Simple Chemiluminescence Determination of Pilocarpine in Pharmaceuticals and Human Serum

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Abstract

A new method using flow injection analysis (FIA) and chemiluminescence (CL) detection has been developed for the rapid, simple and precise determination of pilocarpine hydrochloride (PH). The method is based on the CL reaction of PH with tris(1,10 phenanthroline)ruthenium(II), $[Ru(phen)_3^{2+}]$, and Ce(IV) in sulfuric acid medium. Effects of chemical variables were investigated using a central composite design (CCD) and the response surface methodology (RSM). Under optimum conditions, the CL intensity was proportional to the concentration of the drug in solution over the range 0.12–40.00 µg mL^{-1}. The limit of detection (S/N = 3) was 34 ng mL^{-1}. The method was applied successfully to the determination of PH in pharmaceutical formulations and spiked human serum. The relative standard deviation for determination of 7 replicates at a level of 1.5 µg mL^{-1} of PH was 0.6%. The minimum sampling rate was 70 samples per hour.

Keywords: Flow injection, Chemiluminescence, Pilocarpine, Central Composite Design

1. Introduction

Pilocarpine (Fig. 1) is a muscarinic alkaloid obtained from the leaves of tropical American shrubs from the genus *Pilocarpus*. It is a non-selective muscarinic receptor agonist in the parasympathetic nervous system, which acts therapeutically at the muscarinic acetylcholine receptor M3 due to its topical application. Pilocarpine has been used in the treatment of chronic open-angle glaucoma and acute angle-closure glaucoma for over 100 years.[1] Pilocarpine also acts on the ciliary muscle and causes it to contract.
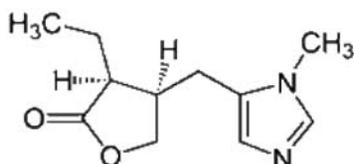
Figure 1. Chemical Structure of Pilocarpine

The monitoring of Pilocarpine Hydrochloride (PH) is important for quality assurance in pharmaceutical industry and for obtaining optimum therapeutic concentrations in body fluids to minimize the risk of toxicity. Therefore, it is important to develop simple and sensitive methods for the determination of this drug.

PH was analyzed by many methods including polarography,[2] spectrophotometry,[3] capillary electrophoresis,[4] mass spectrometry[5–7] and high performance liquid chromatography.[8–12]

According to our best knowledge, this is the first chemiluminescence (CL) method proposed for the determination of pilocarpine. The CL involving $Ru(bpy)_3^{2+}$ is one of the most interesting series of CL reactions. It involves the oxidation of $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$, which is followed by reduction with an analyte species to produce light emission. The luminescence properties of $Ru(phen)_3^{2+}$ are similar to $Ru(bpy)_3^{2+}$ with the exception that $Ru(phen)_3^{2+}$ shows higher sensitivity.[13]

In the experimental design we attempt to monitor the effects of certain inputs or material on the subject mat-

ter of interest. The advantages of the experimental design are many: i) the major advantage is the reduction of the number of experiments and ii) the possibility of studying the interactions among the various factors. A significant interaction implies that changes in one factor may be dependent on the level of the other factor. If this happens, interpretation of the results has to be done cautiously to avoid inaccurate general statements on the individual factors.[14]

In this work a new method has been presented for the determination of PH. It is based on the CL reaction of PH with $Ru(phen)_3^{+2}$ and Ce(IV) in sulfuric acid medium. In the method described, the chemical variables were optimized using a chemometric approach. A central composite design (CCD) was used for the experimental design and response surface methodology (RSM) was used for the modeling. The present method is simple and relatively sensitive; furthermore, it has a wide linear dynamic range and good precision, allowing it has been applied to the determination of PH in drug formulation and spiked human serum.

2. Experimentation

2. 1. Reagents and Preparation of Solutions

All the solutions were prepared by using reagent grade chemicals and doubly distilled water. Acetonitrile was HPLC-grade (Caledon, Canada). PH standard solution (0.400 mg mL^{-1}) was prepared daily by dissolving 40.0 mg PH (Sigma-Aldrich, Steinheim, Germany) in 100.0 mL water. It was stored in a refrigerator and protected from light. Working solutions were prepared by appropriately diluting the stock solution before use. Ru(II) solution (10.0 × 10^{-3} mol L^{-1}) was prepared by dissolving 0.3640 g of dichlorotris (1,10 phenanthroline) ruthenium(II) hydrate (Sigma-Aldrich, Steinheim, Germany) in water and diluting to the mark in a 50.0 mL volumetric flask. Ce(IV) solutions (0.2–6.0 × 10^{-3} mol L^{-1}) were prepared by dissolving proper amounts of ceric ammonium nitrate (Riedel-de Haën, Germany) in calculated volume of H$_2$SO$_4$ (1.0 mol L^{-1}) and diluting to the mark with distilled water in 50.0 mL volumetric flasks.

Serum samples were taken from the health center of the Gorgan University (Iran).

2. 2. Instrumentation

A schematic diagram of the flow system is shown in Fig. 2. The CL signal was measured with a CL analyzer with a head on photomultiplier tube (PMT) and low pass filter whose output was connected to a 16 bit A to D and data processing system. A 12-channels peristaltic pump (Desaga, Model PLG, USA) with three silicon rubber tubes (1.0 mm i.d.) was used. PTFE mixing joints and PTFE tubing (1.0 mm i.d.) was used for the connections.

The flow cell was coil shaped and was kept at a distance of 0.5 cm from the PMT. Sample solutions were injected using a 6-position rotary valve.
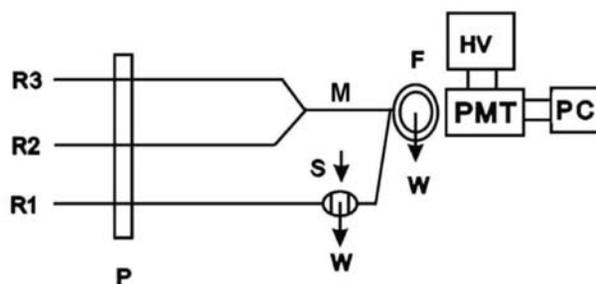
Figure 2. Schematic diagram of flow injection analysis system, R$_1$: H$_2$O, R$_2$: acidic Ce(IV), R$_3$: Ru(II), P: peristaltic pump, S: injection valve, M: mixing coil, F: flow cell, W: waste, HV: high voltage power supply, PMT: photomultiplier tube, PC: computer.

2. 3. Experimental Procedure

As shown in Fig. 2, channels containing carrier H$_2$O (R$_1$), Ce(IV) solution in sulfuric acid (R$_2$) and Ru(II) solution (R$_3$), were pumped by a peristaltic pump. At joint S, an aliquot of standard solution of PH was injected into the carrier stream via a sample injection valve. R$_2$ and R$_3$ solutions were mixed through a mixing coil (silicon tubing, 1.0 mm i.d.) and then mixed with PH exactly in front of the PMT to produce peak-like CL emission that was monitored on a computer to smooth the signal and to determine the peak height.

Before to inject the samples, manifolds were cleaned with a dilute solution of HCl (0.1 mol L^{-1}); subsequently, the reagents including the complex of Ru(II) and acidic Ce(IV) were passed through the manifolds with a low flow rate (0.5 ml min^{-1}) for at least 2 minutes to ensure that adsorption of the reagents on the inner surface of the manifolds had not any effects on the accuracy and precision.

2. 4. Procedure for Preparation of PH Droplets

The content of each eye drop (1% or 2%) was transferred into a small beaker. According to manufacturer's specification, one mL of the drug was diluted in 3–4 steps in order for its concentration to be approximately in the linear range of the calibration graph.

2. 5. Procedure for Spiked Serum

For serum samples only a deproteination pretreatment step using acetonitrile was carried out; an extraction procedure was not necessary.[15] An aliquot of standard solution of PH was added to 1.0 mL of serum sample in a centrifuge tube and mixed for 2 min. Two milliliters of acetonitrile was added, followed by centrifugation at 4000

rpm for 15 min. The protein-free supernatant was transferred into a small conical flask and evaporated to dryness under a stream of nitrogen at room temperature. The dry residue was dissolved in 25.0 mL of water. A blank value was determined by treating drug-free serum in the same way. The absolute recovery was determined by comparing the representative peak height of the serum sample with the peak height of the standard drug at the same concentration.

2. 6. Software

All computations were performed using Matlab (The Math-Works Inc., Natick, MA, USA).

3. Results and Discussion

The method is based on the rapid reduction of $Ru(phen)_3^{3+}$, produced in the reaction between $Ru(phen)_3^{2+}$ and acidic Ce(IV), by PH leading to production of strong CL. The CL reaction mechanism can be expressed by the following reactions according to our previous investigations:[16]

$$Ru(phen)_3^{2+} + Ce(IV) \rightarrow Ce(III) + Ru(phen)_3^{3+}$$

$$PH + Ce(IV) \rightarrow PH^{\bullet+} + Ce(III)$$

$$PH^{\bullet+} \rightarrow PH^{\bullet} + H^+$$

$$Ru(phen)_3^{3+} + PH^{\bullet} + H_2O \rightarrow$$
$$[Ru(phen)_3^{2+}]^* + PH \text{ fragments}$$

$$[Ru(phen)_3^{2+}]^* \rightarrow Ru(phen)_3^{2+} + h\upsilon$$
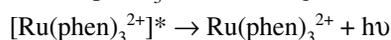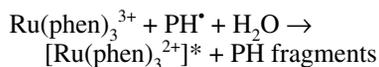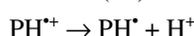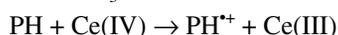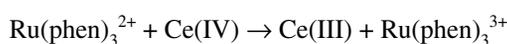
3. 1. Preliminary Investigation

Before undertaking any optimization study it is important to delineate clearly the boundaries of conditions controlling the analysis; therefore initial preliminary experiments using the classical one-factor-at-a-time method served to detect the variables and their respective working ranges that influence the CL intensity. Optimum values obtained by this method for manifold and chemical variables are shown in Table 1.

Table 1. Optimum values for variables obtained by the one-factor-at-a-time method.

Variable	Value
Flow Rate per Channel (mL min^{-1})	3.2
Mixing Coil Length (cm)	10.0
Sample loop Volume (μL)	420
Ce(IV) Conc. ($\times 10^{-3}$ mol L^{-1})	3.0
Ru(II) Conc. ($\times 10^{-3}$ mol L^{-1})	3.0
H$_2$SO$_4$ Conc. (mol L^{-1})	0.09

Concentration of PH: 10 μg mL^{-1}

3. 2. Experimental Design Optimization

After detecting the respective working ranges of the variables, three chemical variables including concentration of Ru(II), Ce(IV) and H$_2$SO$_4$ were studied using RSM. Manifold variables (flow rate, injection volume and mixing coil length) almost had linear changes in the range in which they were studied. Therefore the optimum conditions for the manifold variables gained by the one-factor-at-a-time method were used at RSM optimization of the chemical variables (optimum condition of manifolds supposed to be the same in both one-factor-at-a-time and RSM methods).

A central composite design, including three factors and five levels for each factor ($\alpha = 1.68$), was designed for the experiments. The coded levels of the variables together with their real experimental values are given in Table 2 and experiments are listed in Table 3.

Table 2. Factor levels of the central composite design.

Coded levels	Ce(IV) ($\times 10^{-3}$ mol L^{-1})	Ru(II) ($\times 10^{-3}$ mol L^{-1})	H$_2$SO$_4$ (mol L^{-1})
$-\alpha$	0.48	0.48	0.04
-1	1.5	1.5	0.06
0	3.0	3.0	0.09
$+1$	4.5	4.5	0.12
$+\alpha$	5.52	5.52	0.14

Table 3. Design matrix and responses for the central composite design.

Exp. No.	Ru(II) (X$_1$)	Ce(IV) (X$_2$)	H$_2$SO$_4$ (X$_3$)	Response (mV)
1	-1.68	0	0	62
2	-1	-1	-1	56
3	-1	-1	1	58
4	-1	1	-1	72
5	-1	1	1	92
6	0	-1.68	0	41
7	0	0	-1.68	22
8	0	0	0	121
9	0	0	0	118
10	0	0	0	119
11	0	0	1.68	83
12	0	1.68	0	102
13	1	-1	-1	50
14	1	-1	1	63
15	1	1	-1	44
16	1	1	1	83
17	1.68	0	0	110

In order to describe the way in which the variables are related and the way in which they influence the CL intensity, the Response Surface Methodology (RSM)[17] was

used to assemble the model. Therefore, the data obtained from the set of conditions employed by the CCD were fitted to the following parametric equation (full second order polynomial):

$$I_{CL} = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1X_2 + b_5X_1X_3 + b_6X_2X_3 + b_7X_1^2 + b_8X_2^2 + b_9X_3^2 \quad (1)$$

where I_{CL} is the response, b_0 is the intercept, Xs are the three chemical variables, b_1–b_3 are the linear parameters, b_4–b_6 represent the interaction parameters and b_7–b_9 are the quadratic parameters. Table 4 gives the estimates of 10 parameters contained in Eq. (1), obtained by the least squares.

Table 4. Estimation of 10 parameters including in Eq (1) by least squares.

Source	Parameter	Parameter Estimate	*P* value
Intercept	b_0	105	*< 0.0001*
Ce(IV) A	b_1	12	*0.0067*
Ru(II) B	b_2	4	0.1911
H_2SO_4 C	b_3	12	*0.0513*
AB	b_4	–4	0.4334
AC	b_5	5	0.4125
BC	b_6	6	0.8435
AA	b_7	–7	*0.0038*
BB	b_8	–10	*0.0276*
CC	b_9	–15	*0.0123*
Model		*0.0010*	
Lack of Fit		0.1223	

Factors in italic were found to be statically significant after ANOVA of the results (*p* Value < 0.05 at a 95% confidence level).

Analysis of the residuals from the regression model and the lack-of-fit test revealed that the second order polynomial model, tentatively assumed, would be an adequate description of the surface over the region studied.

The operational optimum was determined using a grid method on Eq. (1); in this method the levels of the factors are submitted into the regression equation while the levels vary gradually from –1.68 to +1.68. The levels corresponding to the maximum response are selected as optimum levels. The optimum levels and their respective actual values are listed in Table 5.

Table 5. The optimum levels and their respective actual values obtained by RSM.

Variable	Coded Level	Optimum Values (mol L^{-1})
Ce(IV)	+0.70	3.46×10^{-3}
Ru(phen)$_3^{+2}$	+0.65	3.43×10^{-3}
H_2SO_4	+0.00	0.09

The results obtained by the experimental design and RSM procedure were better than the one-factor-at-a-time method. The main reason could be due to the interaction between variables. The portion of these interactions in multivariate optimization methods could be calculated and employed in optimization of the variables. But in univariate optimization of variables the effect of these interactions could not be determined. Therefore the optimum conditions obtained by the one-factor-at-a-time method will be different from the real optimum conditions when interactions between variables are important.

3. 3. Analytical Features

A long series of standard solutions of PH were subjected to the optimized CL method for the purpose of calibration. The CL response was found to be linear in the concentration range of 0.12–40.00 μg mL^{-1}, the linear equation was $I_{CL} = 18.96(\pm0.01) C_{PH} + 0.30(\pm0.13)$, ($r^2 = 0.9959$), where I_{CL} is CL intensity and C_{PH} is concentration of PH in μg mL^{-1}. Fig. 3 shows duplicate CL profiles obtained under the optimum conditions of three standard solutions of PH (3.5, 15.0 and 25.0 μg mL^{-1}). The relative standard deviation for determination of 7 replicates of 1.50 μg mL^{-1} of PH was 0.6% and for 0.12 μg mL^{-1} (3 replicates) it was 1.2% (repeatability). The minimum sampling rate obtained was 70 samples per hour. The limit of detection (LOD) was calculated as 3σ/m where σ is the standard deviation obtained from peak to peak noise existing in the blank response and m is the slope in the calibration equation. The LOD obtained was 34 ng mL^{-1}, indicating good detectability. The limit of quantification
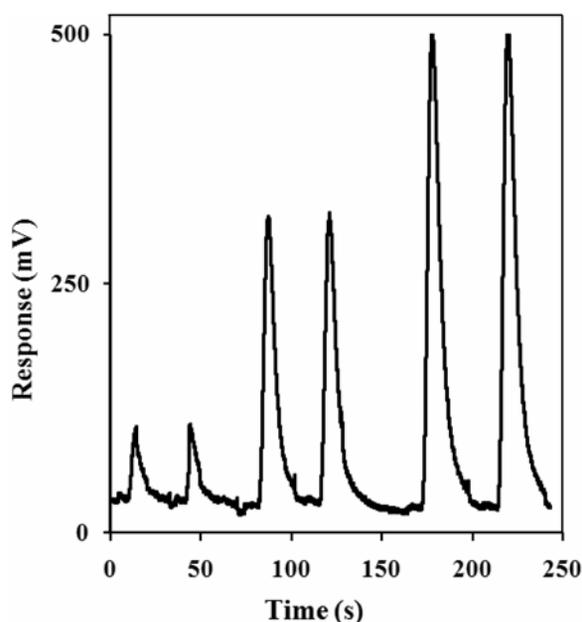
Figure 3. Typical CL profiles obtained by duplicate injection of three standard solutions of PH (3.5, 15, and 25 μg mL^{-1}) under optimum conditions.

(LOQ) was 0.12 µg mL^{-1}. Deviation of the mean (predicted by the calibration equation) from the true value for concentrations of 1.5 µg mL^{-1} (7 replicates) and 0.12 µg mL^{-1} (3 replicates) were 8% and 6%, respectively.

3. 4. Robustness

Robustness was checked by making a slight deliberate change in the experimental procedure leading to the following results: i) injection speed by the user did not have any significant effect on the CL response; ii) flow rate and high voltage parameters must be tightly controlled between experiments (acceptable range for the flow rate is 3.20 ± 0.13 ml min^{-1} and 1000 ± 5 volts for the high voltage); iii) the Ru(II) complex is stable for at least 2 months at 25 °C and stored in the dark; iv) the Ce(IV) solution after preparation must be stored for 24 hrs before being used; v) Ce(IV) solutions are stable for 5 days and vi) the standard and sample solutions were found to be stable for 5 days (at 10 °C).

3. 5. Influence of Interfering Substances

In order to validate the possible analytical application of the method, interference effects of some common ions, excepients in pharmaceutical preparations and some amino acids present commonly in human serum, were studied by recovering 2.0 µg mL^{-1} of PH in the presence of each substance. The tolerance of each substance was taken as the largest amount yielding an error of less than 3σ in the analytical signal of 2.0 µg mL^{-1} PH (σ is the standard deviation in 7-fold determination of 2.0 µg mL^{-1}).

The results in table 6 show that the presence of the common excipients of pharmaceuticals and some amino acids do not interfere in the determination of PH.

3. 6. Application

In order to evaluate the applicability of the proposed method, two eye drops and spiked human serum were an-

Table 6. Maximum limit of foreign substances on the determination of 2 µg mL^{-1} PH under optimized conditions.

Substance	Molar ratio of substance to PH
Sucrose, Glucose, Fructose, Serine, Valine, Threonine, Cl$^-$, Urea	1000
Lactose, Leucine, K$^+$, Na$^+$, NO$_3^-$, Cystine	500
Alanine, Glycine, Proline, HCO$_3^-$, CO$_3^{2-}$, PO$_4^{3-}$, Cu^{2+}, Li$^+$, Cd^{2+}, Ca^{2+}, Uric acid	100
Aspartic acid, Tyrosine, Mg^{2+}	10
Tryptophane, Cysteine, Ascorbic acid	1

Table 7. Determination of PH in drug formulations and human serum

Sample	Sample No.	Added (µg mL^{-1})	Found (µg mL^{-1})	Recovery %
Glupine 2% drop	1	–	2.05 ± 0.01	–
	2	1.00	3.02 ± 0.00	97.0
	3	4.00	6.14 ± 0.01	102.3
	4	8.00	10.32 ± 0.04	103.4
Glupine 1% drop	1	–	0.97 ± 0.00	–
	2	1.00	2.00 ± 0.02	103.0
	3	4.00	5.03 ± 0.03	101.5
	4	8.00	8.91 ± 0.01	98.9
Human Serum	1	0.30	0.33 ± 0.01	110.0
	2	0.80	0.81 ± 0.02	101.3
	3	1.50	1.61 ± 0.02	107.3
	4	4.00	3.83 ± 0.03	95.8

Table 8. Analysis of drug formulations containing PH using the proposed method and the reference (BP) method.

Drug	Nominal value	Analytical Results (g/100 mL)		*t*-test [b]	*F*-test [c]
		Proposed method [a]	Reference method [a]		
Glupine 1% OPH Drop	1g/100mL	0.98 ± 0.01	1.00 ± 0.02	2.53	4.00

[a] mean values of four replicates, [b] Student *t*-test calculated, theoretical value = 3.182 (*P = 0.05*), [c] F-test calculated, theoretical value = 15.44 (*P* = 0.05)

alyzed to determine the PH contents. Table 7 shows the results of the recovery studies of PH from drug formulations and spiked serum. Results show good reproducibility and trueness of the method. The result for one of the eye drops was validated by the British Pharmacopoeia (BP) method using a potentiometric titration method.[18] Statistical analysis of the results using student *t*-test and the variance ratio *F*-test showed no significant difference between the performance of two methods with regards to accuracy and precision (Table 8).

4. Conclusions

A new method was proposed for the flow injection CL determination of PH. The method is simple, sensitive and rapid with a wide linear dynamic range between 0.12 and 40.00 μg mL^{-1} (2.5 orders of magnitudes) and has been used to determine PH in drug formulations and human serum. Common sugars, amino acids and ions do not significantly interference in the determination of PH. Highly accurate and precise determination of PH could be performed in human serum and drug formulations by the proposed method.

5. Acknowledgements

6. References

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Povzetek

Na osnovi pretočne injekcijske analize (FIA) in kemiluminiscenčne detekcije je bila izdelana nova metoda za hitro, preprosto in natančno določevanje pilokarpin hidroklorida (PH). Metoda temelji na kemiluminiscenčni reakciji PH s tris(1,10 fenatrolin)rutenijem(II), [Ru(phen)$_3^{2+}$], in Ce(IV) v žepleno kislem mediju. Za optimizacijo postopka je bil uporabljen sestavljen središčni eksperimentalni načrt z metodologijo odzivne površine. Pri optimalnih eksperimentalnih pogojih je intenziteta kemiluminiscence proporcionalna koncentraciji učinkovine v območju od 0,12 do 40 μg mL^{-1}, meja zaznave izdelane metode je 34 ng mL^{-1}. Metoda je bila uporabljena za določevanje PH v farmacevtskih pripravkih in humanem serumu. Relativni standardni odmik 7 določitev pri koncentraciji PH 1,5 1.5 je bil 0,6 %. Z metodo je možno izmeriti 70 vzorcev v eni uri.