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SIGNAL AMPLIFICATION OF SIA-CHEMILUMINESCENCE PROBE FOR METHOTREXATE DETECTION IN PHARMACEUTICAL FORMULATIONS USING SILVER NANOPARTICLES

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ABSTRACT

This work focused on the development of a sequential injection chemiluminescence (SIA-CL) system luminol-ferricyanide (III) in the presence of silver nanoparticles (AgNPs) for the detection of methotrexate (MTX) in its pharmaceutical formulations. The experimental conditions were optimized and the CL detection was conducted by measuring the high catalytic potential of AgNPs on the CL oxidation reaction. It was found that the increase in CL signal is proportional to the concentration of the target analyte. The calibration graph of the SIA-CL system was constructed covering a linear concentration range of 0.01-100 µg mL^{-1} for MTX in the presence of AgNPs. The influence of possible interfering species such as common cations, amino acids, sugars and coformulated additives was tested. The suggested method was validated in accordance with ICH guidelines. The suggested SIA-CL system was successfully applied to determine the investigated drug in bulk powder and pharmaceutical dosage forms. The overcome results were in good agreement with those obtained by other published method.

KEYWORDS: Sequential injection chemiluminescence; Silver nanoparticles; Methotrexate; Pharmaceutical formulations.

1. INTRODUCTION

Cancer can be defined as a group of diseases involving abnormal cell growth, uncontrollably by disregarding the normal rule of cell division with the potential to invade or spread to other parts of the body.[1] Many cancers can be prevented. Others can be detected early in their development, treated and cured. Even with late stage cancer, the pain can be reduced, the progression of the cancer slowed, and patients and their families helped to cope.[2] The present study, investigated a methotrexate (MTX) which is used to treat certain types of cancer or to control severe psoriasis or rheumatoid arthritis that has not responded to other treatments (Figure 1). It is chemically known as (2S)-2-[[4-[(2,4- diaminopteridin-6-yl) methyl-methylamino] benzoyl] amino] pentanedioic acid. It may also be used to control juvenile rheumatoid arthritis. Methotrexate belongs to a class of drugs known as anti-metabolites. It works by slowing or stopping the growth of cancer cells and suppressing the immune system. Early treatment of rheumatoid arthritis with more aggressive therapy such as methotrexate helps to reduce further joint damage and to preserve joint function.[3]

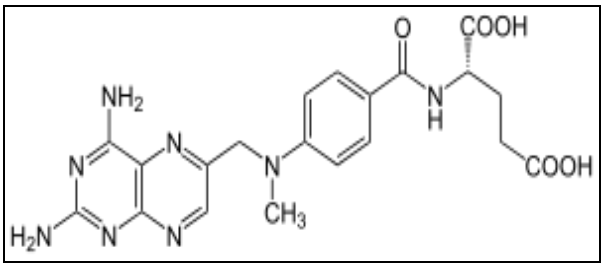


Figure 1: Chemical structure of methotrexate.

Several methods have been addressed for the detection of MTX, including separation methods such as high performance liquid chromatography,[4-7] high performance liquid chromatography coupled with mass spectrometry,[8] capillary electrophoresis.[9,10] Various spectroscopic techniques were reported, including spectrophotometry,[11-14] spectrofluorimetry[15] and chemiluminescence (CL) technique.[16,17] Some electrochemical articles which are concerned with the detection of the investigated drug have been published such as voltammetric method.[18,19]

Although, the chromatographic techniques offer a quick automated and highly accurate quantification of chemical

compounds, they have many drawbacks such as they can be costly, required large quantities of organic solvents and high operator skills. Furthermore, the electrochemical techniques can be also, made very quickly and the results are available as electrical signals, but unfortunately, these techniques displayed some error in analysis, required environmental protection to minimize the toxicity. However, in spectroscopic approaches such as spectrophotometry the stray light that caused by the faulty device design and other factors may influence the spectra measurement accuracy and affect the linear detection range.

Recently, a CL technique as quantitative detection method gained much opportunity and attention, owing to its high sensitivity merits, wider linear range and low background signal.[20]

Nanotechnology becomes one of the most prominent areas of research in science and technology. Many researchers began to concentrate their vision in studying the unique features of nanomaterials and their wide applications in our life.[21] It should be mentioned that, metallic nanoparticles have distinctive properties such as high electrical conductivity, better chemical reactivity, and enhanced catalytic activity.[22]

Several articles which are concerned with the catalytic activity of AgNPs and their impact role in CL reactions have been published in the field of analytical chemistry.[23,24]

This study aimed to focus on the catalytic effect of synthesized AgNPs on the CL signals through the detection of the investigated drug by the SIA-CL system. The suggested CL system was validated and applied for the detection of MTX in its pharmaceutical dosage forms.

2. EXPERIMENTAL

Instruments

All CL measurements were made using SIA-system (FIAlab-3500 instrument, Bellevue, Washington, USA) which is automatically operated by PC equipped with software (FIAlab for windows 5.9.321). The reagents were pumped using 2.5 mL comprised of a CAVRO XL 3000 syringe pump (Cavro Scientific Instrument Int., Bellevue, Washington, USA). The CL signal in the flow cell was obtained using a photomultiplier tube voltage operated at 320 V, autosampler (AML3200). The characterization of the synthesized nanoparticles was carried out by measuring the UV-Vis spectrum of the as-prepared nanoparticles using an Ultrospec 2100-Biochrom spectrophotometer, (Biochrom Ltd, Cambium, Cambridge, UK). The size and shape of AgNPs were measured by transmission electron microscope (TEM) JEOL model 1200EX instrument, (JEOL Ltd, Freising, Germany). Additionally, Siemens D-5000 diffractometer (Siemens, Erfurt, Germany) was used to obtain the XRD pattern of AgNPs. However, PerkinElmer FT-IR spectrophotometer (PerkinElmer Ltd, Yokohama, Japan) was used to record Fourier transforms infrared (FTIR) spectra of the formed nanoparticles.

Materials and reagents

All chemicals used in this study were of analytical grade and the solvents were of HPLC grade. Pure distilled water was used throughout the experiments. Pure grade of methotrexate and its pharmaceutical preparations (Methotrexate sodium® 1g/40 mL) injection and (Methotrexate® 2.5 mg/tablet) were kindly supplied by (MercuryPharma, Ireland). Silver nitrate of purity (99.0 %), sodium borohydride ($NaBH_4$, 98.0 %), sodium hydroxide (NaOH, 98.0 %), potassium ferricyanide (III) (99.0 %), sodium carbonate (99.0 %), sodium bicarbonate (99.0%), Ce (IV) ammonium sulfate, potassium dichromate, and potassium permanganate (99.0 %) were provided by (BDH Ltd., Poole, UK). Trisodium citrate dihydrate (99.5 %) was obtained from (WINLAB, East Midland, UK). Luminol (98.0 %) and hydrochloric acid was purchased from (Sigma-Aldrich, Hamburg, Germany).

Preparation of analytical solutions

Preparation of standard solutions of MTX

A stock solution of 200 µg mL^{-1} of MTX was prepared by dissolving 20 mg of the MTX drug in 100 mL of 0.05 mol L^{-1} hydrochloric acid. The prepared solution was stable for at least 2 weeks when kept in the refrigerator. The working solutions of appropriate concentrations were obtained by further diluted using the same solvent.

Preparation of tablet samples

Ten tablets of MTX were weighed and pulverized. An accurate amount of the powder equivalent to 20 mg of the drug was transferred into a 100-mL volumetric flask. Approximately, 10 mL of 0.05 hydrochloric acid mol L^{-1} was added. The solution was diluted to the mark with distilled water and sonicated for 10 min then filtered. The nominal contents of the drug were calculated from either the plotted calibration graph or using the regression equation.

Preparation of injection samples

The content of one MTX injection 1g/40 mL was transferred into volumetric flask. Approximately, 0.4 mL of one vial content was diluted with 100 mL of 0.05 mol L^{-1} of sodium hydroxide in 100-mL volumetric flask. Serial dilutions in the range of 0.01-100 µg mL^{-1} were prepared using the same solvent. Direct detection of MTX was carried out using the suggested SIA-CL system in the presence of AgNPs.

Synthesis of AgNPs

The synthesis of AgNPs was carried out by the reduction of silver nitrate using an aqueous solution of sodium borohydride as a reducing agent. Approximately, 5 mL of 2.0×10^{-3} mol L^{-1} sodium borohydride was added dropwise with continuous stirring to 30 mL of 1.0×10^{-3} mol L^{-1} silver nitrate. After 5 min, the mixture color

turned yellow. The formation of yellow colored solution indicated the reduction of silver nitrate into Ag• and the formation of AgNPs. Then, 5 mL of 2.0×10^{-3} mol L^{-1} trisodium citrate dihydrate was added to the resultant solution to stabilize the prepared AgNPs.

General CL procedure

The SIA-CL measurements were carried out under PC control to verify the optimum precision and control the valves and pump movement (Figure 2). All lines of the system were filled with the carrier solution (distilled water) to remove the air bubbles, then the studied drug solution and reagents were aspirated.

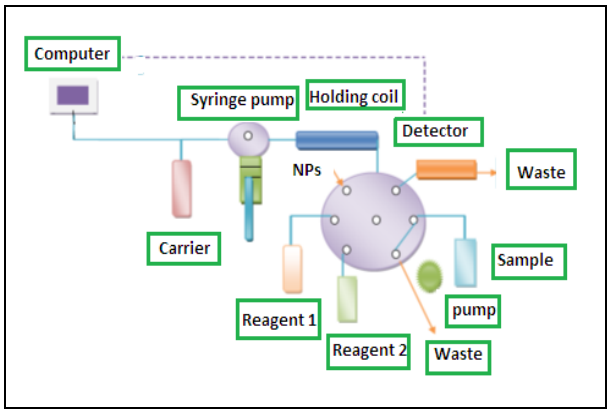


Figure 2: Schematic diagram of SIA-CL system.

The sequence of the aspirated sample and reagents was automatically controlled. SIA-CL system of luminol-ferricyanide (III) is used in the presence of AgNPs for the detection of MTX. Mixture of 40 µL of luminol, 60 µL of potassium ferricyanide and 50 µL of MTX in the presence of 1.0 mL of AgNPs were aspirated into the holding coil through the eight-way injection valve at a flow rate 100 µL s^{-1} and then the mixed solution was flushed continuously into the flowthrough cell located in front of detection cell of the photomultiplier tube (FIALab-PMT). The CL signals were monitored with triplicate analysis cycles for each test solution. The average CL intensity was used to plot the calibration graph against drug concentrations. Then, the regression equation was derived. All measurements were carried out at ambient temperature 25±1˚C.

3. RESULTS AND DISCUSSION

Characterization of AgNPs

UV-Vis spectroscopy

AgNPs have optical features that are very sensitive to size, shape and concentration, which facilitate the use of UV-Vis spectroscopy as a valuable tool for identifying, and characterizing these nanoparticles. The as-prepared AgNPs was characterized by recording UV-Vis absorption spectrum. A significant absorption peak at 420 nm was observed for AgNPs (Figures 3). According to the literature values[25] this distinct peak represents the formation of AgNPs with particle sizes of approximately 20 nm.

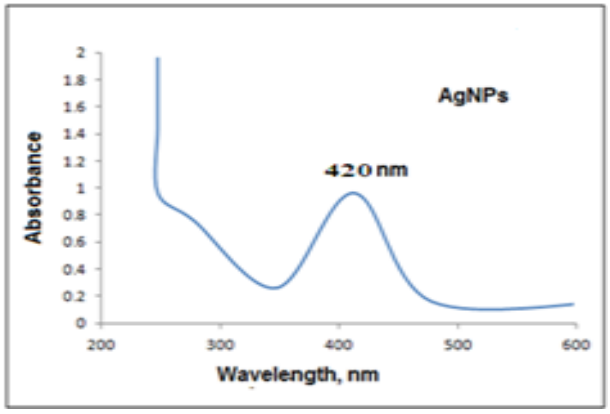


Figure 3: UV-Vis absorption spectrum of AgNPs.

FT-IR spectroscopy

To evaluate the purity and nature of AgNPs surface, FT-IR spectrum was recorded. As shown in Figure 4, two main bands were detected at 530 and 1360 cm^{-1}. These two bands were attributed to the presence of Ag^{+} and N-O from $AgNO_3$. However, the presence of other bands in 3310 and 2910 cm^{-1} were for N-H stretching and O-H stretching bands, respectively, due to the adsorption of water on the surface of AgNPs. Another vibrational band was observed at 1632 cm^{-1} may be attributed to carbonyl group C=O stretching vibration band. The result of this FT-IR spectroscopic study confirmed the reduction of silver nitrate into AgNPs by using $NaBH_4$ as reducing agent.

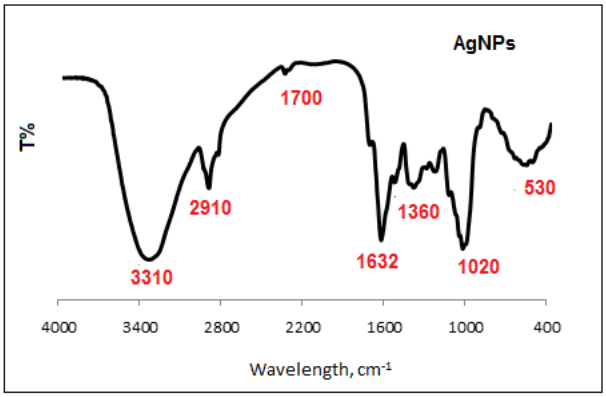


Figure 4: FT-IR spectrum of AgNPs.

Characterization using TEM and SEM

The TEM image of the as-prepared AgNPs (Figures 5a) indicated that the prepared nanoparticles are fairly uniformly distributed, spherical in shape and their sizes are 20 nm. Additionally, SEM image was used to characterize the surface morphology of the formed AgNPs (Figures 5b).

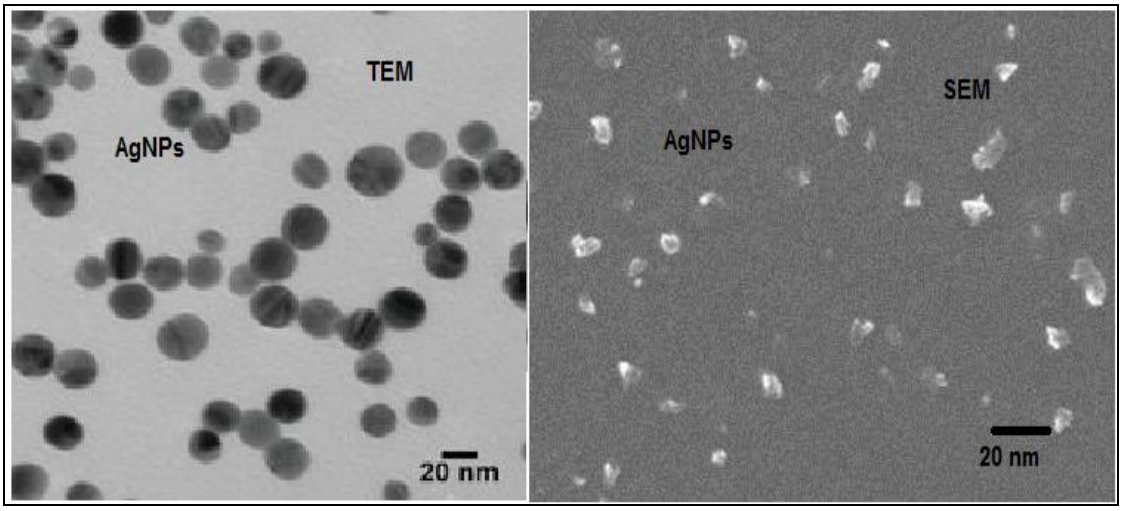


Figure 5: (a) TEM image of AgNPs (b) SEM image of AgNPs.

XRD patterns of AgNPs

X-ray diffraction pattern of AgNPs was studied over a 2Θ range of 20-80° to investigate the crystal structure of the as-prepared nanoparticles. The diffracted patterns were compared with the standard database recommended by the International Centre for Diffraction Data (ICDD). XRD pattern of AgNPs displayed different significant peaks at 38.5°, 44.4°, 64.6° and 78.8° indicating the presence of Ag (111), Ag (200), Ag (220) and Ag (311), respectively (Figure 6).

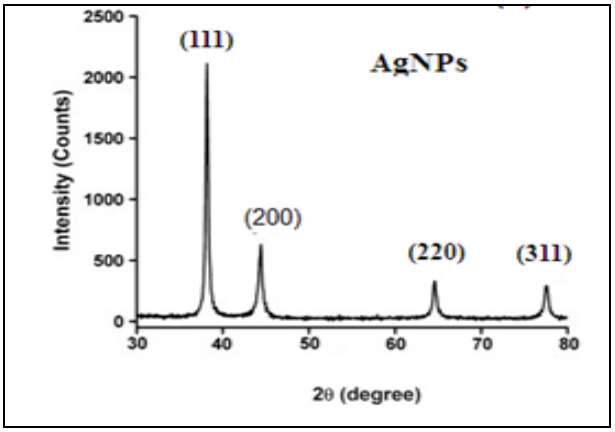


Figure 6: XRD spectrum of AgNPs.

Optimization of experimental conditions

Selection of chemiluminescent reagents

To select the suitable CL reagent for the suggested CL system, four different types of CL reagents such as luminol, lucigenin, sodium sulfite and Ru (bpy) $_3^{-2}$ have been tested. It was found that luminol was the most suitable reagent for the determination of MTX (Figure 7).

The CL activity of luminol system was accomplished in alkaline medium. Therefore, different kinds of alkaline media in the concentration range of 1.0×10^{-4}- 1.0×10^{-1} mol L^{-1} were examined, including sodium hydroxide, sodium carbonate and sodium bicarbonate. The strongest signal was obtained by using luminol in the presence of 1.0×10^{-2} mol L^{-1} sodium hydroxide solution (Figure 8).

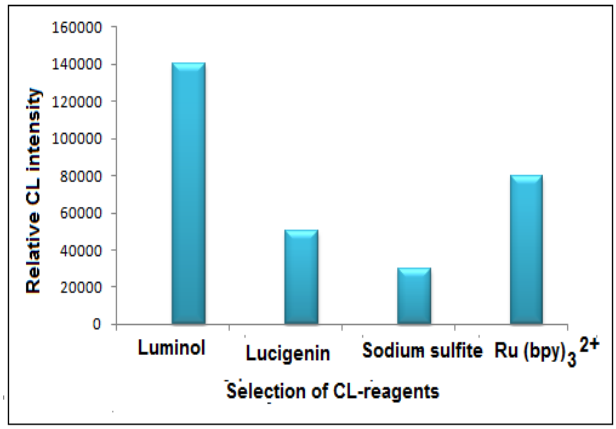


Figure 7: Selection of CL reagents using luminol, lucigenin, sodium sulfite and $Ru(bpy)_3^{2-}$.

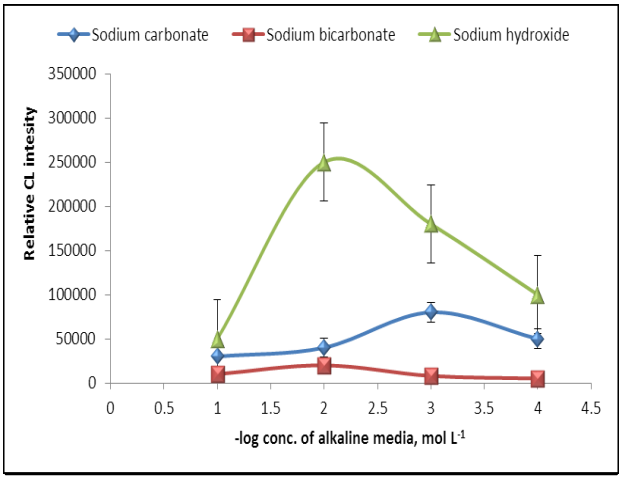


Figure 8: Selection of suitable alkaline medium using sodium hydroxide, sodium carbonate and sodium bicarbonate for the CL reaction of luminol.

Selection of oxidizing agents

Different types of oxidants including, potassium permanganate, Ce (IV) ammonium sulfate, potassium

dichromate and potassium ferricyanide (III) were tested for the CL reaction of the investigated drug. The CL signal was recorded for each oxidant and it was found that the higher signal was obtained by using luminol-potassium ferricyanide (III) system for determination of MTX in the presence of AgNPs (Figure 9).

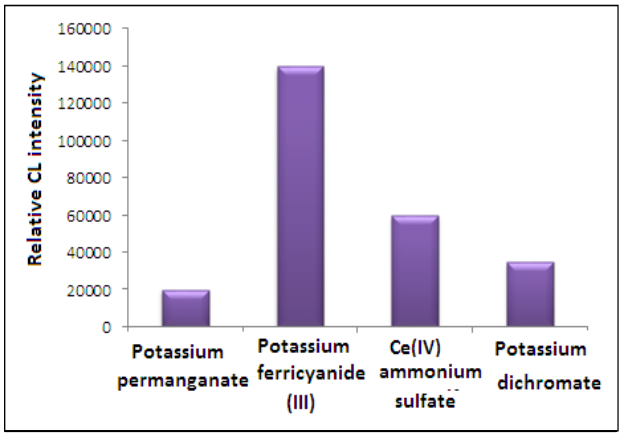


Figure 9: Selection of suitable oxidizing agent to be reacted with luminol and 1.0 µg mL^{-1} of MTX in the presence of AgNPs.

Effect of CL reagent and oxidizing agent concentration

The influence of luminol concentration as CL reagent and potassium ferricyanide (III) as an oxidizing agent on the CL signal was studied. Different concentrations in the range of 1.0×10^{-6} - 1.0×10^{-2} mol L^{-1}of each of luminol and potassium ferricyanide (III) were investigated. Maximum CL signals were obtained by using 1.0×10^{-4} mol L^{-1} of luminol and 1.0×10^{-3} mol L^{-1} of potassium ferricyanide (III) for the detection of MTX in the presence of AgNPs (Figures 10).

Effect of flow and aspirate rates

Herein, some physical parameters such as AgNPs volume, flow rate and aspirate rate of the sample and reagents were evaluated and carefully optimized to accomplish the SIA-CL detection of MTX.

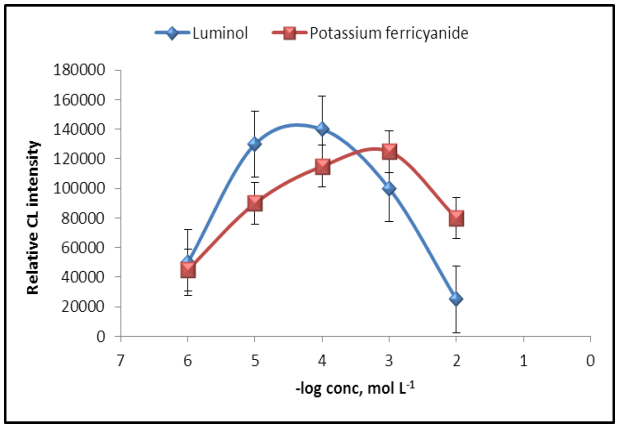
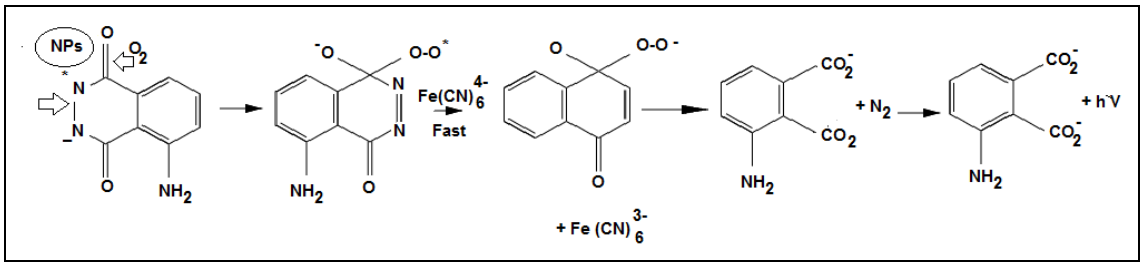


Figure 10: Effect of concentrations of CL reagent and oxidizing agent for the detection of MTX.

FIAlab software version 5.9.321 was used for the automatic control of the aspirated volume of both samples and reagents. The obtained data of SIA-CL determination of the studied drug were 40 µL of luminol, 60 µL of potassium ferricyanide and 50 µL of MTX in the presence of 1.0 mL of AgNPs at a flow rate 100 µL s^{-1}.

Possible CL reaction mechanisms

The CL reactions differ from fluorescence reactions in which they emit light in the visible or infrared region. However, in fluorescence reactions an excitation source is needed. In the present study the CL reaction involves a highly oxidizing species such as (potassium ferricyanide). In the this system luminol-potassium ferricyanide, under the optimum CL reaction conditions, emitted light intensity was increased with short duration time in the presence of AgNPs indicating the catalytic activity of the added nanoparticles. As shown in Scheme 1, the reduction of luminol radicals was accomplished on the surface of the nanoparticles during the exchange interaction between the unpaired electrons of luminol and the conduction band electrons on the nanoparticles.



Scheme 1: The possible chemical reaction mechanism of luminol-potassium ferricyanide (III) in the presence of AgNPs

Under optimal conditions, AgNPs of 20 nm, size displayed the highest CL intensity lasting for long duration of time. This effect can be attributed to the increase in surface area and surface electron density in the catalytic reaction involving NPs. It is also being reported that organic compounds containing hydroxyl (OH) and amino (NH_2) groups can greatly interact with nanoparticles leading to the enhancement or inhibition of CL signal amplification.[25] Therefore, the present study focused on the possibility of MTX to react with the as-prepared nanoparticles and enhance the CL signal.

Study of interference

To examine the selectivity of the suggested SIA-CL system towards the detection of the selected MTX, various possible cations, amino acids, sugars and some excipients were used. Under the same optimum conditions the detection of 1.0 µg mL^{-1} of MTX was treated with a sample containing 1.0 µg mL^{-1} of interfering species such as some possible cations Na^{+}, K^{+}, Mg^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, NH_4^{+} and Zn^{2+}, anions including Cl^{-}, PO_4^{3-}, NO_3^{-}, and SO_4^{2-}, sugars such as glucose, sucrose and lactose and some common acids such as citric, oxalic, tartaric and ascorbic acid. Also the effect of some amino acids such as cysteine, histamine, tyrosine and glucosamine was investigated. The results in Table 3 showed that no interference was observed. The tolerance of foreign species gave an error ≤ ±5.

Table 3: Tolerable concentration level of interference to 1.0 µg mL^{-1} of MTX in the presence of AgNPs.

Interference	Tolerable level
Co^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2}, Zn^{2+} Na^{+}, K^{+} and Mg^{2+}	1000
Cl^{-}, PO_4^{3-}, NO_3^{-}, NH_4^{+} and SO_4^{2-}	350
Glucose, sucrose and lactose	800
Citric acid, oxalic acid, tartaric acid, ascorbic acid	240
Cysteine, histamine, tyrosine, glucosamine	460

Method validation

The developed SIA-CL method for the determination of the studied drug was validated according to ICH[26] guidelines.

Linearity, limits of detection, and quantification

The linear relationship was tested by plotting the calibration graph of the CL intensity of the SIA-CL system vs. the investigated MTX concentrations. Linear regression was calculated by least-square regression analysis. The developed SIA-CL system exhibits a linear relationship in the presence of AgNPs over the concentration range of 0.01 – 100 µg mL^{-1} for MTX.

The limits of detection and quantification (LOD and LOQ) of the studied drug by the proposed SIA-CL system was calculated using S/N = 3 and S/N =10, respectively.[27] The recorded signals showed lower LOD values of 0.003 µg mL^{-1} for MTX in the presence of AgNPs However, the obtained LOQ were found to be 0.001 µg mL^{-1} (Table 4).

Accuracy and precision

The accuracy of the proposed SIA-CL system was expressed as percentage recovery calculated from the obtained results. The developed method showed good accuracy with % recovery, 99.10 % for MTX detection. In order to estimate the precision of the proposed SIA-CL system, intra-day and inter-day assay were applied.

Table 4: Performance data of the studied drug using the proposed SIA-CL method in the presence of AgNPs

Parameter	SIA-CL system
Linear range, µg mL^{-1}	0.01-100
LOD, µg mL^{-1}	0.003
LOQ, µg mL^{-1}	0.01
Regression equation	I_{CL}=230.54 C +152.76
Correlation coefficient, r	0.9997
% RSD	0.9

Three different concentrations of MTX were investigated in triplicate (n = 3) the recorded data were calculated as % RSD. As summarized in (Table 5), the calculated % RSD were found to be ranged from 0.3-0.7 %, 0.3-0.6 % for intra-day and inter-day assay, respectively. These results revealed high precision of the suggested SIA-CL system.

Table 5: Intra-day and Inter-day precision data using the proposed SIA-CL system for the detection of MTX in the presence of AgNPs.

System		Conc., µg mL^{-1}	% Recovery	% RSD
Luminol-ferricyanide-MTX-AgNPs	Intra-day assay	0.01	99.7±0.5	0.5
		50	99.4±0.7	0.7
		100	99.5±0.3	0.3
	Inter-day assay	0.01	99.3±0.5	0.5
		50	99.9±0.3	0.3
		100	99.6±0.6	0.6

Analytical application

The developed SIA-CL system was employed to detect MTX in its bulk powder (Table 6). Also, in the commercially available formulations (Methotrexate®2.5 mg/tablet) and (Methotrexate sodium® 1g/40 mL injection). The developed SIA-CL method was applied and the results summarized in Table 7 were compared with those obtained from other spectrophotometric previously reported method.[14] To evaluate the performance of the proposed method, the obtained results were statistically treated using Student's t-test and variance ratio F-test.[27]

Table 6: Determination of MTX in its bulk powder using SIA-CL system in the presence of AgNPs.

Luminol-ferricyanide- AgNPs system			
Sample	**Taken µg mL^{-1}**	**Found µg mL^{-1}**	**% Recovery**
Pure Drug	0.01	0.01	100.0
	1.0	0.99	99.0
	5	4.98	99.6
	10	9.99	99.9
	50	49.76	99.5
	100	99.22	99.2
Mean±SD	99.6±0.3		
n	6		
Variance	0.09		
%SE	0.12		
% RSD	0.30		

Table 7: Determination of the studied drug in its pharmaceutical formulations by the SIA-CL methods in the presence of AgNPs.

Sample	**(MTX® 2.5 mg/tablet)**			**(MTX sodium ® 1 g/40 mL) injection**			
	Taken µg mL^{-1}	**Found µg mL^{-1}**	**% Recovery**	**Taken µg mL^{-1}**	**Found µg mL^{-1}**	**% Recovery**	**Reported Method [14]**
MTX	0.01	0.01	100.00	0.01	0.0089	98.0	
	0.05	0.049	98.00	0.05	0.05	100.0	
	10	9.96	99.60	10	9.89	98.9	
	50	49.87	99.74	50	49.95	99.9	
	80	79.15	98.93	80	79.84	99.8	
	100	99.63	99.63	100	99.76	99.8	
Mean±SD	99.32±0.7			Mean±SD	99.4±0.8		99.18±0.5
n	6			n	6		6
Variance	0.49			Variance	0.64		0.25
% SE	0.29			% SE	0.32		0.20
t-test	0.397 (2.228) *			t-test	0.583 (2.228)*		
F-test	1.96 (5.05)*			F-test	2.56 (5.05)*		

Figures in parentheses are the tabulated values of t- and F- tests at 95% confidence limit

4. CONCLUSION

The present study described a new sensitive and selective enhanced SIA-CL system luminol-ferricyanide (III)-AgNPs for the detection of MTX in its bulk powder and pharmaceutical formulations. The outcome of the investigation revealed that the increase in CL signals is proportional to the selected analyte concentrations. The suggested system exhibits a linear behavior over concentration range of 0.01-100 µg mL^{-1} for MTX in the presence of AgNPs. The obtained results were treated statically and revealed good agreement with those obtained from a previously reported method. The proposed method was successfully applied to determine the studied drug in its bulk powder and pharmaceutical preparations.

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