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Development of AgNPs-tragacanth Conjugated Gel as a Novel Green Membrane in Electro-membrane Extraction: Tenofovir Disoproxil Fumarate Assay in Human Plasma Samples

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AgNPs-tragacanth conjugate gel as an eco-friendly and low cost membrane was applied for the effective electromembrane extraction (EME) of tenofovir disoproxil fumarate, an important acidic and high polar (logp = 1.25) antiviral drug, followed by HPLC-UV determination. The effect of various parameters on the extraction efficiency including the presence of AgNPs, tragacanth gum concentration, gel thickness, pH values of the donor and acceptor phases, applied voltage, extraction time and agitation rate were investigated and optimized. The best extraction efficiency was obtained at 2.5% w/v of tragacanth gum, 4.0 mm gel thickness, donor phase pH = 7.0, acceptor phase pH = 8.0, applied voltage: 30 V, extraction time: 15 min and agitation rate: 500 rpm. During the method validation, under the optimized conditions, a good linearity dynamic range (LDR), ranging in 10-750 ng ml^{-1}, with coefficient of determination (R^2) = 0.999 was obtained. Limit of detection (LOD) and limit of quantitation (LOQ) were estimated to be 5.55 ng ml^{-1} and 10 ng ml^{-1}, respectively. According to the verified results, the RSD values for intra and inter-day precisions were in the range of 0.53-10.04% and the relative error (RE%) ranged between -2.31 and 8.20%. Finally the applicability of this method in real samples was confirmed by an acceptable performance in extraction and determination of TDF in human plasma samples.

Abbreviations used: AgNPs, Ag nanoparticles; HPV, Hepatitis B virus; ART, antiretroviral therapy; HBsAg, Hepatitis B surface antigen; HIV, Human immunodeficiency virus; TDM, Therapeutic drug monitoring; SPE, Solid phase extraction; LLE, Liquid-liquid extraction; SPME, Solid phase micro extraction; LPME, Liquid phase microextraction; EME, Electro-membrane extraction; SLM, Supported liquid membrane; PPT, Protein precipitation; HPLC, High performance liquid chromatography; ATR, Attenuated total reflection; SD, Standard deviation; LOD, Limit of detection; LDR, Linear dynamic range; FESEM, Field emission scanning electron microscope; TDF, Tenofovir disproxil fumarate.

Keywords: Electro-membrane extraction, AgNPs-Tragacanth, Green method, Tenofovir disproxil fumarate, HPLC-UV

INTRODUCTION

Hepatitis B infection caused by the hepatitis B virus (HBV) is a major, common problem eventually leading to liver cirrhosis and cancer [1]. The HBV infection occurs in 5-10% of HIV-infected patients and increases the risk of death [2]. The HIV infection accelerates the progression of HBV-related liver diseases in individuals exposed to HBV and increases proportion of deaths amongst HIV mono-infected patients [3]. The global guidelines recommend initiation of antiretroviral therapy (ART) with tenofovir as the first-line treatment for all HBs Ag-positive HIV/HBV co-infected individuals. Tenofovir disoproxil fumarate (TDF), regardless of its impact on HIV, has led to significant improvements in HBV viral suppress [4]. Also, TDF in combination with other antiretroviral drugs is used to treat HIV infection and have a remarkable effect on reduction in global deaths from AIDS [5].

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The determination of antiviral drugs in biological samples (plasma and serum) can provide the means to construct pharmacokinetic profiles, pharmacodynamics and drug interaction mechanism studies, to investigate the response of therapy (positive or negative) and is a vital key factor to evaluate if target concentrations are reached as well as preventing accidental poisoning. Indeed, there is a high demand to therapeutic drug monitoring (TDM) of antiviral agents and their pharmacokinetic interaction studies to determine drug exposure during treatment; thereby, allowing for optimal dosing, reduced toxicity/drug resistance and consequently treatment management between currently-used and new antiviral agents [6].

Sample preparation/pretreatment is one of the most critical and commonly required steps to achieve higher sensitivity and selectivity in analytical determinations. In recent decades, miniaturization of traditional sample preparation approaches has motivated new ideas, leading to developing innovative solutions to solve their fundamental limitations for application in various matrices/conditions. For instance, classical sample preparation techniques such as solid phase extraction (SPE) and liquid-liquid extraction (LLE) have been replaced with solid phase micro extraction (SPME) and liquid-phase micro extraction (LPME) [7-10]. However, despite their excellent performances, some limitations such as high cost of the SPME commercial micro-fibers and longer extraction times in hollow fiber-based liquid phase microextraction method (HF-LPME) have enhanced the motivation to improve these conventional sample pretreatment strategies.

Electro-membrane extraction (EME) was initially developed by Pedersen-Bjergaard and Rasmussen [11] in 2006 as an alternative sample clean-up approach to tackle the drawbacks of the above-mentioned conventional techniques. In EME, the target analyte(s) are migrated from a donor phase (DP), through an organic solvent immobilized as a supported liquid membrane (SLM), into an acceptor phase (AP) located in the lumen of a hollow fiber. For the effective migration, the analyte(s) must be in the charged state. As an important principle of EME, pH in the sample and acceptor solutions (phases) should be adjusted to basic conditions for acidic analytes and *vice versa*, to acidic conditions for basic samples to ensure that the analytes are charged. EME is a promising and efficient sample clean-up/preparation concept for selective extraction of biological and pharmaceutical compounds from their complex matrices [12-16]. Due to having the unique properties of EME technique, the most focused application of this method has been extensively reported for the extraction of drug substances and much effort has been made on improving the concept of EME for efficient pretreatment of pharmaceutical analytes from their complicated matrices. Even though EME has become a popular technique, because of low consumption of organic solvents, extending its solvent-free platform through elimination of even low aliquots of organic solvents is promising. For instance, Nojavan *et al*. [17] have reported a simple and solvent-free EME setup for the simultaneous extraction of basic drugs with a wide polarity window using agarose gel as a membrane without any organic solvent usage. Also, polyacrylamide gel, as another new membrane in electro-membrane extraction of model basic drugs, was introduced by the same research group, which these new membranes offer the advantages of low-cost and fabrication simplicity [18]. Besides, the use of these materials as the ultra-safe membranes makes the EME method more environmentally-friendly.

So far, a few sample pretreatment approaches including SPE [19-21], LLE [22] and protein precipitation (PPT) [23] have been proposed for the extraction/isolation of TDF from plasma and other biological matrices. A thorough survey of literature clearly shows that the application of EME and/or any other novel clean-up techniques for the extraction of TDF has not been studied yet. Considering the excellent efficiency of the EME technique, for the first time, we utilized an improved, cheaper and easier solvent-free electro-membrane extraction approach as a novel environmentally-friendly platform for the extraction and determination of TDF in a complicated biological matrix. For this purpose, tragacanth (katira) containing silver nanoparticles (tragacanth gel-AgNPs conjugate gel; by immobilizing the Ag nanoparticles on its surface and structure), without using any organic solvent or reagent, were used as a green membrane gel for the selective extraction of TDF from human plasma samples. Tragacanth is derived from several Astragalus herb genus/species widely growing in the Middle East and in particular in IRAN. The most fractions of tragacanth are soluble in

water, however, it contains some compounds termed as Bassorin which have the capacity to swell and form a gel when exposure to water (though insoluble in water). Accordingly, and considering the prior reports on superior impact of silver nanoparticles in improving the mass transfer and enhancement of the extraction performance of EME [24], tragacanth were prepared in aqueous silver nanoparticles medium (synthesized via a chemical reduction method). Next, the polar TDF (logP, 1.25) as an acidic model drug analyte (pKa = 3.75) was successfully extracted using the designed membrane (AgNPs-tragacanth conjugate gel) followed by determination with HPLC. More importantly, this new EME approach uses neither organic solvent nor carrier agents for the extraction of the target analyte. Moreover, both of the AP and DP solutions are aqueous and the procedures for the membrane fabrication/shaping as well as its thickness determination are quite facile. The proposed method provides a green alternative to traditional EME methods, since no hazardous and/or expensive material was used in the extraction methodology. To evaluate the advantages and disadvantages of the proposed method for determination of TDF levels, a comparison with other reported HPLC-based techniques [25-30], including LOD, LOQ, linear range, matrix type, sample treatment and sample stacking method is presented in Table 1.

EXPERIMENTAL

Reagents and Materials

The standard of tenofovir disoproxil fumarate was kindly donated by Bakhtar Biochemistry Pharmaceutical Co (BBpharmaco, Kermanshah, Iran). Silver nitrate ($AgNO_3$, 99.8%) and sodium borohydride ($NaBH_4$, 98.0%) were purchased from Merck (Darmstadt, Germany). Tragacanth (natural gum) was acquired from a local medicinal herbs market (West Azarbaijan province, Mahabad, Iran). All HPLC grade solvents including water and methanol were obtained from Daejung-Chemical and Metals Co. Ltd. (Kyeonggi-Do, South Korea). All compounds, reagents and solvents were in analytical grade or of the highest and most available purity. Deionized water was used for all sample preparations. The pH adjustments were performed using HCl (0.1 M) and NaOH (0.1 M) solutions.

The stock solution of TDF (1000 mg l^{-1}) was prepared by dissolving a required amount of standard substance in HPLC grade water and stored at 4 °C wrapped in an aluminum paper sheet and used within 1 month. All required standard solutions were prepared daily by diluting an appropriate amount of each stock solution with HPLC grade water to the required concentrations in the range of 10-500000 ng ml^{-1}.

Chromatographic Condition

All chromatographic separation and measurements were performed using an Agilent 1200 series HPLC system (Agilent Technologies, CA, and USA) equipped with an online vacuum degasser unit, quaternary pump, auto-sampler and UV detector. The analytical column used was XDB-C18 (15 cm × 0.46 cm i.d., 5 μm, Agilent, USA) connected with a guard column (4.6 cm × 12.5 cm i.d., 5 μm) to protect the analytic column. The ChemStation (Chem32) software was applied for the instrument control and data acquisition/processing. The mobile phase was 60:40 (V/V) methanol/water, and the injection volume was 10 μl. The flow rate was 1.0 ml min^{-1}. The detection and reference wavelengths were set as 254 nm.

Preparation of Tragacanth Gel and AgNPs-Tragacanth Conjugate Gel

Tragacanth gel was prepared by dissolving tragacanth powder in boiling DI water (2.5% w/v) followed by shaking it for several times until a clear solution was observed and stored at room temperature for 24 h. The resultant tragacanth gel was used for primary tests.

The AgNPs-tragacanth conjugate gel was prepared according to the following steps: First, AgNPs were synthesized by the chemical reduction of metal salt precursor (silver nitrate, $AgNO_3$) in aqueous solution with $NaBH_4$. For this purpose, 0.0038 g of $NaBH_4$ was dissolved in 50 ml DI water. Separately, 50 ml $AgNO_3$ solution (2×10^{-4} M) was prepared by dissolving 0.0017 g $AgNO_3$ in 50 ml DI water. AgNPs were produced by a drop-wise addition of the $AgNO_3$ solution into the $NaBH_4$ solution followed by stirring for 1 h at ambient conditions. Second, the milled tragacanth powder was dispersed in the synthesized AgNPs suspension with a concentration of 2.5% (w/v) to immobilize the silver nanoparticles in the

Table 1. Proposed Method for Determination of TDF, a Comparison with other Reported Methods

Analytical method	Sample preparation	Matrix	LOD (ng ml^{-1})	LDR (ng ml^{-1})	Ref.
HPLC-UV	LLE	Tablet dosage form	427.7	27000-162000	[25]
HPLC-UV	LLE	Tablet dosage form	30	100-150	[26]
HPLC-UV	LLE	Tablet dosage form	2.18	42-126	[27]
HPLC-UV	MSPE	Tablet dosage form	0.67	4-400	[28]
HPLC-UV	LLE	Human plasma	No reported	5-1000	[28]
LC-MS/MS	Protein precipitation	Human plasma	No reported	0.4 -40	[29]
LC-MS/MS	SPE	Human plasma and spinal fluid	No reported	0.5-500 0.1-50	[30]
HPLC-UV	Proposed EME in this work	Human plasma	5.55	10-750	This work

surface and pores of the gel network and kept for 24 h at room temperature until the gelation completed and the homogenized gel was formed. Finally, the fabricated yellowish AgNPs-tragacanth gel was stored at room temperature being protected from light and used as a membrane for the electro-membrane extraction of the target analyte.

Proposed EME Procedure

The setup and equipment used for the proposed EME procedure as well as overall extraction steps are illustrated in Fig. 1. The DC power supply used was a PV-600 model (Mobtaker Aryaei J., Zanjan, IRAN) with programmable voltage in the range of 0-600 V and maximum current output of 500 mA. Two platinum wires with a diameter of 0.2 mm were used as electrodes in the sample and acceptor solutions, and were connected to the power supply. The sample solution (5 ml with pH = 7.0) containing TDF was introduced into a 6 ml glass vial as a donor compartment. The Eppendorf containing 50 µl of aqueous AP (pH = 8.0) and 4 mm thickness of AgNPs-tragacanth gel in the bottom were inserted in the EME sample vial. The positive electrode was placed in the AP and the negative electrode was immersed in the sample solution. During the extraction, the sample solution was agitated under a stirring rate of 500 rpm and an applied voltage of 30 V for 15 min. Finally, after the extraction was completed, the voltage was cut-off and the AP was carefully collected using an HPLC micro-syringe and subsequently injected into the HPLC for further analysis.

RESULTS AND DISCUSSION

Characterization of AgNPs-tragacanth Gel

To demonstrate the morphological structure and

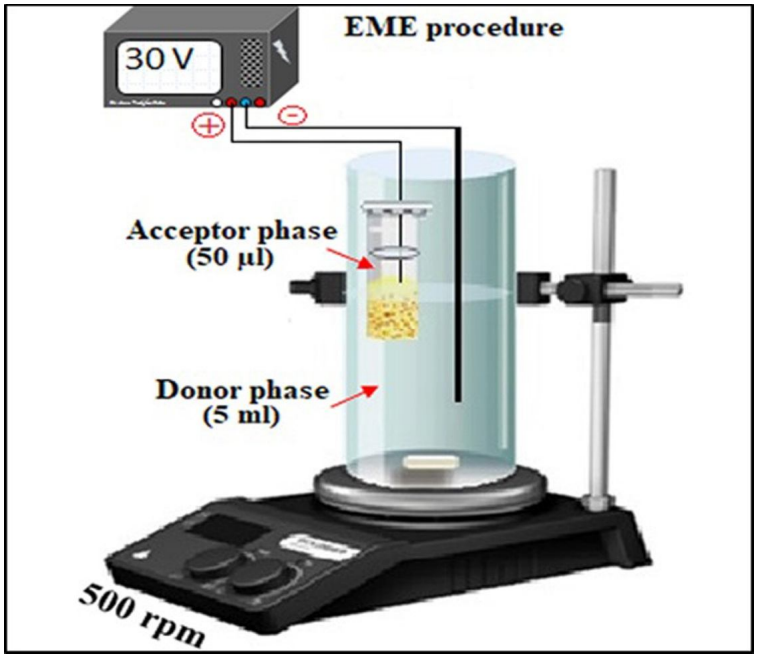

Fig. 1. Schematic representation of the proposed EME procedure for the determination of TDF. Extraction conditions: concentration of TDF in water: 300 ng ml^{-1}, sample volume: 5.0 ml, acceptor solution volume: 50 µl, applied voltage: 30 V, pH of sample solution: 7.0, pH of acceptor solution: 8.0, extraction time: 15 min, agitation speed: 500 rpm, gel thickness: 4.0 mm, concentration of tragacanth: 2.5% W/V.

porosity of the tragacanth gel and also to exhibit the presence of silver nanoparticles within the prepared membrane gel network, FESEM images were captured. For doing so, a piece (approximately 1 × 1 cm) of the both prepared samples (tragacanth gel and tragacanth-AgNPs conjugate gel; see section 2.3) were manually cut and allowed to dry for about 48 h prior to measurement. The recorded FESEM images are shown in Fig. 2A. A look at the recorded micrographs reveals not only the high porosity of the prepared gel (which can facilitate the transportation of the target analyte from the DP to AP) but also the presence of AgNPs embedded in the structure and porosity of the gel (Fig. 2B). The arrowed portions of the FESEM image in Fig.2B show the presence of spherical AgNPs with an average size of about 20 nm which are well distributed/immobilized within the membrane. Therefore, morphological analyses offer a strong evidence for the formation of tragacanth-AgNPs conjugate gel.

Effect of Silver Nanoparticles on the Extraction Performance of TDF

Due to the unique and special physicochemical properties of silver nanoparticles, their effect on the extraction of TDF was investigated using tragacanth gel as a novel supported membrane in EME. So, in the present work, electro-membrane extraction of the target analyte was evaluated on three concentration levels of TDF (10, 300 and 750 ng ml^{-1}) under the optimized conditions (using tragacanth gel). Next, the extraction procedure was carried out according to the described procedure using AgNPs-tragacanth conjugate gel as a membrane (Fig. 3A), and finally all the extracted samples were subjected to the developed HPLC procedure. Following that, the extraction recoveries (ER%) were calculated; the obtained results clearly showed that the extraction of analyte into the AP (extraction recovery of TDF) increased up to 20% when AgNPs-tragacanth conjugate gel was used as a membrane.

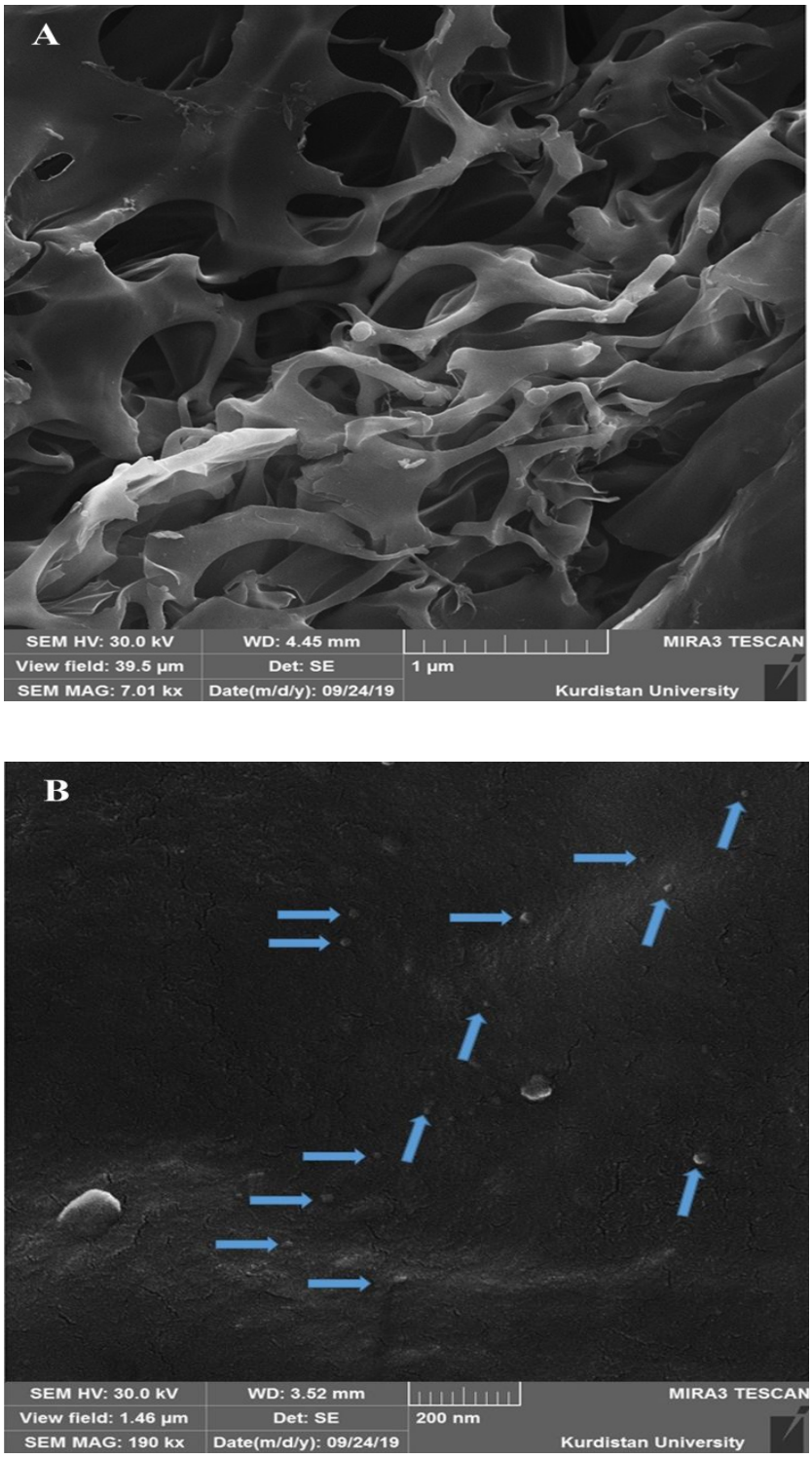

Fig. 2. FESEM of AgNPs-tragacanth conjugate gel, (A**)** the high porosity of the AgNPs tragacanth gel and (B) AgNPs embedded in tragacanth.

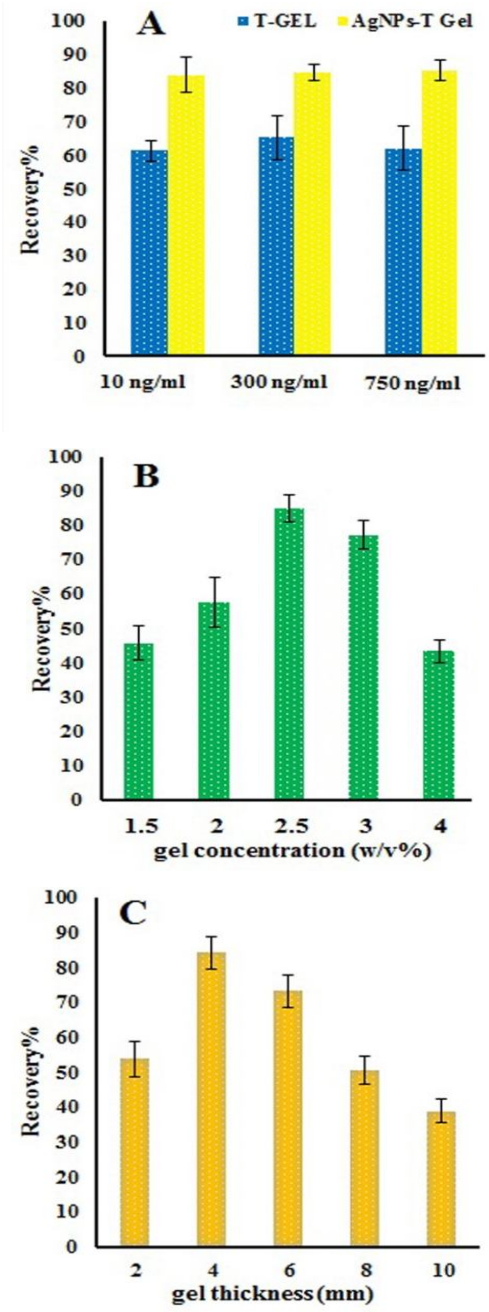

Fig. 3. (A) Effect of Ag NPs on the extraction efficiency of TDF. Extraction conditions: concentration of TDF in water: 300 ng ml^{-1}, sample volume: 5.0 ml, acceptor solution volume: 50 µl, applied voltage: 30 V, pH of sample solution: 7.0, pH of acceptor solution: 8.0, extraction time: 15 min, agitation speed: 500 rpm, gel thickness: 4.0 mm, concentration of tragacanth: 2.5% w/v. (B) Effect of tragacanth content on the gel stability and extraction of TDF; extraction conditions: concentration of TDF in water: 300 ng ml^{-1}, sample volume: 5 ml, acceptor solution volume: 50 µl, the others were the same as given in Fig. 3A. (C) Effect of AgNPs-tragacanth conjugated gel thickness on the TDF extraction by the proposed method. Extraction conditions: concentration of TDF in water: 300 ng ml^{-1}, sample volume: 5 ml, acceptor solution volume: 50 µl, the others parameters were the same as given in Fig. 3A.

This is probably due to the presence of AgNPs in the structure of the tragacanth gel which could improve the homogeneity of electric field across the gel surface, which in turn, may enhance the stability of gel and ameliorate the electric field strength (under the high voltages) during the extraction process and consequently increase the extraction performance. Furthermore, the presence of AgNPs in the gel pores results in a more effective surface area and enhances overall partition coefficient of the target compound; hence, the transportation of the analyte over the gel will be improved [24]. Indeed, metallic nanoparticles have superior advantageous which modification of their surface with appropriate functional groups would allow them to selective and specific binding to certain analytes. Moreover, in-situ fabrication of metallic nanoparticles is a facile process, whereas, other common supports such as hollow fibers and flat sheet membranes need the commercial production lines.

Effect of Tragacanth Content on Gel stability and TDF Extraction

To achieve the maximum extraction efficiency, AgNPs-tragacanth conjugate gels were prepared at different concentrations of tragacanth ranged in 1.5-4.0% w/v and the target analyte was extracted at the optimized conditions using the fabricated gels. During the extraction, the gel prepared at low concentration of 1.5% (w/v) was not stable, which led to destroying and leakage into the sample vial under stirring due to its low viscosity. So, the initial tragacanth gel concentration was gradually increased and the obtained results (Fig. 3B) revealed that the stability of the gel as well as the extraction performance have improved in the concentration of 2.5% (w/v). At higher concentrations (3.0-4.0%), the extraction efficiency (the signal intensity) deceased probably due to the fact that the density of the membrane (gel) and consequently the lower pore sizes at these concentrations can affect the electro-migration and transportation of the analyte ions from the sample solution into the AP. Therefore, 2.5% (w/v) of tragacanth gel was selected as the optimal gel concentration for the membrane fabrication in the further experiments.

Effect of AgNPs-tragacanth conjugate gel thickness. Gel thickness plays a remarkable role in achieving higher and appropriate extraction performances of the system. Therefore, selection of a proper thickness of membrane is very crucial to optimize the extraction procedure. Hence, the effect of membrane thickness on extraction efficiency of TDF was investigated by varying the membrane (gel) thickness in the range of 2-10 mm. Various thicknesses of the membrane gel were tested by variation the volume of the gel in the Eppendorf tube and extraction recoveries were calculated at these conditions. As displayed in Fig. 3C, the maximum extraction performance was achieved at a gel thickness of 4 mm, wheres the recoveries and the extraction efficiencies decreased significantly when the AgNPs-tragacanth gel thickness was higher than 4 mm. It should be noted that the extracction recovery decresed when AgNPs-tragacanth gel with 2 mm in thickness was used as a membrane due to the instability of the gel and leakage into the sample during extraction. At higher thicknesses of gel, the analyte ions are trapeed in the membrane pores which inhibits the analyte comes into the AP from the DP and diminishes the extraction effecincy. Therefore, 4 mm was selected as the optimal membrane thickness for the extraction of TDF using the developed EME technique for the further experiments.

Effect of the pH values of donor and acceptor phases. As mentioned earlier and as a main principle of EME technique, the analyte(s) should be in the ionic form(s) to achive the maximum electrokinetic migration into the AP in presence of an electrical field. By adjusting the pH values in the sample and acceptor solutions (phases) the ionization of target compound(s) could take place. So, the pH adjustments have a significant impact on the electro extraction of a target analyte and achieving the maximum extraction efficiency. Reasonably, in this study, the influence of pH of donor and acceptor solutions on extraction recovery of the target analyte was evaluated. Considering the fact that TDF is an acidic drug (pKa = 3.75), the pH value of AP should be high enough (basic) to maintain the target analyte in the anionic form to migrate toward the positive electrode under the predetermined voltage. Therefore, the pH of AP was kept constant at 8.0 while the pH of DP was changed within the range of 5.0-10.0 and the extraction of TDF was performed. As shown in Fig. 4, the results of TDF determination and calculated recoveries indicated that with increasing the pH of sample solution up to 7.0, the extraction performance of the target drug increased significantly and the maximum

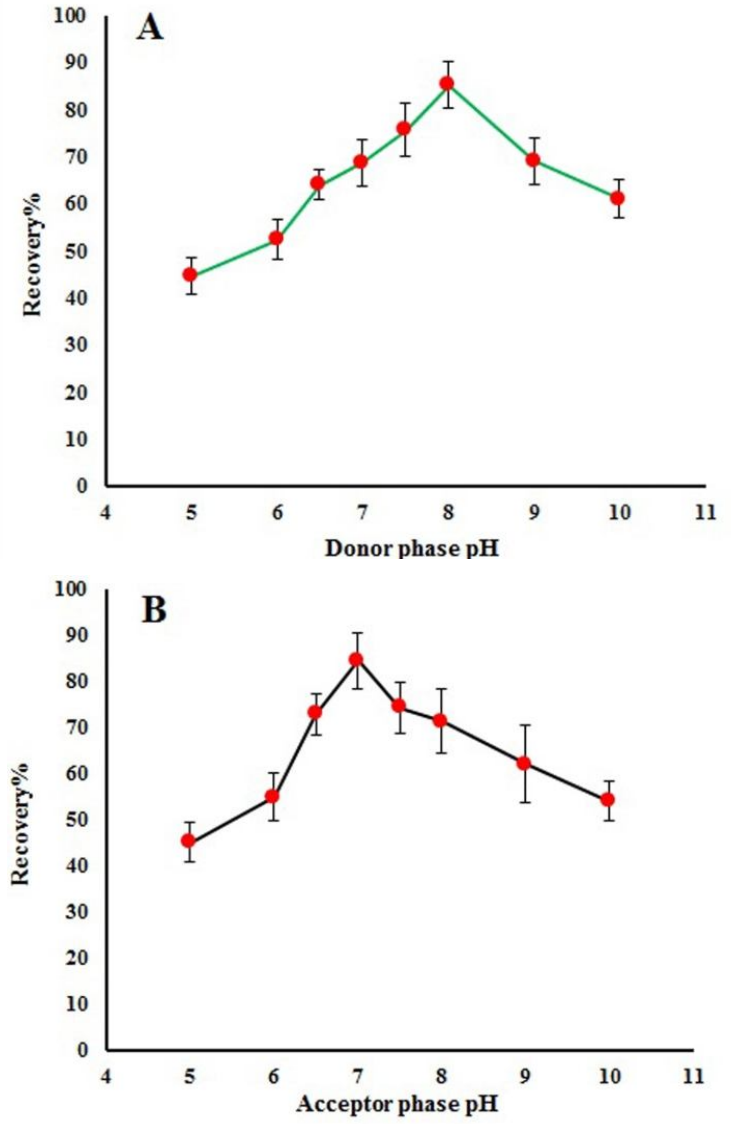

Fig. 4. (A) Effect of donor phases pH on the TDF extraction. Extraction conditions: concentration of TDF in water: 300 ng ml^{-1}, sample volume: 5 ml, acceptor solution volume: 50 µl, applied voltage: 30 v, pH of acceptor solution: 8, extraction time: 15 min, agitation speed: 500 rpm, thickness gel: 4 mm, concentration of tragacanth: 2.5% w/v. (B) Effect of the acceptor phases pH values on the TDF extraction. Extraction conditions: concentration of TDF in water: 300 ng mL^{-1}, sample volume: 5mL, acceptor solution volume: 50 µl, the others were the same as given in Fig. 3A.

recovery were obtained at this value. This is due to the fact that TDF is completely charged at pH values equal to 7.0 and greater. On the other hand, the recoveries of the analyte decreased at pH > 7.0 values. Probably, relative enhancing of the ion balance under these conditions as well as hydroxide ion competition with analyte ion in electro-migration could impress the extraction flux over the membrane and are responsible for the observed decrease in the extraction performances. Also, the pH value of the AP plays a vital role in the proposed EME method because

of the critical need to keep the charged state of the extracted analyte during electro-migration to guarantee the successful extraction. Accordingly and considering the acidity of the drug, the impact of different pH values in the AP (within the range of 5.0-10.0) was examined while the pH of DP was kept constant at 7.0. Figure 4B clearly reveals that the extraction recovery gradually increased by increasing the pH of AP and reached its maximum percentage at pH = 8.0. The reduced extraction recoveries at lower pH values of AP might be due to the back-extraction of the analyte into the sample solution as a result of analyte protonation and converting to naturalized form, whereas, at higher pH values of AP the target drug is completely in its ionized form which in turn may enhance the migration of analyte toward the negative electrode (anode) and prevent it from being back-extracted. Moreover, at pH > 8.0 values, bubble formation during the extraction and an increase in the volume of the AP as a result of high concentration of OH^- ion make the extraction system instable, thereby decreasing extraction recoveries of the analyte [31]. Thus, the pH(s) of 7.0 and 8.0 in the DP and AP were selected as proper values for the further experiments, respectively.

Effect of the Applied Voltage

In EME, applied voltage and the electrokinetic migration are two major factors for migration and transportation of analyte from the DP to the AP due to the significant dependence of mass transfer to the electrical field. Therefore, applied voltage has an extreme and vital impact on the extraction recovery. To investigate and determine the most appropriate voltage, a series of extraction experiments were performed by applying various potentials in the range of 0-60 V for 15 min. Figure 5A demonstrates that in the absence of electrical field, the migration of analyte from the DP to the AP does not take place and thus the extraction of the analyte is almost null. By increasing the applied voltage from 0 up to 30 V, the extraction efficiency increased, whereas a decrease in extraction recovery was observed at the voltages above this value. This decrease in the performance of the proposed extraction method (by further increase in applied voltage from 30 to 60 V) could be explained considering two main reasons. The first reason is the water electrolysis at higher voltages during extraction according to the following reactions:

$$DP: 2H_2O + 2e^- \rightarrow H_2 + 2OH^- \text{ (cathode-reduction)} \quad (1)$$

$$AP: 2OH^- \rightarrow \tfrac{1}{2} O_2 + H_2O + 2e^- \text{ (anode-oxidation)} \quad (2)$$

This phenomenon could negatively affect the electro-migration of the analyte(s) through formation of bubbles at the electrodes, leading to instability in the migration of the analyte [32]. Also, changing the pH of the AP during the extraction due to electrolysis reactions will cause a decrease in the extraction performance. The second factor is an increase in the volume of the AP at higher voltages as a result of EOF effect which can cause a decrease in the extraction recovery. It should be noted that at lower voltages the EOF phenomenon has a negligible effect on the volume of the AP. Therefore, based on the obtained results, 30 V was set as the optimal voltage for the subsequent experiments to achieve an efficient EME efficiency.

Effect of Extraction Ttime

Extraction time at a constant voltage magnitude has a significant effect on the quantitative migration of the target compound(s) from DP into the AP [33]. Also, much shorter time in sample preparation step is considered as one of the main advantages for sample analysis. Accordingly, extraction time is another operational key parameter in EME that has to be evaluated thoroughly for achieving maximum extraction performance. To establish extraction-time profile of the target analyte, the effect of extraction time on the efficiency of the proposed EME method was studied from 5 to 30 min keeping the applied voltage at 30 V. As displayed in Fig 5B, by increasing the extraction time up to 15 min, the extraction performance increased and reached its highest value. However, at extraction times longer than 15 min, a slight decrease in extraction efficiency was observed probably due to the instability and destroying the membrane gel at such longer times. Also, bubble formation at electrodes and pH change in AP as a result of electrolysis reaction (at times beyond 15 min) cause a decrease in the extraction efficiency. Hence, 15 min was employed as the optimum extraction time for the subsequent

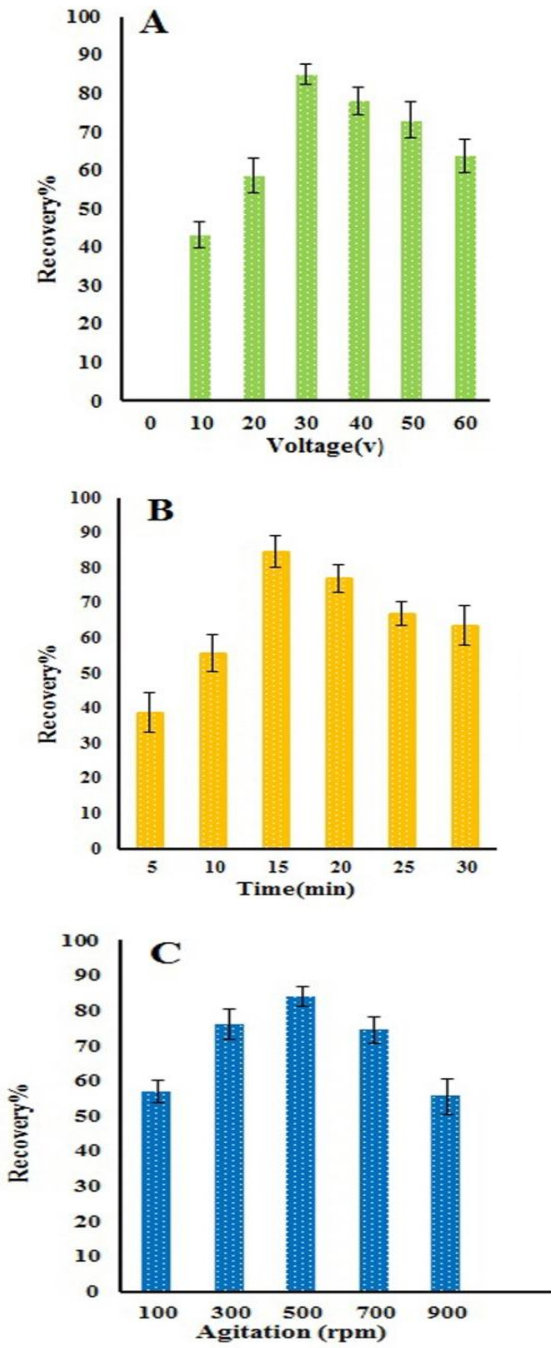

Fig. 5. (A) Effect of applied voltage; extraction conditions: concentration of TDF in water: ng ml^{-1}, sample volume: 5 ml, acceptor solution volume: 50 µl, pH of sample solution: 7, pH of acceptor solution: 8, extraction time: 15 min, agitation speed: 500 rpm, thickness gel: 4 mm, concentration of tragacanth: 2.5% w/v. (B) Effect of the extraction time. Extraction conditions: concentration of TDF in water: 300 ng ml^{-1}, sample volume: 5 ml, acceptor solution volume: 50 µl, the others were the same as given in Fig. 3A. (C) Effect of the agitation rate on the extraction of TDF. Extraction conditions: concentration of TDF in water: ng ml^{-1}, sample volume: 5 ml, acceptor solution volume: 50 µl, the others were the same as given in Fig. 3A.

experiments.

Effect of the Agitation Rate

Agitation of sample solution accelerates the mass transfer (due to the convection effect) and minimizes the time required to reach the thermodynamic equilibrium. As a final step of the optimization processes of the proposed EME procedure, the agitation speed effect on the extraction performance was investigated in the range of 100-900 rpm, keeping the other extraction parameters at the optimal values (Fig. 5C). As it is clear, the extraction efficiency of the target compound increased with increasing the agitation rate up to 500 rpm as a result of convection effect and acceleration in reaching the thermodynamic equilibrium [34], correspondingly. At higher agitation speeds, the extraction recoveries decreased which can be attributed to the formation of gas bubbles (which can destabilize the migration of the target drug) [35] as well as destroying the gel membrane during the extraction. Eventually, 500 rpm was selected as the most appropriate agitation rate for the upcoming analyses.

Method Validation

As a partial validation of the proposed method and to investigate its efficiency in the determination of TDF, analytical figures of merit including calibration linearity or linear dynamic range (LDR), coefficient of determination (R^2), LOD (limit of detection), LOQ (limit of quantification), intra- and inter-day precisions (estimated by relative standard deviation of the mean; RSD%), accuracy (estimated by relative error; RE%), extraction recovery (ER%) and enrichment factor (EF) were evaluated under the optimized conditions (all experiments were replicated three times). The obtained results were tabulated in Table 1. The calibration linearity was evaluated by plotting the mean of three calibration curves (that were constructed on three nonconsecutive days) using spiked samples treated with different concentrations of TDF which was linear over the range of 10-750 ng ml^{-1} (R^2 = 0.999). Also, LOD (5.55 ng ml^{-1}) was calculated using the following equation (Eq. (1)), where "δ" is the standard error of the intercept and "a" is the slope of the calibration curve. Also, LOQ (10 ng ml^{-1}) was reported as the lowest concentration point of the constructed calibration curve.

$$LOD = 3.3 \times (\delta/a) \quad (1)$$

To evaluate the reproducibility (n = 3) of the proposed method, inter- and intra-day precisions were examined under the optimized conditions by analyzing 3 replicates of each sample (through determination of three concentration levels of 10, 300 and 750 ng ml^{-1}) in one analytical run and one sample of each concentration in three consecutive days, respectively. As summarized in Table 2, the results demonstrated that the intra- and inter-day precisions (RSD%) of different concentrations were in the range of 0.53-10.04% which are within acceptable and valid range confirming that the developed method is precise. The accuracy of the proposed method was also assessed in the same procedure as described above and the REs (%) were found to be between satisfactory values of -2.31 and 8.20%. In addition, the EF and ER% were calculated using the following equations (Eq. (2) and Eq. (3)), respectively:

$$EF = \frac{C_a}{C_d} \quad (2)$$

$$ER\,(\%) = EF \times \frac{V_a}{V_d} \times 100 \quad (3)$$

where C_a and C_d are the concentration of analyte in the AP and within the sample, respectively. V_a and V_d are also the volumes of the acceptor (50 µl) and the donor phases (5000 µl), respectively. As seen, the calculated EF and ER (%) are both 85.0-(%) which demonstrates the suitability of the proposed EME method for the extraction of the examined compound.

Method Application and Real Sample Analysis

In order to test the applicability of the developed EME method for the extraction and determination of TDF, the analysis of real samples was performed in spiked human plasma samples under the optimized conditions. The frozen drug-free human plasma samples were provided by Iran Blood Transfusion Organization (Mahabad, IRAN) and immediately transferred to the laboratory and stored at below -20 °C until use. Prior to daily analysis, plasma samples thawed at room temperature for about 30 min and then an aliquot of 500 µl of the samples were spiked with

Table 2. Analytical Characteristics of the Proposed Method for the Determination of TDF

Evaluation parameter			Value
Calibration curve equation			y = 2.1991x + 17.206
Correlation coefficient (R^2)			0.999
Limit of detection (LOD)			5.55 a
Limit of quantification (LOQ)			10 a
Linear dynamic range (LDR)			10.0-750.0 a
Precision (repeatability) (RSD%)	Intraday	10 a	10.04
		300 a	0.64
		750 a	0.53
	Interday	10 a	9.58
		300 a	3.08
		750 a	1.47
Accuracy (Error %)	Intraday	10 a	8.21
		300 a	-2.31
		750 a	0.83
	Interday	10 a	6.92
		300 a	-0.074
		750 a	1.40
Enrichment factor			85
Extraction recovery (%)			85

a: ng ml^{-1}.

Table 3. The results of Determination of TDF in Human Plasma Samples

Sample	C_{real}	C_{added}	$C_{Found} \pm SD$
Plasma1	nd	300 a	291.8 ± 3.7
Plasma2	nd	300 a	303.3 ± 4.4
Plasma3	nd	300 a	286.8.3 ± 4.3
Plasma4	nd	300 a	309.8 ± 1.4

nd: no detectable; a: ng ml^{-1}.

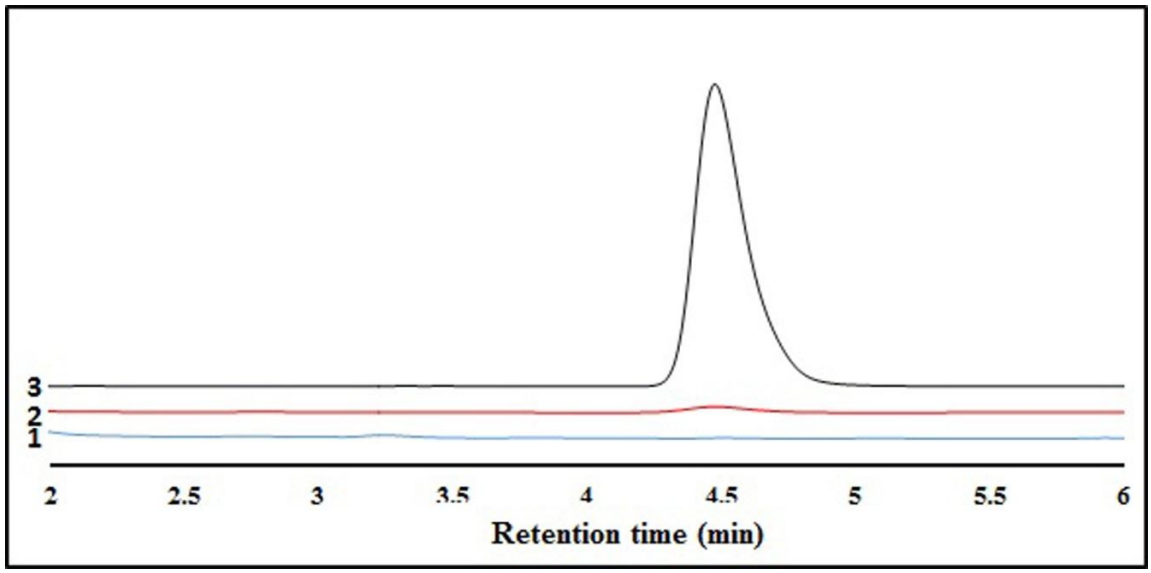

Fig. 6. Typical chromatogram for (1) plasma sample, (2) plasma sample spiked with 300 ng ml^{-1} of TDF before extraction and (3) plasma sample spiked with 300 ng ml^{-1} of TDF after extraction.

300 ng ml^{-1} level of TDF and vortex-mixed (Dragon Lab, MX-S, Connecticut, USA) for 1 min. The spiked samples were next diluted with deionized water and their pH values were adjusted to 7.0 by addition of NaOH (final volume: 5.0 ml) and finally subjected to the developed EME procedure under the optimized conditions. All experiments were replicated three times and the results obtained using the calibration curve are summarized in Table 2. Also, relative recoveries percentages (RR, %) determined using Eq. (4) were in the ranges of 95.9-103.3% (Table 3).

$$RR\,(\%) = 100 \times \frac{Found\ \ value}{No\min al\ \ value} \qquad (4)$$

The obtained typical chromatograms of the blank (drug-free) and spiked plasma sample are displayed in Fig. 6, respectively. Overall, no interference peak (arising from any significant matrix-effect) was found during analyzing the TDF in real samples indicating the reliability of the proposed method for real sample analyses of TDF.

CONCLUSIONS

The main focus of this study was on development and application of a solvent-free EME platform using AgNPs-tragacanth conjugate gel (as a membrane) for the extraction of tenofovir disoproxil fumarate (TDF) as one of the important currently-used antiviral agents. To our knowledge, this is the first report describing the application of natural materials (instead of chemical gels) as membrane in electro-membrane extraction of polar acidic compounds and represents a simple, green, low-cost and rapid assay for the preconcentration and determination (HPLC-UV) of TDF without using any organic solvent and/or carrier agents. The method validation studies revealed that under the optimized conditions, this technique provides a low limit of detection (5.55 ng ml^{-1}), good intra-and inter-day precisions (RSDs%: in the range of 0.53-10.04%), desired accuracy (REs%: between -2.31 to 8.20%) and satisfactory results in terms of enrichment factor and extraction recovery. Finally, the real sample analyses data using spiked human plasma samples demonstrated the applicability of the proposed method for the extraction and determination of the target drug without any potential matrix effect.

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