Flow Injection Determination of Antibacterial Drug Trimethoprim in Pharmaceutical Preparations with *Tris*(2,2'-Bipyridyl)Ruthenium(III) Chemiluminescence

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Summary: In the present study, a simple and sensitive chemiluminescence method using *tris*(2,2'-bipyridyl)ruthenium(III) with flow injection detection is reported for the analysis of trimethoprim in pharmaceutical preparations. The calibration graph was linear over the concentration range of 0.05-5.0 $\mu g\ mL^{-1}$ with relative standard deviations ($n = 4$) in the range 1.0 – 2.5%. The limit of detection (3σ blank) was 0.008 $\mu g\ mL^{-1}$ with a sample throughput of 100 h^{-1}. The effect of some organic compounds, common excipients used in pharmaceutical preparations, cations & anions were studied for trimethoprim determination. $Ru(bipy)_3^{2+}$ was chemically oxidized to $Ru(bipy)_3^{3+}$ online using lead dioxide mini column.

Introduction

Trimethoprim, 2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine, having molecular formula ($C_{14}H_{18}N_4O_3$), is a white to cream colored, odorless, bitter compound (Fig.1). Trimethoprim is an antibacterial drug; it blocks the production of tetrahydrofolic acid from dihydrofolic acid by binding to and reversibly inhibiting the required enzyme, dihydrofolate reductase. This binding is much stronger for the bacterial enzyme than for the corresponding mammalian enzyme. Thus, trimethoprim selectively interferes with bacterial biosynthesis of nucleic acids and proteins. Trimethoprim is often combined with sulphonamides (*e.g.* sulphamethoxazole) in compound pharmaceutical preparations to achieve synergistic antibacterial effect but it is also utilized as a single active substance particularly in the treatment of urinary tract infections.

Numerous methods such as high performance liquid chromatography [1-3], capillary zone electrophoresis [4, 5], thin layer chromatography [6], spectrophotometry [7-9], voltammetry [10], mass spectrometry [11] and piezoelectric ion-selective sensors [12] have been reported for the determination of trimethoprim in multi-component pharmaceutical preparations or in body fluids, foodstuffs and biological tissues. Flow-injection analysis (FIA) with on-line extraction of trimethoprim into chloroform and UV detection at 280 nm has been reported [13] for trimethoprim determination in compound tablets with sulphamethoxazole or tetracycline and in compound injection solutions with berberine; the calibration graph was linear over the concentration range 25-150 mg mL^{-1}.

Analytical interest in liquid-phase chemiluminescence (CL) is continuously growing over the last three decades; specially when coupled with FIA, the best demonstration of this interest is the large number of recent manuscripts dealing with analytical applications for inorganic and organic (like drugs or pesticides) substances in a variety of industrial, clinical and environmental matrices. An analytical advantage of CL includes high sensitivity, very low limits of detection and a wide linear range [14-19].

Tris(2,2'-bipyridyl)ruthenium(II) complex is one of the most frequently used CL system, it produces light emission from the excited state $[Ru(bipy)_3^{2+}]^*$ that can be obtained in different reactions which imply electron transfer and regeneration of the $Ru(bipy)_3^{2+}$ species. For these reactions the CL intensity is linearly proportional to the concentration of any of the reagents, enabling their determination by suitable adjustment of the concentrations of the other reagents. $Ru(bipy)_3^{2+}$ is the most studied and exploited inorganic compound used in electrogenerated CL; its analytical usefulness as an ECL label. Analytical applications of $Ru(bipy)_3^{2+}$ has recently been reviewed [20].

Miroslav *et al.* [21] reported the CL method for the determination of trimethoprim by automated sequential injection technique with permanganate and

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hexametophophate as reagents. The calibration graph relating to the intensity of luminescence to concentration of trimethoprim was parabolic (r=0.9994) over the concentration range of 0.5-100 μg mL^{-1} and with rectilinear part (r= 0.9999) over the concentration range of 20 - 100 mg mL^{-1}. The limit of detection was 0.1 μg mL^{-1} and method was used for the assay of trimethoprim in pharmaceutical preparations. Yunhua He *et al.* [22] reported the molecular imprinting-chemiluminescence method for the determination of trimethoprim, in which trimethoprim-imprinted polymer was used as the molecular recognition material and the CL reaction of trimethoprim with potassium permanganate in acidic medium was used as the detection system. The CL intensity responds linearly to the concentration range from 0.05 to 5.0 μg mL^{-1} of trimethoprim, with a detection limit of 0.02 μg mL^{-1}. The method was applied for the determination of trimethoprim in pharmaceutical preparations.

The aim of the present work was to establish a simple and sensitive FI-CL method for the determination of trimethoprim. This method is based on the reaction of trimethoprim with $Ru(bipy)_3^{3+}$ to produce excited state $[Ru(bipy)_3^{2+}]^*$ which undergoes relaxation by generating CL. $Ru(bipy)_3^{3+}$ is generated online from $Ru(bipy)_3^{2+}$ in sulphuric acid medium by lead dioxide mini column [23]. After manifold parameters optimization, the method was applied for the determination of trimethoprim in pharmaceutical preparations. The limit of detection (3σ blank) was 0.008 μg mL^{-1}, with a sample throughput of 100 h^{-1}.

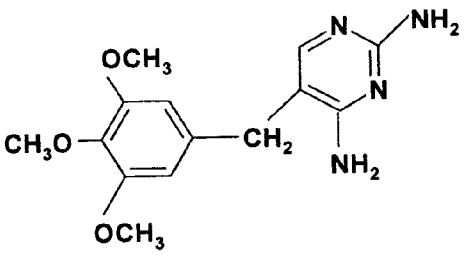

Fig. 1: Trimethoprim structure.

Results and Discussion

Selection of Parameters for FI-manifold

In order to select the best conditions for the determination of trimethoprim, various experimental parameters were investigated using a univariate approach. The key parameters optimized were phosphate buffer pH, sulfuric acid and $Ru(bipy)_3^{2+}$ concentration, flow rate of sample carrier and reagent streams. All of these studies were performed with 0.1 μg mL^{-1} trimethoprim standard solution (60 μL) and a PMT voltage of 800 V.

The efficiency of chemiluminescence emission is highly dependent on reaction pH; therefore the use of buffer is critically important. Initially water was used as sample carrier stream for trimethoprim but due to sulphuric acid from CL reagent stream cause the drop of pH (pH 1.8) after merging with sample carrier stream and cause very weak CL signal, therefore the use of buffer was decided for sample carrier steam. The effect of phosphate buffer pH (0.05 M) was investigated over the range of 6.0-8.0. By increasing the pH of buffer the signal increases gradually and at pH 7.5 the CL response was reproducible with smooth base line, therefore the phosphate buffer (0.05 M) pH of 7.5 is selected for further studies. The sulphuric acid concentration for CL reagent stream was studied in the range of (0.001 - 0.05 mol L^{-1}) and maximum CL response was observed at 0.01 mol L^{-1} and selected for further studies. The effect of $Ru(bipy)_3^{2+}$ concentration was then studied in the range 1.0×10^{-6}-1.0×10^{-4} mol L^{-1} using the optimized sulphuric acid concentration. The suitable CL response was observed at 1.0×10^{-5} mol L^{-1} of $Ru(bipy)_3^{2+}$ concentration and further increase resulted in non-reproducible CL signals with high baseline and blank values. Therefore, a $Ru(bpy)_3^{2+}$ solution of 1.0×10^{-5} mol L^{-1} was selected for subsequent studies (Fig. 2). Flow rate for each of the two channels were simultaneously investigated over the range 1.25-2.9 mL min^{-1} in terms of sensitivity, sample throughput and reagent consumption. Increase in flow rates increases the background CL and a flow rate of 2.1 mL min^{-1} gave almost the maximum CL response (signal-to-noise ratio) with a steady base line and reproducible peak height. The optimum flow rate (4.2 mL min^{-1} total flow rate) depends on the distance and the residence time of analyte from the T-piece to the reaction glass coil placed in front of the PMT; which is in this case is 1.5 cm and 10 seconds respectively. The effect of the sample injection volume on CL intensity were investigated over the range of 30-180 μL. increase in sample volume increase the sensitivity and decreases the calibration range; as at higher sample injection volumes the CL reagents are

saturated with analyte therefore cause decrease in calibration range. Therefore sample injection volume of 60 μL was selected for lower limit of detection, large calibration range and for the economy of sample consumption.

Figures of Merit

Under the optimum conditions, calibration graph was prepared from the results of quadruplicate 60 μL injections of the trimethoprim standard solutions. A linear calibration graph of CL intensity vs. trimethoprim concentration over the range of 0.05-5.0 μg mL^{-1} (n = 8) was obtained (r^2 = 0.999) with regression equation I = 782.05c-0.1337 (where I is the CL intensity in mV and c is the concentration in μg mL^{-1}). The relative standard deviations (RSD) were 1.0-2.5 %. (n = 4) over the range studied. The limit of detection (3σ blank) was 0.008 μg mL^{-1} with sample throughput of 100 h^{-1}. A representative chart recorder outputs for the determination of various concentrations of trimethoprim is shown in Fig. 3.

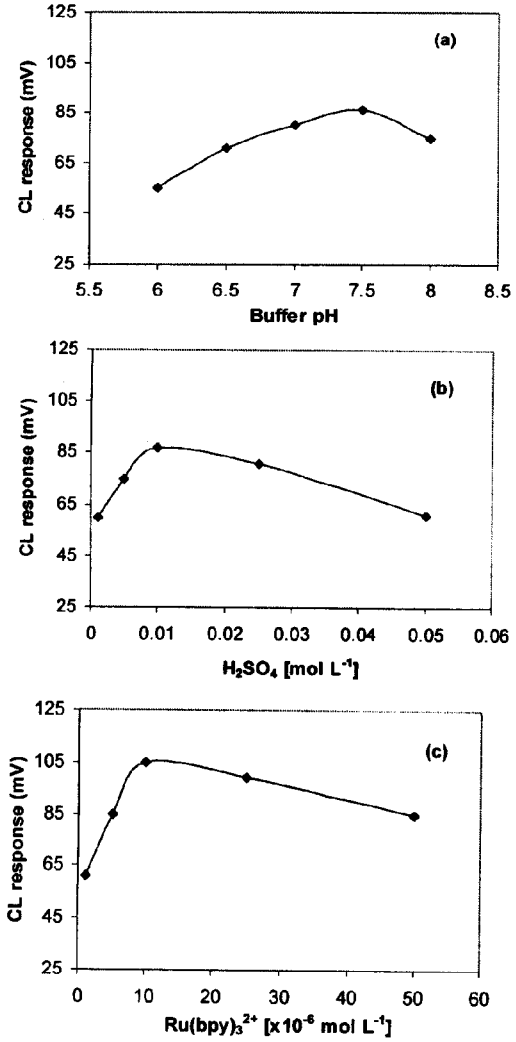

Fig. 2: Variation of CL intensity with Buffer pH (a), H_2SO_4 (b), $Ru(bipy)_3^{2+}$ (c) concentrations with 0.1 μg mL^{-1} trimethoprim (60μL).

Study of Foreign Species

In this study, other foreign species were added to the sample solution and their effects on the CL signals were investigated. The study was carried out with 0.1 μg mL^{-1} of trimethoprim. The effect of each species was considered as interference when caused a relative error in the signal of ±5% in comparison with the signal obtained for each analyte in absence of the species. A 1000-fold of starch, sucrose, glucose, glycerol, ethanol, cellulose, gum arabic, polysorbate 80, gelatin, magnesium stearate, Ca^{2+}, Mg^{2+}, Cl^-, CO_3^{2-}, SO_4^{2-}, 500 folds of povidone, Fe^{2+}, NO_3^-, and 100 fold of sulphamethoxazole did not interfere.

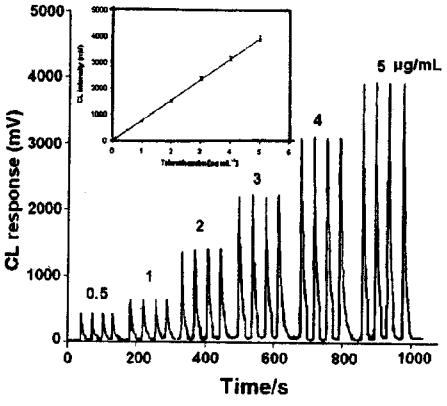

Fig. 3: A representative recorder outputs for series of standards under the proposed conditions by FI-CL system. Inset: the calibration curve of trimethoprim.

Applications

Five tablets from commercially available formulations were weighed and powdered. An accurate weight of the powder conivalent to one

tablet was mixed with 100 mL of methanol in a 100 ml calibrated flask, stirred for about 10 min and filtered to separate any insoluble matter. The filtrate was collected in a clean flask. Appropriate aliquots were taken and diluted with phosphate buffer. An infusion ampoule (5 mL) was taken and diluted appropriately in phosphate buffer (pH 7.5). The samples were injected to the CL system; the amount of trimethoprim per tablet and injection was calculated from related linear regression equation respectively. The data is shown in Table-1. The results obtained were in good agreement with the amount labeled and with the reference method [24]. The determination results of recovery test are shown in Table-2.

Experimental

Reagents and Solutions

All solutions were prepared using ultra-high purity (UHP) water (Elga, Purelab Option, UK) and all reagents were of analytical grade, unless stated otherwise. *Tris*(2,2'-bipyridyl)ruthenium(II)chloride hexahydrate (Fluka, UK) stock solution (1.0×10^{-3} mol L^{-1}) was prepared in sulfuric acid (1.0×10^{-2} mol L^{-1}). A working $Ru(bpy)_3^{2+}$ solution (1.0×10^{-5} mol L^{-1}) was prepared by diluting the stock solution with an aqueous solution of sulfuric acid (1.0×10^{-2} mol L^{-1}). Trimethoprim (Dr. Ehrenstorfer GmbH, 99.5%) stock solution of 500 $\mu g\ mL^{-1}$ was prepared in ethanol and stored at -4°C in brown bottle in dark. Subsequent standard solutions were prepared daily by serial dilution of the stock solution with phosphate buffer. Septran® infusion injection, and Syraprim® tablets (GlaxoSmithKline, Pakistan) were purchased from local market.

Table-1: FI-CL determination of trimethoprim in pharmaceutical preparations (n = 5).

Sample	labeled	Proposed method found	Reference method [24] found
Injection	16 mg mL^{-1}	15.8 ± 0.1	15.75 ± 0.1
Tablet	300 (mg / tablet)	298.4 ± 0.1	296.5 ± 0.5

Table-2: FI-CL determination of trimethoprim in pharmaceutical preparations (n = 6) (recovery test results).

Sample	Actual ($\mu g\ mL^{-1}$)	Added ($\mu g\ mL^{-1}$)	Found ($\mu g\ mL^{-1}$)	Recovery (%)
Injection	1.0	0	1.02±0.04	102
		1.0	1.92±0.05	96
		2.5	3.65±0.10	104
		4.0	5.30±0.08	106
Tablet	2.0	0	2.10±0.07	105
		1	3.11±0.06	104
		2	3.92±0.11	98
		3	4.82±0.20	96

Instrumentation and Procedures

The two channel flow injection chemiluminescence manifold used for this work is shown in Fig. 4. A peristaltic pump (Ismatec Reglo, 4 channels, Switzerland) was used to deliver the sample carrier and reagent solutions at a flow rate of 2.1 mL min^{-1}. A rotary injection valve (Rheodyne 5020, Anachem, Luton, UK) was used to inject standards (60 μL) into phosphate buffer stream and merged at a T-piece with the $Ru(bipy)_3^{2+}$ reagent stream (after passing through PbO_2 column). The merged stream traveled 1.5 cm before passing through a glass spiral flow cell (2 mm i.d., 25 mm dia) placed directly in front of an end window photomultiplier tube (9798B, Electron Tubes, Ruislip, UK). The PMT, glass coil and T-piece were enclosed in a light-tight housing. The PMT was attached to a 2 kV power supply (Burle, PF1053, USA). The detector output was recorded using a chart recorder (Kipp & Zonen BD11, Holland).

Lead Dioxide Column Preparation

An acid-washed glass column (12 mm x 4.0 mm, i.d.) was packed with solid lead dioxide (5 mm x 4 mm packing area) plugged with cleaned cotton wool at both ends and connected to the FI manifold with silicone tubing. The packed column was washed with a stream of water for 15 minutes and incorporated on-line within the FI-CL manifold for $Ru(bipy)_3^{2+}$ oxidation. The life of PbO_2 column was checked by passing 2000 mL solution of $Ru(bipy)_3^{2+}$ (1.0×10^{-5} mol L^{-1}) continuously, the efficiency of the column was found to be unchanged.

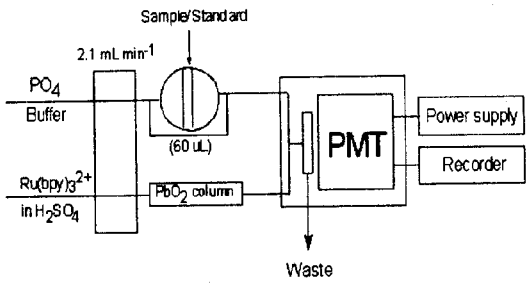

Fig. 4: FI-CL manifold for the determination of trimethoprim.

Conclusions

The proposed FI-CL method for the determination of trimethoprim is simple and rapid

(100 h^{-1} sample throughput). The method is better in the term of sensitivity and sample throughput with the most of the reported methods including FI-CL/ECL. The method has the limit of detection of 0.008 $\mu g\ mL^{-1}$. The method was applied to commercially available pharmaceuticals and the results are in good agreement with the amount of trimethoprim labelled.

Acknowledgments

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