltammetry [5,14], fluorescence [15–17], surface-enhanced Raman scattering (SERS) [18], and ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) analysis [19]. Most of the analytical methods reported for MTX are tedious, time-consuming, and need highly skilled operators. UV–Vis spectroscopy is one of the inexpensive, low cost, and simplest analytical tools routinely used in almost all biomedical laboratories [20,21].

Liquid-liquid extraction (LLE) and solid phase extraction (SPE) are the most popular sample preparation processes [22,23]. The sorbent is the heart of SPE. In conventional SPE, solid particles are immobilized generally on a supporting phase such as a cartridge, fiber, and disk. The liquid phase might percolate through the solid particles; hence, SPE devices are usually recommended only for single use [24]. Complete removal of the sample residuals from the sorbents is of prime importance in SPE. Therefore, obtaining a clean extract requires several washing cycles by testing a variety of eluents. Another problem that SPE method encounters, in particular when dealing with biological matrices, is clogging the sorbent pores, which consequently affects the analyte retention time and sorbent capacity. Dispersive micro-solid phase extraction (D-μ-SPE) is a new, rapid, simple, and effective type of SPE used for pre-concentration of trace amounts of target analytes. In this technique, the adsorbent is dispersed into the sample solution and separation process is achieved by centrifugation or an external magnetic field [25]. Magnetic solid phase extraction (MSPE) is a promising technique in which extraction is performed by sorbents with magnetic or magnetizable properties [26]. Graphene and graphene-based materials are good candidates for sample preparation as in many studies they have been directly used as an adsorbent in the D-μ-SPE process because of their unique properties; *e.g.,* having a high surface area to weight ($2630\ m^2\ g^{-1}$), thermal and chemical stability, and a simple synthesis process [27]. Graphene oxide (GO) is a functionalized form of graphene with different epoxy, hydroxyl, carbonyl, and carboxyl surface groups [28]. It is easily prepared from natural graphite by Hummer's method [29]. GO with polar functional groups and delocalized π-electron system is an appropriate adsorbent for extraction of polar and aromatic organic compounds. GO, as an adsorbent, shows difficulty in its recovery and separation from aqueous suspensions after the extraction process; therefore, a great number of efforts have been made to solve this problem by functionalization of the graphene surface [30,31]. Polypyrrole (PPy), one of the most common conductive polymers, has some unique characteristics such as a high conductivity, good stability, non-toxicity, low cost, and ease of preparation making it suitable for modification of GO surface. Mixed hemimicelles are recently used in the structure of SPE adsorbents due to providing high extraction efficiency. Mixed hemimicelles provide a proper interaction according to the electrostatic interaction between ionic surfactants and opposite charges on metal oxides [32]. Ionic liquids (ILs) have received attention because of their promising properties in terms of their great physicochemical characteristics such as low volatility, good thermal stability, high conductivity, and tunable miscibility [33]. Some ILs such as imidazolium-based ILs have long alkyl chains, which enable them to aggregate and form a surfactant-like compound in aqueous solutions. This behavior has resulted in their successful application in different sample preparation procedures including magnetic mixed hemimicelles solid phase extraction [34]. Moreover, this performance has attracted great attention because of its several advantages such as high extraction efficiency, easy elution of analytes, and a high flow rate for sample loading. Furthermore, because of the possibilities of fabricating ILs with diverse polarity, the degree of hydrophobicity can be easily matched according to the nature of analytes [35].

More recently, magnetic materials have received attention because of the high surface to volume ratio that provides a high density of binding sites and their excellent magnetic properties for easy separation of adsorbent from sample solution [36]. There are advantages to being inspired by the simultaneous use of GO coated with magnetic PPy coupled with hemimicelles in the extraction efficiency of analyte because of simple magnetic separation and providing a higher surface for extraction.

In this work, ionic liquid-based magnetic mixed hemimicelles D-μ-SPE using 1-hexadecyl-3-methylimidazolium bromide coated magnetic GO/PPy as an adsorbent was designed for extraction and pre-concentration of MTX from urine samples. HPLC-UV and UV spectroscopy were used for determination of the analyte concentration. The developed method was validated according to FDA guideline and applied for the determination of MTX levels in two patients with acute lymphoblastic leukemia. The combination study of experiment and computational strategy was applied for the interpretation of adsorption mechanism.

2. Experimental

2.1. Materials and instruments

MTX powder, pyrrole (Py), and 1-hexadecyl-3-methylimidazolium bromide (C16mimBr) were purchased from Sigma (Sigma, USA). Acetonitrile (ACN), methanol, ethanol and acetone were obtained from Scharlau (Barcelona, Spain). Sodium hydroxide (NaOH), hydrochloric acid (HCl), expanded graphite powder, sulforic acid (H_2SO_4, 98%), hydrogen peroxide (H_2O_2, 30%), $KMnO_4$, $FeCl_2{\cdot}4H_2O$, $FeCl_3{\cdot}6H_2O$, ammonia solution and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany).

The UV–Vis spectrophotometer (Thermo, USA) accompanied with Thermo Insight software was used to determine the concentration of MTX. A 350 μL quartz cell was used to record spectrums and absorbance measurements. The absorbance was recorded at the wavelength of 375 nm. Fourier transform infrared (FT-IR) spectrometry (Tensor 27; Bruker; Germany) was applied at 400–4000 cm^{-1} to characterize the synthesized GO and its magnetic form. Scanning electron microscopy (SEM; Mira 3 FEG-SEM; Tescan; Czech Republic) was used for the morphologic survey. Magnetization curves were recorded using (VSM—4 in., Daghigh Meghnatis Kashan Co., Iran) at room temperature. Zeta potential was measured using a Zetasizer (Nanotrac Wave; Microtrac; Germany). Powder X-ray diffraction (XRD) was carried out using a D5000 (Siemens; Germany) with a Cu tube anode. A vortex from Ika Company (Genius 3, Germany) was used in sample preparation. A Sigma centrifuge (Osterode, Germany) was used in the precipitation step. The pH was adjusted using a Meterohm® pH meter (Herisau, Switzerland). A Sonica Ultrasonic Cleaner machine (Milan, Italy) was used to ultrasonic performance. HPLC–UV analyses were performed using a HPLC apparatus (Agilent, Germany) equipped with a UV detector and Agilent C18 (150 × 4.6 mm, 5 μm) column.

2.2. Standard solutions and urine samples

Stock standard solution of MTX (1000 μg/mL) was constructed in methanol and stored in −4 °C. Drug-free urine samples were provided by volunteer donors and transferred in polypropylene microtubes and

frozen. Urine samples thawed at room temperature unassisted before daily experiments. Daily standard MTX solutions were prepared in drug-free urine sample and diluted with the same matrix. The required calibrator solutions (10, 100, 500, 1000 ng/mL) were prepared in the same manner. Two samples were obtained from patients receiving MTX who had signed consent forms approved by the ethics committee, Tabriz University of Medical Sciences. These Samples were also collected in polypropylene tubes and stored in refrigerator until analysis.

2.3. Synthesis of magnetic graphene oxide/polypyrrole nanocomposite (magnetic GO/PPy)

Preparation of GO was completely described in our previous work [23]. Briefly, GO synthesized *via* modified and green Hummer's method. The mixture of graphite powder (0.5 g) and concentrated H_2SO_4 (12 mL) were prepared in a conical flask and put it in an ice bath while maintaining mix. Then, $KMnO_4$ (1.5 g) was added gradually into this mixture and the resultant was stirred using magnetic stir bars while the temperature adjusted at 35 °C. The formation of brown color solution was reached after stirring for 30 min. Then 15 mL distilled water was added to the mixture and temperature of the reaction was elevated up to 90 °C solution was mixed for another 30 min at. After that, 1 mL of H_2O_2 was introduced into the reaction mixture as a reaction terminator and the color of the solution was changed into yellowish brown. The solid phase was sedimented after centrifugation and washed thoroughly with HCl 3% (3 × 15 mL) in order to remove the contaminates. Then, the particles were washed by distilled water until the pH reached to neutral. The resultant particles were oven dried at 70 °C and the dark brown GO particles was resulted.

GO (0.2 g) was dispersed in ultra-pure water (200 mL) with ultrasonication for 1 h. Py monomer (0.5 mL) was then added and stirred for 1 h at room temperature. Subsequently, GO/PPy solution was prepared by adding $FeCl_3.6H_2O$ (3 g) into this solution and continues agitation for 2 h. The as-synthesized GO/PPy solution was washed with ultrapure water to eliminate impurities.

$FeSO_4.7H_2O$ (0.667 g) and $FeCl_3.6H_2O$ (1.000 g) were added into the GO/PPy solution (250 mL) produced above and mechanically agitated at 80 °C for 30 min to completely load ferric and ferrous salts onto the GO/PPy. Ammonia solution (15 mL, 30%, *v*/v) was added dropwise after 2 h of reaction at 80 °C to form magnetic nanoparticles. This solution was subsequently cooled, filtered and washed to eliminate residual base. The resulting IL coated magnetic GO/PPy composite was dried and stocked.

2.4. Extraction procedure

The D-μ-SPE performance was done as follows: First, 0.5 mL of the urine sample was put in a 15 mL tube spiked with MTX and diluted six times with deionized water. Then, 100 mL C16mimBr solution (4 g/L) was added to this solution. The pH of this solution adjusted to 4 with 30 μL of HCl 0.1 M. Next, 3 mg IL coated magnetic GO/PPy weighed and dispersed into the mixture. The tube was placed in an ultrasonic bath for 6 min. Subsequently, the adsorption procedure was completed by vortexing for 1 min in order to accelerate the mass transfer of analytes by increasing the interfacial area between the solid sorbent and sample solution. The MTX-loaded sorbent was separated by an external magnetic field and the supernatant was filtered off. Afterward, 700 μL of acetone as desorption solvent was added to the sedimented MTX/sorbent, this mixture sonicated for 3 min and then filtered by a magnetic field. The supernatant was kept for the drug content analysis. The acetone was evaporated by nitrogen gas and the residue was dissolved in 200 μL ACN. Finally, the amount of MTX was determined by UV spectrophotometer at 375 nm.

2.5. Confirmation the method with HPLC

The pH of urine samples was set at 10 and then it was centrifuged for 15 min until solid residuals precipitated at the bottom of the tube. The supernatants were transferred into another tube and diluted for 5 times. A previously reported HPLC method was used for the determination of MTX in urine samples [6]. The mobile phase consisted of a mixture of 50 mM sodium acetate buffer (pH 3.6)–acetonitrile (89:11, v/v). Isocratic elution was performed with a flow rate of 1 mL/min and the detection wavelength was set at 375 nm. Urine samples were provided by a healthy volunteer and spiked with MTX. Detector response was linear in the range of 0.1–5.0 μg/mL.

2.6. Molecular modeling

AutoDock Vina, as one of the widely used simulation programs in molecular docking studies, was used in present work. The crystal structure of MTX containing information about MTX composition and atoms positions were taken from the protein databank (PDB) website. The structure of sorbent was designed and saved as “pdb” data file. The types of interactions considered were hydrogen bonds, electrostatic forces, and hydrophobic forces. An 80 × 80 × 80 Å grid box (grid spacing = 0.75 Å) was made to cover the whole surface of sorbent and MTX.

3. Results and discussion

3.1. Confirmation of synthesized IL coated magnetic GO/PPy

Zeta potential analysis is a promising tool for determining the differential potential between the surface charge of sorbent and those of opposite sign coming from the medium that is organized around the sorbent. Knowing the zeta potential of sorbent helps one realize the stabilization of sorbent by understanding the balance between the attractive and repulsive forces. The zeta potential of the prepared IL coated magnetic GO/PPy is the characteristic of its stability in solution media. The zeta potential of the 1 mg/mL IL coated magnetic GO/PPy dispersions was 20 mV. Usually, it is accepted that the zeta values more than ± 15 mV fall into a moderately stable system category. The high absolute positive zeta potential values proved that repulsion force overcomes π-π stacking and van der Waals forces interactions between the sheets and prevents the particles aggregation.

The crystalline structures and interlayer changes of GO and IL coated magnetic GO/PPy were confirmed by XRD patterns and depicted in Fig. 1b. The GO shows a typical peak at 2θ = 11.3° indicating the distance between GO sheets due to the presence of functional groups such as hydroxyl, epoxy, and carboxyl groups attached on inter-planner sheets from both sides [37]. This peak disappeared in IL coated magnetic GO/PPy nanocomposite, suggesting that GO has been reduced by PPy. As described, the space between the GO layers is related to oxygen-containing groups but in the reduction process, it is expected that the inter planner distance was diminished (Fig. 2a). Reflections for intense peaks (220), (311), (4 0 0), (4 2 2), (5 1 1), and (4 4 0) are indexed to plans at 2θ = 30.15, 36.27, 43.32, 53.89, 57.13, and 62.29°, respectively. This values represent the successful synthesis of GO/PPy and are in accordance with the reported literature [38]. Moreover, no peaks of PPy appeared because they may have been covered by the strong diffraction peaks of Fe_3O_4 [27].

In order to examine the morphology of the prepared nanocomposites, SEM analysis was performed (Fig. 2c). As can be seen, GO has a wrinkled morphology. In comparison with pristine GO sheets, uniform decoration of the magnetic particles can be sharply seen on the surface of GO sheets.

The FT-IR spectrum (Fig. 3) of GO before coating presents peaks at 1739, 1625, 1396, and 1049 cm^{-1}, which are assigned to the C=O stretching of COOH groups, C=C stretching vibration, C-OH stretching

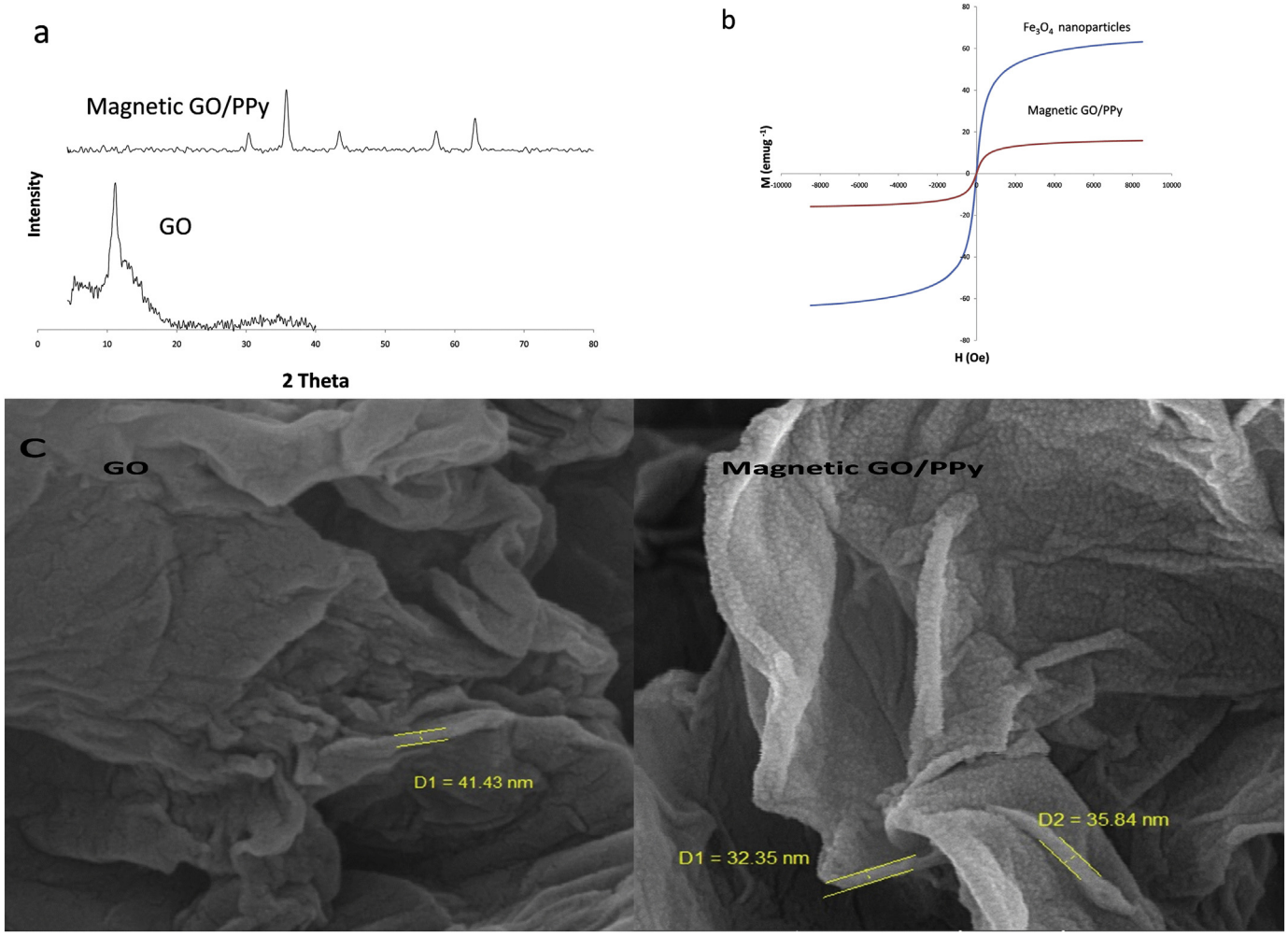

Fig. 2. X-ray diffraction patterns (a), magnetization hysteresis loops of IL coated magnetic GO/PPy (b) and scanning electron microscope images (c) of GO and IL coated magnetic GO/PPy.

vibration, and C—O vibrations from alkoxy groups, respectively. The broad peak appeared at 3397 and 1261 $cm^{-1}$are assigned to the stretching vibration of the O—H and CO—H bonds. The peaks at 2926 and 285 cm^{-1} originate from asymmetric stretching and symmetric vibrations of C—H bond in $–CH_2$, respectively. This evidence confirms the successful oxidation of graphite to GO by Hummer's method. The FT-IR spectrum of the GO sample accorded well with the published reports [39–41]. After coating with Py, the intensity of these peaks becomes much less confirming that the oxygen-containing groups are mostly reduced by Py. Peak at 1557 cm^{-1} observed in GO/PPy spectrum is assigned to C—N stretching vibration present in PPy ring. Moreover, the attachment of Fe_3O_4 nanoparticles on the GO surface was evidenced by FT-IR spectrum. The unique peak at 568 cm^{-1} related to the Fe—O group proves the presence of Fe_3O_4 nanoparticles in magnetic GO/PPy. This peak implies the existence of an interaction between and Fe_3O_4 and GO. Also, the magnetic property of the adsorbent is clearly shown in Fig. 3b.

3.2. Binding mode between GO and MTX

Because of the advantages of the molecular docking procedure in host-guest chemistry, the computational docking studies were performed by placing MTX on the surface of sorbent. The studies were carried out to predict the binding energy between sorbent and analyte. Docking calculation was performed by AutoDock Vina. Fig. 4 depicts the interaction modes between sorbent and MTX. Considering the discussion on the effect of pH, it is reasonable to speculate that electrostatic attraction made a minor contribution to the extraction. The accessible surface in sorbent has adsorption affinity with hydrophobic effects and π–π interactions between sorbent and MTX. Other probable interactions between adsorbent and MTX would be derived from the intermolecular hydrogen bonds between the nitrogenous group in MTX structure and sorbent. Moreover, van der Waals interaction also made a contribution to adsorption. The excellent extraction capacity obtained from sorbent could be attributed to all these interactions. The binding energy indicates the binding strength coming out of adsorption simulation procedure, which serves as the standard for the binding strength. Among all possible interaction models calculated by AutoDock Vina, the overall binding energy of the extraction process was predicted to be −8.35 kcal/mol for the most suitable interaction at which MTX attaches on sorbent from the wide surface. A negative value of binding energy represents a large exothermic adsorption of the adsorbate molecule over sorbent nanosheet.

3.3. Optimization of extraction parameters

In order to improve the extraction recovery, a set of effective parameters were studied including sample volume, pH of sample solution, amount of adsorbent, adsorption and desorption times, and type of eluting solution. There are some computational approaches for modeling and optimization of independent variables but herein preliminary studies were performed in human urine samples by the one

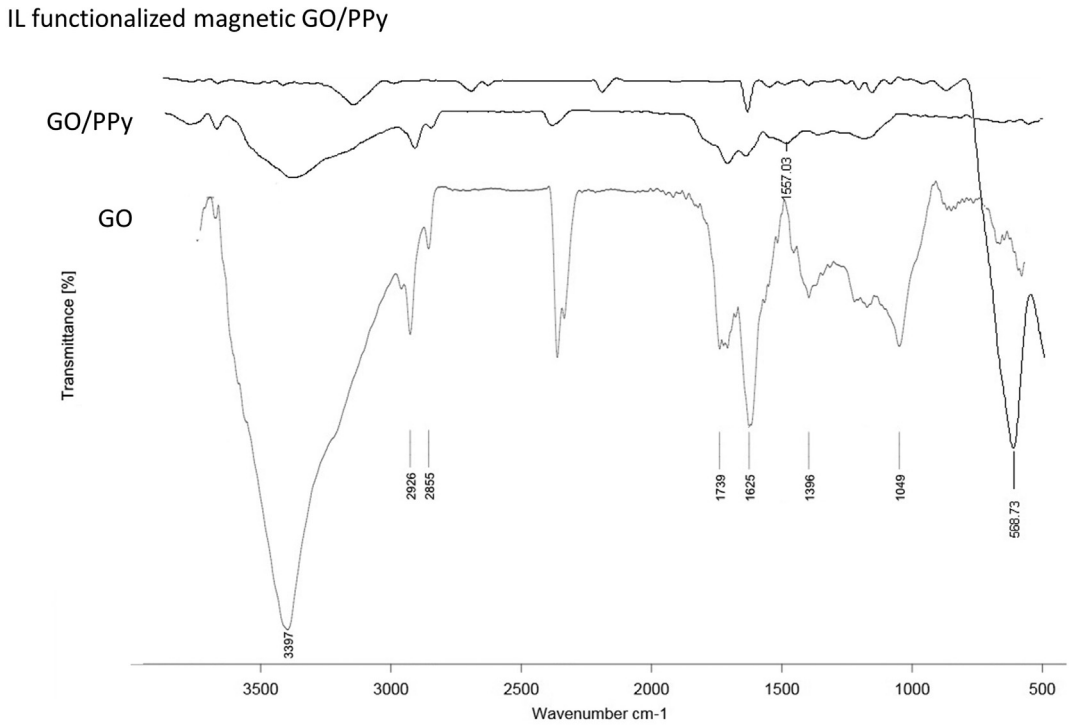

Fig. 3. Fourier transform spectroscopy (FT-IR) spectra of GO, GO/PPy and IL functionalized magnetic GO/PPy.

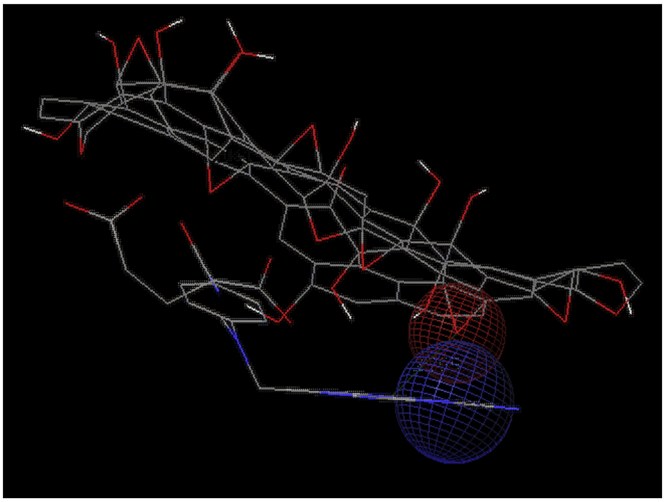

Fig. 4. Molecular docking study between sorbent and MTX.

parameter at a time approach [42–45]. Extraction of MTX performed through π-π interaction between aromatic moiety on the drug structure and delocalized π-electron system of GO/PPy surface and also formation of the hydrogen bonding between carboxyl and amine group of MTX with carboxyl and hydroxyl groups on the surface of GO/PPy were considered.

3.4. Sample volume

The effect of sample volume on the microextraction procedure, taking into account constant dilution for all urine samples, was studied. For this purpose, 0.5 mL of urine after MTX spiking was used in all studied volumes. Volumes of sample solutions were varied from 1 to 10 mL in test tubes. The highest analytical response was achieved at the sample volume of 3.0 mL. Therefore, 3.0 mL was chosen as the optimum sample volume.

3.5. pH optimization

The pH of the sample solution plays an important role in the extraction process because it could determine the ionization state of the target analyte as well as a surface charge of the adsorbent. The pH values of the sample solution were set between 3.0 and 11.0 by the addition of 0.1 M HCl or 0.1 M NaOH solutions, respectively. From Fig. 5A, it is revealed that the extraction has occurred best in acidic conditions. The MTX has three pK_a values of 3.8, 4.8, and 5.6 due to the different chemical sites. In pH = 4, the MTX is mostly in its undissociated form, thus the solubility is reduced and thereupon maximum extraction is reached. It can be concluded that the electrostatic interactions between MTX and adsorbent play a moderate role in the adsorption process. Since the pH of the real urine samples was generally in the range of 6.5–7.0, it was needed to adjust the pH of the sample solution before the adsorption process.

3.6. Amount of IL coated magnetic GO/PPy sorbent

To check the maximum recovery of the analyte, the amount of sorbent was investigated in different amounts of adsorbent (*i.e.,* 2, 3, 4, and 5 mg). As depicted in Fig. 5B, by exceeding the sorbent amount of 2 mg, no improvement in extraction occurred. This result is probably due to the aggregation and a decrease in the effective adsorption surface area for drug loading. So, 3 mg was considered to be the best amount.

3.7. Adsorption time

The adsorption time means the contact time between adsorbent and analyte in order to obtain the maximum drug loading on the sorbent. The time of adsorption is a necessary parameter to ensure the sufficient contact between sorbent and analyte. In the present stage, the effect of time on extraction efficiency was checked in different contact times; *i.e.,*2, 4, 6, 8, and 10 min. As shown in Fig. 5C, the equilibrium of the extraction process was reached at 6 min so recovery increased during this time. Such a fast equilibrium between sorbent and analyte is

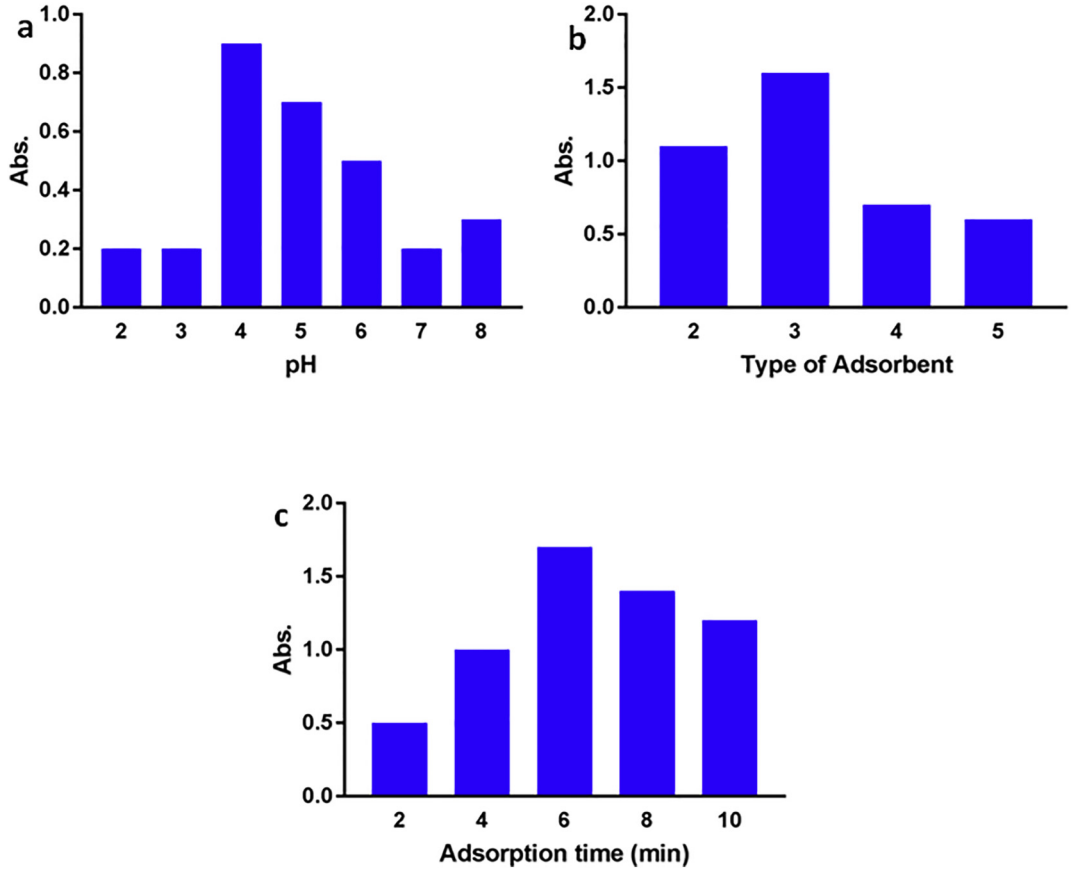

Fig. 5. Effect of pH (A) various amounts of IL coated magnetic GO/PPy adsorbent(mg/mL) (B) and contact time (C) on extraction efficiency.

reasonable due to the high hydrophilicity of adsorbent and its large accessible surface for extraction. With extending the time over 6 min, no significant change in analytical response was observed and subsequent experiments were set at 6 min. This observation could be as the result of nanoparticle agglomeration upper than the equilibrium point then reduction of surface area for drug adsorption.

3.8. Desorption conditions

The efficiency of the extraction process is highly dependent on the solvent used for the elution. ACN, acetone, methanol, and ethanol were tested and found that (Fig. 6A) the desorption capacity of acetone is much better than that other eluents. Acetone with the highest extraction efficiency is selected as the desorption solvent.

In order to complete desorption of analyte from GO, the effect of desorption time on desorption capacity was also evaluated. Desorption time (1, 3, 5, 7, and 9 min) was also investigated. As shown in Fig. 6B, 3 min is sufficient to desorb the MTX.

3.9. Method validation

Implemented methods of analysis are sometimes modified to supply the requirements of the laboratories that utilize these methods. Therefore, these modifications should be checked in details to ensure the proper performance of the developed method. ***FDA*** has issued a procedure for method validation [46]. In the present paper, the

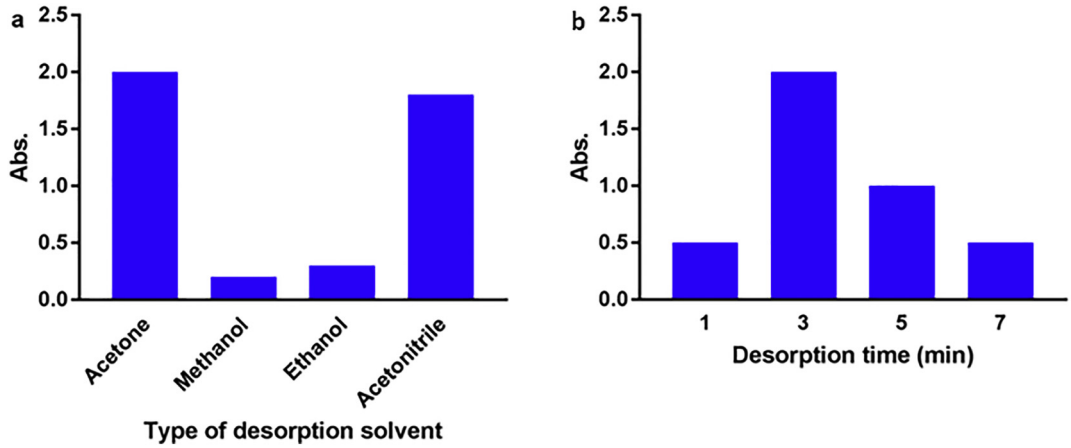

Fig. 6. Effect of type of elution solvent (A) and desorption solvent volume (B) on extraction efficiency of IL coated magnetic GO/PPy adsorbent.

required aspects for an analytical method have been covered; *i.e.,* linearity, accuracy, precision, recovery, stability, robustness and selectivity.

3.10. Linearity

The calibration curve generated over the MTX concentration range of 10–1000 ng/mL, with three replicates at each concentration level. For spiked urine samples, the correlation coefficient was greater than 0.99. Linear regression analysis of the results yielded an equation of $y = 0.803\ C_{MTX}$ (μg/mL) + 0.032.

3.11. Precision, accuracy, and recovery

Precision was calculated in accordance with the FDA recommendation in two stages. Intra-day repeatability was assessed by analyzing five consecutive injections of MTX-spiked samples in three varying concentrations. The inter-day analysis was performed by quantification of the same concentrations on five consecutive days.

According to the FDA guideline, the RSD% values lower than 20% for lower limit of quantification (LLOQ) and 15% for other occasions, which are acceptable for bioanalysis. The accuracy of the method was defined as a relative error (RE%) calculated using the following equation:

$$RE\% = 100 \times \left(\frac{Found\ value - Nominal\ value}{Nominal\ value}\right) \quad (1)$$

The relative recoveries (RR%) are defined as the measured concentration of the drug divided by its actual concentration. The intra-day and inter-day precisions, accuracy, and recovery were performed by five replicates of 100, 500, and 1000 ng/mL. The precision, accuracy, and recovery calculations are detailed in Table 1.

3.12. Stability

In this work, the stability tests were conducted to investigate the stability of the components. Since samples are frozen and thawed for several times, the effect of multi-freeze/thaw cycles should be accounted. The stability of the analyte should also be assessed in the sample preparation step until the analysis was completed to figure out any degradation during the sample preparation. Table 2 presents the results of stability evaluation.

3.13. Robustness

Robustness is included in the FDA guideline as a characteristic required for validation. The proposed method needs to be robust that can be easily transferred inter-laboratory if necessary. There are a set of variables related to a developed method including pH, the volume of acetone, and the contact time between adsorbent and MTX. In a robust method, the method should remain unaffected by slight changes of the variables and be reliable for routine usage. This procedure is apart from the optimization phase and evaluates the parameters by deliberate variations. The factors to be assessed here are related to operational parameters. The method was remained unchanged through the small changes applied to the method including pH value in ± 0.1 unit, acetone volume in ± 5 μL, and contact time between sorbent and MTX at ± 1 min. A series of experiments was carried out to cover all combination of variations and analytical signals were recorded. The proposed method showed no significant variation in the presence of negligible changes of the mentioned parameters.

Table 1
Assay precision, accuracy and recovery of urine samples spiked with MTX.

Analyte	Nominal concentration (ng/mL)	Intra-day RSD %; n = 5[a]	Inter-day RSD%; $n = 5$[b]	Accuracy (RE%)[c]	Recovery[d] (%)
MTX	100	10.2	8.4	11.0-	89.0
	500	7.8	10.0	10.0-	90.0
	1000	2.0	4.0	6.8-	93.2

[a] Number of replicates.
[b] Number of days.
[c] RE% = 100 × ((Found value-Nominal value)/Nominal value).
[d] Recovery (%): Found value/Nominal concentration × 100.

Table 2
Evaluation of method stability of MTX in urine.

Analyte	Concentration added (ng/mL)	Recovery (%RR)	
		Freeze-thaw stability	Room temperature stability
MTX	100	84.0	80.0
	500	96.4	94.0
	1000	92.7	91.0

Table 3
Comparison of the two methods for the analysis of MTX in the urine samples of patients treated with MTX.

Real sample #	MTX level in urine		t-value[b]	F-value
	Given method[a]	Reference method		
1	286.6 ± 32.1 ng/mL	300 ± 26.4 ng/mL	2.9	1.4
2	124.3 ± 21.8 ng/mL	122.6 ± 14.0 μg/mL	2.9	2.3

[a] The found values are average of three determinations ± standard deviation.
[b] Critical t-value and F-value at.%95 confidence level is 4.30 and 19.00, respectively. Experimental condition for reference method; Mobile phase: phosphate buffer (pH = 3.6): acetonitrile (89:11, *v*/v); flow rate = 1 mL/min; λ = 375 nm.

3.14. Selectivity

For spectrophotometric determination, it is necessary to investigate the acceptable levels of selectivity. Sample preparation is the first step to control the sensitivity and selectivity of the analytical method. Selectivity demonstrates the ability of the developed method to quantify the intended analyte among the compounds present in the sample. According to the FDA guideline, the drug-free urine sample was collected from three different sources and applied into the spectrophotometer after performing D-μ-SPE. The blank samples showed no suspected interferences at absorbance area of the intended analyte and/or additional compounds did not have UV absorption at that wavelength. It means that routine constituents of extracted residual had no interferes with the analysis performed. So, the proposed method differentiates the target analyte in the presence of potential interferences.

Selectivity of the method was ensured by adding 100 ng/mL of different probable co-administrated drugs (*e.g.,*vancomycin, morphine, clonazepam, aspirin, and and cyclosporine) and most routinely administrated drugs such as acetaminophen and diazepam in urine samples. The acceptable tolerance of accuracy should not exceed 20%, as was observed in the present study. The obtained results confirmed that MTX could be successfully determined in real human urine samples without the interference of a variety of routine compounds such as proteins, salts, and usual co-administrated substances.

3.15. Application of D-μ-SPE procedure to real sample

In the present study, the MTX concentration in urine was determined using the validated spectrophotometric method following an intravenous administration of 1 mg/kg/dose of MTX to two patients

Table 4
Comparison of different methods for the determination of MTX.

Matrix	Matrix volume	Extraction method	Detection method	Linearity	LOD	Validation	Ref.
Plasma	100 μL	On line SPE	HPLC/UV	50–500 ng/mL	–	Recovery Accuracy Sensitivity	[3]
Serum	–	–	CV	7.0×10^{-7}–1.0×10^{-4} mol/L	70 nM	Recovery Accuracy Sensitivity	[5]
Plasma	–	LLE	HPLC/UV	–	0.01 μM	Recovery Precision Accuracy Sensitivity	[11]
Serum	800 μL	SPE	HPLC	0.025–5.00 mM	0.003 mM	Recovery Precision Accuracy Sensitivity Selectivity	[6]
Serum	1 mL	–	CV	5 nM– 0.2 μM	1.7 nM	Recovery Selectivity Stability	[14]
Serum and urine	–	SPE	Fluorimetry	4×10^{-9}-5×10^{-7} g/mL	8.2×10^{-10} g/mL	Recovery Sensitivity Selectivity	[15]
Serum and urine	50–100 μL	–	Fluorimetry	0.02–10 μg/mL	0.015 μg/mL	Recovery Precision Accuracy Specificity	[16]
Urine	10 mL	D-SPE	UPLC-ESI-MS/MS	0.25–12.5 ng/mL	0.04 μg/L	Recovery Precision Accuracy Sensitivity	[19]
Urine	5 mL	SPE	HPLC-MS/MS	0.5–16 μg/L	0.2 μg/L	Accuracy Precision Accuracy	[47]
Plasma	1 mL	LLE	LC-MS/MS	0.05–25.0 μmol/L	LOQ 0.05 μmol/L	Recovery Precision Accuracy Specificity Stability	[48]
Blood	5 mL	–	CV	0.02–100 μM	10 nM	Selectivity Sensitivity Stability	[49]
Saliva	20 μL	LLE	LC-MS/MS	2–2000 ng/mL	1 ng/mL	Recovery Precision Accuracy Stability Selectivity	[50]
Plasma and saliva	1 mL and 10 μL	SPE	HPLC/UV	0.2–500 nmol/L	0.1 nmol/L	Recovery Specificity	[51]
Urine	250 μL	SPE	HPLC/UV	5–1000 ng/mL	2.5 ng/mL	Recovery Precision Accuracy Sensitivity	[52]
Plasma	1 mL	SPE	CE	0.1–10 μM	0.05 μM	Recovery Precision Accuracy	[53]
Urine	0.5 mL	D-μ-SPE	Spectrophotometry	10–1000 ng/mL	7 ng/mL	Precision Accuracy Recovery Selectivity	This work

with acute lymphoblastic leukemia. To evaluate the application of the present method, two urine samples were taken from patients under MTX therapy and then analyzed by the optimum D-μ-SPE condition. Table 3 presents the patient urine MTX levels for both samples. The accuracy of the method was confirmed by HPLC measurements. Comparing the results obtained using the current and the reference methods by statistical analysis of the using Student's *t*-test and variance ratio F-test showed no statistically significant difference between the performance of the two methods regarding the accuracy and precision, respectively.

3.16. Comparison of the proposed method with others

The performance of the developed D-μ-SPE/spectrophotometric method is comparable with other techniques from the viewpoints of a straightforward technique, LLOQ, apparatus, and validation tests. MTX determination in biological samples has been addressed several times and shown a considerable success in covering the therapeutic window. For a number of MTX determination LC methods cited in Table 4, sophisticated detection systems such as MS or fluorescence were used that are not easily available in routine bioanalytical laboratories. The present method provided comparable LLOQ (10 ng/mL) in comparison with such sophisticated detectors.

In addition, the sample preparation step in most of the methods is performed by tedious and time-consuming SPE or LLE techniques. The present D-μ-SPE method is an improved form of extraction in terms of sensitivity, rapidity, and facility to fix the limitation of both LLE and SPE methods to a great extent. By applying this method, one of the significant limitations of LLE methods (*i.e.,* having a clean extraction phase) was completely bypassed.

Prepared carbon-based nanosorbent, IL coated magnetic GO/PPy, presents favorable properties owing to its unique architecture. GO planner morphology provides an exclusive surface – because carbon atoms are in contact with the surrounding matrix – and enables both surfaces of it to be available for extraction. Furthermore, the molecular docking technique was adapted to explain the adsorption mechanisms of MTX toward sorbent. So, it confirms better performance in terms of spontaneously and fast adsorption equilibrium procedure. Less consumption of organic solvents, no use of water-immiscible hazardous solvents, and excellent purification ability compared to traditional LLE and SPE sample preparation are the advantages of this method.

About the analytical device, the spectrophotometer is superior to other state-of-the-art techniques in terms of analysis time, flexibility, and chemical consumption. When a mass spectrometer is utilized as the LC detector (LC-MS), the buffer system must be volatile, suggesting that the most popular buffer for LC work, such as phosphate, cannot be used.

Finally, most of the methods have insufficient validation tests, which lead to some problem such as false positive results. As biological matrices have enough complexity and interference problems, the present method afforded co-extraction and co-elution interfering very well and selectivity tests were adequate. The obtained results confirmed that MTX could be successfully determined in real human urine samples without the interference of a variety of routine compounds such as proteins, salts, and the usual co-administrated substances.

4. Conclusion

MTX is the only antimetabolite for which therapeutic drug monitoring is recommended. The IL coated magnetic GO/PPy was used as the sorbent and the method was successfully applied to the separation and preconcentration of MTX from urine matrices. The present D-μ-SPE is a promising technique by which extraction is performed by nanosheets. These carbon-based nanosorbents hold a special place because of their unique architecture. IL coated magnetic GO/PPy sorbent possesses ultra-large surface area and abundant delocalized π-electron system, therefore, could be used to extracts MTX mostly ***via*** strong π–π interactions, van der Waals interactions, and hydrogen bonding. In addition, the interaction mechanism and adsorption capacity of sorbent toward MTX was successfully illuminated with computational simulation. The extracted analyte was quantified by the spectrophotometer and the results were compared by HPLC. Less sample treatment time, reduced hazardous and water-immiscible solvents consumption, sensitivity, and minimal transferring steps are most highlighted points in the use of present D-μ-SPE. The limit of sensitivity is superior to or comparable with that of other listed methods, especially those coupled with sophisticated detectors. This method is rapid, easy to operate, and importantly well suited to handle biological matrices, urine for TDM, and pharmacokinetic studies.

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