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Research Article

Development of a Nanocluster-Based Platform for Determination of Sofosbuvir

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Abstract

Background: Sofosbuvir is a potent direct-acting antivirus agent that has been listed as a promising medicine for the treatment of all genotypes of hepatitis C virus. As antiviral drugs could be metabolized to their associated compounds and toxicologically and pharmacologically interfere with the parent drugs, identifying the therapeutic range of drugs would be notable.

Methods: In the current study, copper nanoclusters (Cu NCs) are synthesized during the reduction of copper nitrate with hydrazine hydrate in a protected media and used as a nanoprobe for the determination of sofosbuvir in plasma samples. Herein, synchronous fluorescence spectroscopy (SFS) is used for monitoring of fluorescence variation of nanoprobe owing to the excessive benefits compared with the traditional fluorescence.

Results: SFS peak of Cu NCs has appeared at 355 nm with $\Delta\lambda$=80 nm which is decreased in the presence of sofosbuvir. To optimize the reaction factors, a response surface methodology is used and in the optimized conditions, a linear concentration-response plot is obtained in a range of 0.05-6.0 μg mL^{-1} with a limit of detection of 0.0147 μg mL^{-1}.

Conclusion: The developed method also reveals good repeatability and selectivity for sofosbuvir in plasma samples.

Introduction

Hepatitis C virus (HCV) responsible for chronic and acute liver infection is one of the significant worldwide health problems. The severity of this single-stranded RNA virus can be serious or mild enduring for weeks or a whole lifetime.[1] Until recently, HCV was treated by ribavirin and interferon therapy that could cause serious-lethal adverse effects and high pill burden.[2] Regarding various drawbacks of the previous therapies, more effective and tolerated therapy called direct-acting antivirus agents (DDA) has been recently established for the treatment of HCV. Sofosbuvir is a potent DDA that was listed as a promising medicine for the treatment of all genotypes of HCV. Owing to their numerous merits including high efficacy, few side effects, a high rate, and a short period of cure, sofosbuvir has been extensively adopted as an important drug for HCV treatment.[3] Sofosbuvir acts by inhibiting the NS5B nucleotide polymerase through interfering with the RNA replication process[4] and reach to maximum plasma concentration in about 0.5 to 2 h with a maximal concentration of 567 μg.L^{-1} as a therapeutic level.[5]

As antiviral drugs could be metabolized to their associated compounds and toxicologically and pharmacologically interfere with the parent drugs, identifying the therapeutic range of drugs would be notable. Furthermore, an enhancement in the efficacy of therapy and a decrease in the side effect and care costs would be occurred by knowing the concentration of drugs in the biological samples.[6,7] Therefore, developing a sensitive, selective, fast, easy, and eco-friendly method for the determination of sofosbuvir in biological fluids would be highly encouraging. So far, there are some published techniques for sofosbuvir determination alone or with other metabolites such as reverse phase high performance liquid chromatography (HPLC)-UV,[8,9] UPLC-mass spectrophotometry (MS)-MS,[10,11] thin-layer chromatography (TLC),[12-14] voltammetry[15] and spectrophotometry.[7,16-18] Despite the advantages of each method for employment in clinical courses, most of the previous techniques need more time, expensive instrumentation, extensive extraction procedures, and a high rate of solvent consumption. In this regard, developing a reproducible, stable, reliable, and fast sensor for the analytical determination of drugs has aroused massive attention by virtue of their importance in the biological samples.

Recently, nanoclusters (NCs) as a group of nanomaterial

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have ignited a surge of unprecedented interest in various fields including bioassays, sensing, and single-molecule optoelectronics due to their small particle size. The high sensitivity, stability, efficiency, easy operation, fast, and low-cost features of NCs offer proper conditions for being used in fluorescent sensing protocols.[19,20] Metal NCs especially Cu NCs have been extensively adopted as fluorescent probes for sensing due to their not only abundant, available, and inexpensive source; but also, their good fluorescence properties.[21,22] Thus, the use of Cu NCs advocates great potential for clinical applications. In the present study, a Cu NCs-based probe is developed for the determination of sofosbuvir in plasma samples. Herein, synchronous fluorescence spectroscopy (SFS) is used for monitoring of fluorescence variation of nanoprobe owing to the excessive benefits compared with traditional fluorescence. SFS is an attractive multidimensional fluorescence, simple, and sensitive technique for biomolecules quantitative determination which includes scanning by emission and excitation monochromators simultaneously.[23] The narrow-sharp spectrum obtained in SFS results in the simplification of the emission spectra, a decrease of interferences, and enhancement of the resolution and selectivity of the spectra.[24] The reaction conditions are optimized by response surface methodology (RSM) and the proposed method is fully validated for the determination of sofosbuvir in plasma samples.

Materials and Methods

Reagents and solutions

Citric acid ($C_6H_8O_7 \bullet H_2O$) and cetyltrimethyl ammonium bromide (CTAB, $C_{19}H_{42}BrN$) received from Merck and hydrazine hydrate ($H_4N_2.H_2O$) and copper nitrate ($Cu(NO_3)_2 \bullet 3H_2O$ purchased from Sigma-Aldrich in analytical grade are used as reagents for Cu NCs preparation. A solution of 1000 mg.L^{-1} of sofosbuvir (Khorshid Kadous Pharmaceutical Co., Iran) is prepared by dissolving it in a proper amount of methanol and working solutions are freshly prepared daily by diluting the stock solution with deionized water.

Apparatus and software

A FP-750 spectrofluorometer (Jasco Corp., Japan) at synchronous mode with $\Delta\lambda$=80 nm, medium sensitivity, 10 nm slit width in emission and 5 nm slit width in the excitation paths at room temperature is applied for recording the SFS spectrum. For pH adjustments, a digital pH-meter model 744 (Metrohm Ltd., Switzerland) and for characterization of nanoparticles, a CM30 Transmission electron microscopy (Philips, The Netherlands, www.philips.com) are used. Furthermore, MINITAB (Minitab Inc. Release 17.0) software is employed for ANOVA analysis and experimental design.

Synthesis of Cu NCs

The fluorescent Cu NCs are prepared by the reduction of copper nitrate using hydrazine in the presence of CTAB and citric acid.[25] For this purpose, first, 0.05 mL citric acid of 0.1 mol L^{-1} (0.5 mmol L^{-1} in 10 mL) and 0.0182 g of CTAB are inserted into the 10 mL of 2 mol L^{-1} aqueous solution of hydrazine hydrate and a colorless solution is obtained which is stirred for 30 minutes. Besides, another solution is prepared by inserting 0.05 mL citric acid of 0.1 mol L^{-1} and 0.0182 g of CTAB, and 0.0482 g of $Cu(NO_3)_2.3H_2O$ into the deionized water following the stirring process. Then, the as-prepared solutions are mixed and stirred at room temperature for 3 h. The color change to reddish-brown indicates the synthesis of Cu NCs. The prepared Cu NCs are kept at 4 °C in a dark place for usage.

Samples preparation

Human plasma samples are used for assaying the sofosbuvir. In this regard, 500 µL of human plasma sample is poured into a 2.0 mL microtubes and spiked with different concentrations of sofosbuvir. For protein precipitation, about 500 µL of acetonitrile is added to the desired plasma sample and vortexed for 2 min. The tube contents are centrifuged at 10000 rpm for 5 min and clear supernatant is transferred to a clean microtube.

General procedure

The analysis procedure is made in a 2 mL microtube by a batch method. 50 µL of 0.1 mol.L^{-1} phosphate buffer (pH 7.0) is added into the microtube containing prepared plasma (~ 800 µL obtained from the previous section) and finally, 20 µL of Cu NPs is added to it. After incubation of the sample for 10 min, the SFS spectrum is recorded by the simultaneous scanning of both λ_{ex} and λ_{em} at a constant $\Delta\lambda$ of 80 nm.

Results and Discussion

Characterization of Cu-NCs

The size and shape of the prepared NCs are characterized using TEM. According to Figure 1, the morphology of the Cu NCs is almost spherical and uniform in shape and size with an average diameter of less than 6 nm.

Detection mechanism discussion

The quenching mechanism for a SFS probe arises from two way: (i) interaction with nanoparticles surface and a change in the nature of nanoparticles which is characterized by a decrease in the intensity of the SFS peak of nanoparticles; and (ii) the inner filter effect of the analyte which is the re-absorption of excited or emitted light by a quencher.[26] In both cases, the SFS signal of nanoprobe is decreased proportionally to analyte concentration increase. In the present study, a strong SFS emission at 355 nm is achieved at a $\Delta\lambda$ of 80 nm; on the other hand, the simultaneous scanning of both λ_{ex} and λ_{em} at a constant $\Delta\lambda$ of 80 nm gives a maximum emission intensity at 355 nm which shows that maximum excitation intensity is observed in 80 nm lower than this wavelength (*i.e.* 275 nm). According to the maximum absorbance of sofosbuvir which is observed at 260 nm, the excitation spectrum of Cu NCs is completely

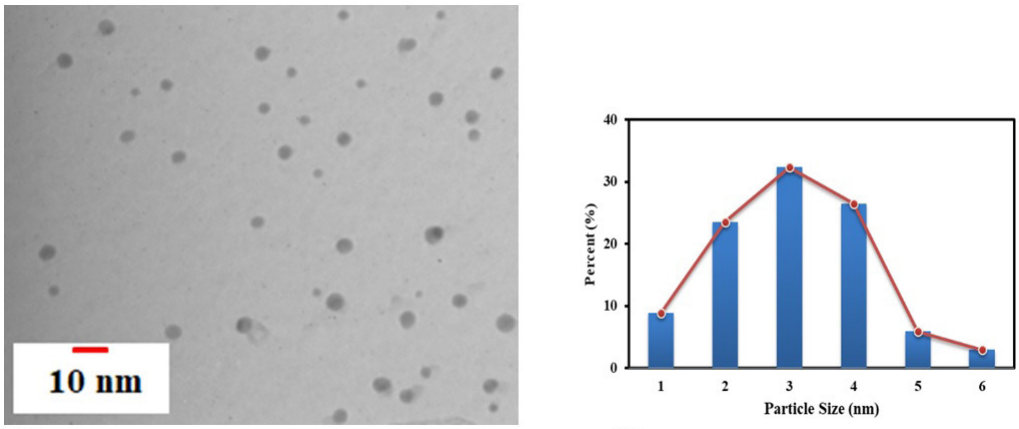

Figure 1. TEM image of the Cu NCs (A) and particle size distribution curve (B).

overlapped by the UV absorption of sofosbuvir (Figure 2) and interprets their SFS spectrum and consequently quenchs the SFS intensity of NCs. As can be seen in Figure 3, Cu NCs show a strong SFS peak centered at 355 nm with Δλ=80 that is quenched with sofosbuvir adding and shows a linear relationship in the concentration range of 0.05-6.0 μg mL^{-1}.

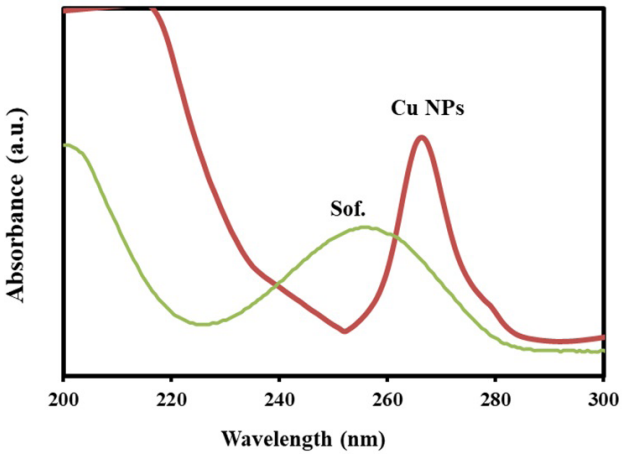

Figure 2. Absorbance spectra of the Cu NCs and sofosbuvir.

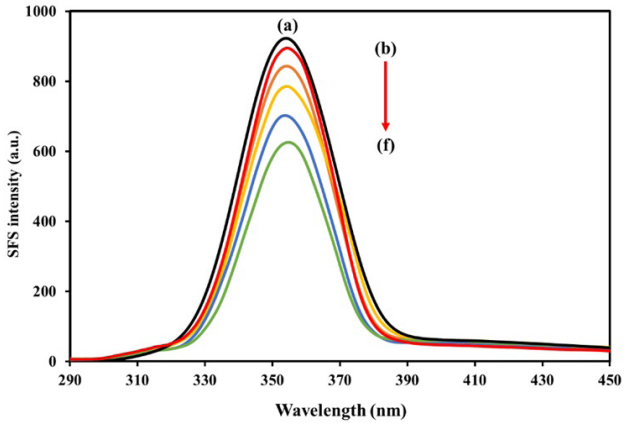

Figure 3. SFS spectra of the Cu NCs in the absence (a) and presence of sofosbuvir in the concentration range of 0.05-6.0 μg mL^{-1} (b-f). Conditions: pH 7, Cu NCs volume= 20.0 μL, [phosphate buffer]=0.005 mol L^{-1}, and time = 10 min. λ_{max}= 355 nm with Δλ=80.

Optimization of reaction conditions by an experimental design

The relation between variables and response functions is investigated by central composite design (CCD) which considers the interactions between the independent parameters. These parameters include pH, reaction time, the concentration of phosphate buffer, and the volume of Cu NCs. Five levels of +α (maximum), +1, 0 (central) -1, and −α (minimum) are established for the above-mentioned parameters. The difference between SFS intensity in the presence and absence of sofosbuvir is considered as analytical response and a plasma sample spiked with 2.0 μg.mL^{-1} sofosbuvir is used for all measurements. The corresponding tables and data are given in Supplementary Data. Each parameter effect on the response is plotted by three-dimensional (3D) graphs and shown in Figure 4 As can be seen in Figure 4a, the maximum intensity for the analytical response is observed at pH 7.0 which can be supplied with 0.05 μL of 0.1 mol.L^{-1} phosphate buffer (0.005 mol.L^{-1}). On the other hand, a better result in the response signal is obtained as the Cu NCs volume raised to 20.0 μL and increase more than this value leads to a decrease in the response due to self-quenching phenomena (Figure 4b). Furthermore, fluorescence intensity increases with an increase in time and remains relatively constant after 10 min (Figure 4c). According to these results, the optimum values are pH of 7.0, buffer phosphate concentration of 0.005 mol.L^{-1}, Cu NCs volume of 20 μL, and reaction time of 10 min. The obtained equation for the dependency of SFS response to the investigated parameters in an uncoded unit is shown as:

$$Y = -368.099 + 99.162\,X_1 + 5708.804\,X_2 + 9.459\,X_3 - 8.411\,X_1^2 - 0.108\,X_3^2 - 0.401\,X_4^2 + 1.400\,X_{14} - 276.190\,X_{23} - 1.179\,X_{24} - 0.031\,X_{34} \quad (1)$$

Where X_1, X_2, X_3, and X_4 stand for pH, buffer concentration, Cu NCs volume, and reaction time, respectively. Sign and coefficient values represent the positive/negative effect of each parameter and their interactions.

Interference study

The selectivity of the Cu NCs based nanoprobe to sofosbuvir is assessed by adding different concentrations of other over-the-counter or co-administrated drugs in a solution with a constant concentration of sofosbuvir (1.0 $\mu g.mL^{-1}$) and recording the signal variation. The results are listed in Table 1. The results demonstrate that the presence of all investigated drugs (except for caffeine) does not affect the response of the nanoprobe. Although, the tolerance limit for ibuprofen, nicotinamide, and losartan is reported to be 10 times which means 10.0 $\mu g.mL^{-1}$ for each drug. It should be noted that the therapeutic level in plasma for all of them is reported to be higher than 10 $\mu g.mL^{-1}$.[27] So it can be concluded that the developed nanoprobe has acceptable selectivity for sofosbuvir determination in plasma samples.

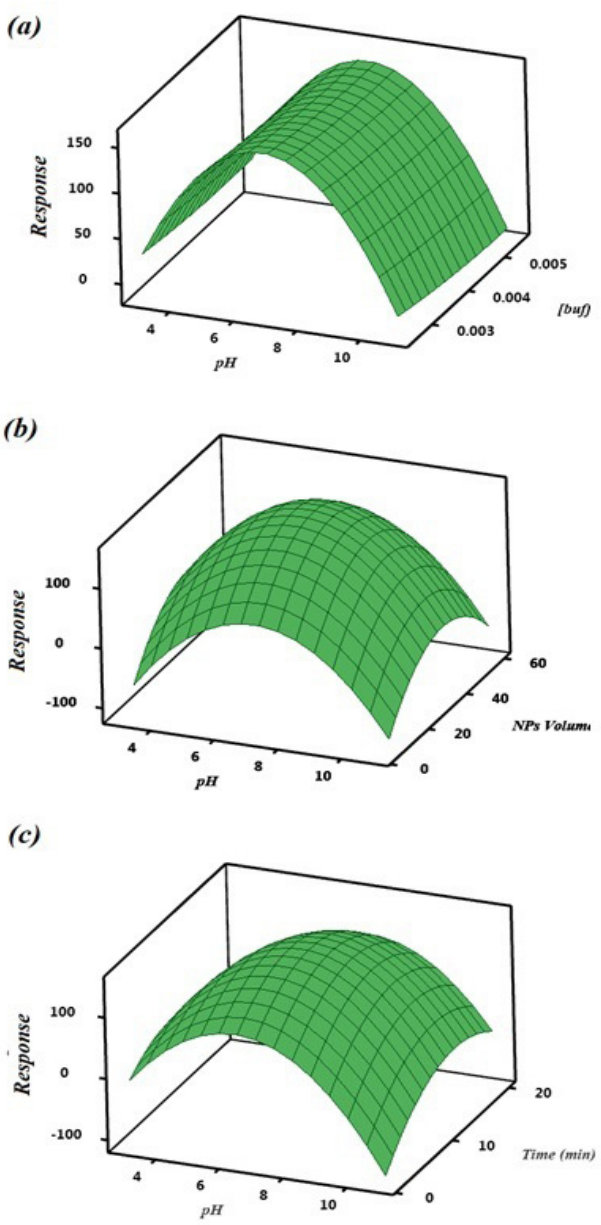

Figure 4. The response surface of the analytical signals as a function of (a) pH and [Buffer], (b) pH and Cu NCs volume, (c) pH and time, respectively.

Table 1. Tolerance limits of some co-administrated and over-the-counter drugs in the determination of 1.0 $\mu g\ mL^{-1}$ of sofosbuvir.

Interfering substances	Tolerance limit (Times)
Clonazepam, amoxicillin, aspirin	100
Diazepam, oxazepam, celecoxib, diltiazem, daclatasivir, naproxen, chlordiazepoxide, paracetamol, pantoprozole	20
Ibuprofen, nicotinamide, losartan, caffeine	10

Analytical figures of merit

In the optimum conditions obtained by CCD, a linear relationship between sofosbuvir concentration and SFS response is observed in the range of 0.05–6.0 $\mu g.mL^{-1}$ with a regression coefficient of 0.9972 (Figure 5). ΔI_{SFS} (difference of SFS intensity in the presence and absence of sofosbuvir) is considered as an analytical signal in these calculations. The limit of detection (LOD) and limit of quantification (LOQ) for this method are reported to be 0.0147 and 0.044 $\mu g.mL^{-1}$, respectively. LOD and LOQ are defined as $3S_b/m$ and $10S_b/m$ where S_b is the blank standard deviation and m is the slope for the calibration curve. S_b is obtained by measuring six blank samples. To evaluate the precision of the developed methodology, the analysis is repeated on the same and different days for a constant concentration of sofosbuvir (2.0 $\mu g.mL^{-1}$). The relative standard deviations (*RSDs*%) for inter-day and intra-day are calculated as 0.6 % and 1.8 %, respectively that points out the good repeatability and reproducibility of the validated method. A comparison of the current method with some other reported methods in the literature is summarized in Table 2. As can be seen, the analytical characterization of the validated method is good and comparable with that of other reported methods.

Analytical application

The validate method is successfully applied for the analysis of spiked plasma samples with sofosbuvir. In order to investigate the method's accuracy, recovery tests are performed by spiking the proper concentrations of sofosbuvir. The obtained results are given in Table 3. The

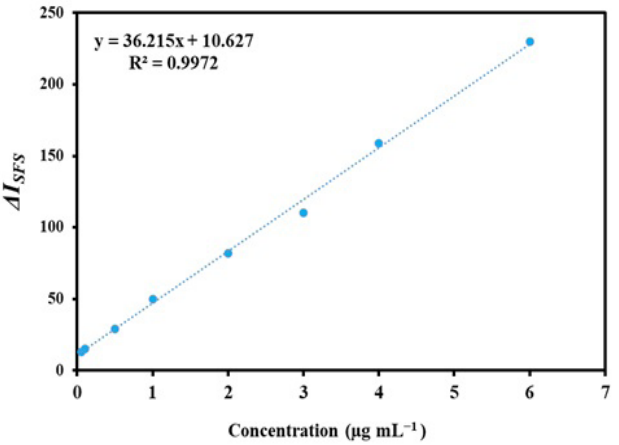

Figure 5. The calibration curve for response of proposed nanoprobe toward sofosbuvir.

Table 2. Comparison of analytical characteristics of the presented method with other reported techniques for determination of sofosbuvir.

Method	Sample	LOD (μg mL^{-1})	Linear range(μg mL^{-1})	Reference
RP-HPLC[a]-UV	Pharmaceutical formulation	0.02	40-240	[7]
RP-HPLC-UV	Pharmaceutical formulation	2.7	9.0-56.0	[8]
Spectrophotometery	Pharmaceutical formulation	5.6	20.4–207.5	[6]
Spectrophotometery	Pharmaceutical formulation	1.21	5.0–90.0	[17]
Voltametry	plasma	0.0003	0.001–0.4	[14]
TLC[b]	Pharmaceutical formulation	0.8[c]	2.5–24.0[c]	[13]
Cu NCs-based SFS	plasma	0.44	0.05−6.0	This work

[a] Reverse phase high performance liquid chromatography
[b] Thin-layer chromatography
[c] Unit is μg/band

Table 3. Determination of sofosbuvir in spiked plasma samples.

Sample	Added (μg·mL^{-1})	Found ± SD (μg·mL^{-1})	Recovery (%)[a]	*t*-test[b]
Plasma 1				
	0.50	0.51 ± 0.01	103.30	1.73
	1.00	1.05 ± 0.04	105.95	2.16
	2.00	1.98 ± 0.04	99.00	0.87
	4.00	4.03 ± 0.16	100.81	0.32
	6.00	6.15 ± 0.25	102.64	1.04
Plasma 2				
	0.50	0.51 ± 0.01	103.30	1.73
	1.00	1.05 ± 0.03	105.03	2.88
	2.00	1.92 ± 0.04	96.23	3.46
	4.00	4.08 ± 0.09	102.19	1.54
	6.00	6.22 ± 0.19	103.71	2.00

[a] Mean of three determination ± standard deviation
[b] *t*-critical =4.3 for n=3 and *P* = 0.05.

recoveries ranged from 96.23% to 105.95%. Numerical analyses of these results by Student's *t*-test shows that there is no significant difference between added and found values.

Conclusion

In the current study, an inexpensive, eco-friendly, and simple SFS approach based on Cu NCs is developed for the determination of sofosbuvir. The validated nanoparticle-based technique has been exposed to be promising for sofosbuvir detection in plasma samples with desired features such as high sensitivity, fast response time, easy operation, and low interfering substances. This method is successfully used for the sofosbuvir determination in plasma samples with recoveries ranged from 96.23% to 105.95%. This is the first effort on the nanoparticle-based optical probe for the quantification of sofosbuvir level and it can be a highly in-demand method in clinical experiments.

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Author Contributions

ZK: The acquisition, analysis, drafting the work, AJ: Design of the work, interpretation of data for the work, ER: design of the work, interpretation of data for the work. All authors read and approved the final manuscript.

Conflict of Interest

The authors report no conflicts of interest.

Supplementary Data

Supporting information is available on the journal's web site along with the published article.

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