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Chemiluminescence Determination of Hydroxyzine and its Metabolite Cetirizine

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A chemiluminescence (CL) method has been developed for the determination of Hydroxyzine (Hyd) and its metabolite Cetirizine (Cet). The method is based on sensitizing effect of Cet and Hyd on the weak CL reaction between $Ru(phen)_3^{2+}$ and acidic Ce(IV). A mechanism for the CL reaction has been proposed on the basis of UV-Vis, fluorescent and CL spectra. By using the recommended procedure, the calibration graphs were linear over 0.14 to 14.0 μg ml^{-1} (r^2 = 0.9966) and 0.07 to 10.0 μg ml^{-1} (r^2 = 0.9984) for Hyd and Cet, respectively. The limits of detections were 0.06 μg ml^{-1} and 0.03 μg ml^{-1} for Hyd and Cet, respectively. The percent of relative standard deviations (n = 11) for 1.0 μg ml^{-1} of Hyd and 0.74 μg ml^{-1} of Cet were 4.5 and 2.3%, respectively. The broad time profile of Hyd and Cet allowed us to determine Hyd contents in plasma samples with minimum blank interferences from cysteine and ascorbic acid. The method has been satisfactorily used for the determination of Hyd and Cet in syrups, tablets and human plasma.

Keywords: Chemiluminescence, Hydroxyzine, Cetirizine, Pharmaceuticals, Plasma

INTRODUCTION

Hydroxyzine (Hyd) is a first-generation antihistamine of the diphenylmethane and piperazine class (Fig. 1) [1]. Due to its antagonistic effects on several receptor systems in the brain, hydroxyzine is claimed to have anticholinergic, sedative, tranquilizing, antispasmodic, local anesthetic, mild bronchodilative and antiemetic activities as well as antipsychotic properties [2,3]. Cetirizine (Cet) is a chemically active oxidative metabolite of Hyd on the structural basis of a dimer of ethylenediamine (Fig. 1) [4]. Cet has the advantage of lacking the central nervous system depressant effects often encountered in antihistamines [5].

Determination of Hyd and Cet in plasma is necessary for pharmacokinetic studies that require a reliable method with low limit of detection to enable accurate and precise measurements. Moreover, monitoring of Hyd and Cet is important for the quality assurance in pharmaceutical industry. In last decade several methods have been developed for determination of Hyd [5-20] and Cet [5,21-33]. Some characteristics of these methods are shown in Table 1. The methods which use a separation technique before the detection of analytes, besides detection of Hyd and Cet, can provide information about concomitants and related substances such as metabolites in the sample; however, some disadvantages could be along with them, such as time-consuming manipulations, need to superior trainings, cost of instruments and difficulties for miniaturization. Compared to the methods listed in Table 1, the proposed method is a simple and fast analytical tool for the determination of Hyd and Cet in pharmaceuticals and human plasma prior to the use of more complex instrumental techniques.

Intrinsic advantages of chemiluminescence (CL) methods in terms of high sensitivity, wide linear dynamic range, and simple instrumentation have turned them into an

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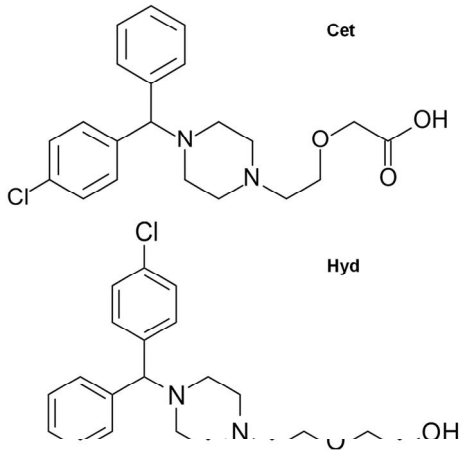

Fig. 1. Chemical structure of Hyd and Cet.

Table 1. Analytical Features of the Methods Proposed for the Determination of Hyd and Cet in Last Decade

Drug	Method	D.L. ($\mu g\ ml^{-1}$)	L.D.R. ($\mu g\ ml^{-1}$)	Sample	Speed (h^{-1})	Ref.
Hyd	HPLC[a]	0.0083	5.01-50.10	-	-	[12]
		-	50-250	Tablet	-	[13]
		-	10-200	Tablet	15	[15]
		0.015	0.04-0.6	Plasma	9	[16]
		-	0.01-10	bulk, Tablet, Serum	-	[17]
	LC[b]	0.2	5-50	Tablet, Syrup	13	[19]
	LC/MS[c]	0.0005	0.00156-0.200	Plasma	17	[14,18]
	Electrochemistry	0.313	0.448-44800	Tablet, Urine	-	[7]
		0.002	0.022-11.2	Tablet	-	[8]
	Spectrophotometry	0.13	1.25-15	Tablet, Urine	-	[9]
		0.62	3.75-45	Tablet, Urine	-	[9]
	CZE[d]	0.00542	1-20	Urine, Plasma	9	[20]
	Titrimetry	-	2-20 (mg)	Tablet	-	[10]
		-	1-9 (mg)	Tablet	-	[11]

Table 1. Continued

	Fluorescence	0.01	0.025-2.00	Plasma	3	[5]
	CL[e]	0.06	0.14-14.0	Tablet, Syrup, Plasma	30	Proposed method
Cet	HPLC	0.10	1.25-10	Capsule	-	[22]
		0.04	0.5-3.0	Tablet, Syrup	15	[24]
		0.26	80-120	Bulk	6	[33]
	HPLC/MS	0.005	-	Plasma	-	[28]
	LC/MS/MS	-	2.5-250 (ng g^{-1})	Brain tissue	4	[25]
		-	0.00025-5	Plasma	4	[25]
		-	0.00025-0.05	Microdialysis	4	[25]
		-	0.001-0.4	Plasma	-	[26]
	LC/MS	-	0.0005-0.3	Plasma	5	[30]
	Electrochemistry	0.033	0.231-4.62	Tablet, Urine	20	[29]
		0.323	0.462-4620	Tablet, Urine	-	[27]
	Spectrophotometry	0.112	2-20	Tablet	-	[32]
		1.0	2.5-20	Tablet	-	[52]
	CZE	0.6	2-50	Tablet	6	[23]
	Fluorescence	0.005	0.025-2.00	Plasma	3	[5]
	CL	0.015	0.05-6.4	Tablet, Syrup, Plasma, Urine	180	[21]
		2.3×10^{-5}	4.6×10^{-4}-0.46	Injection		[53]
		0.03	0.07-10.0	Tablet, Syrup, Plasma	30	Proposed method

[a]HPLC: high performance liquid chromatography. [b]LC: liquid chromatography. [c]LC/MS: liquid chromatography/ mass spectrometry. [d]CZE: capillary zone electrophoresis. [e]CL: chemiluminescence.

important and valuable detection method in analytical chemistry [34]. Accordingly, CL has received much attention in various fields for analysis of substances in pharmaceutical and biological samples [35-41]. After the observations reported by Hercules and Lytle (1966) [42], CL reactions with ruthenium(II) complexes such as tris(2,2-bipyridyl)-ruthenium(II), $Ru(bpy)_3^{2+}$, and tris(1,10-phenanthroline)-ruthenium(II), $Ru(phen)_3^{2+}$ have been extensively studied and exploited in a wide range of analytical applications [43-47]. Compared to $Ru(bpy)_3^{2+}$,

$Ru(phen)_3^{2+}$ produces more intense CL and electrochemiluminescence (ECL) in aqueous solutions [48-51].

In this work, we show that the weak CL emission intensity in the CL system of $Ru(phen)_3^{2+}$-Ce(IV) is greatly enhanced in the presence of Hyd or Cet. This method is the first CL method proposed for the determination of Hyd. The proposed method was successfully used for the quantification of Hyd and Cet in pharmaceuticals and human plasma. The broad time profile of Hyd and Cet in this CL system allowed us to have benefit of time resolve CL to reduce the effect of the blank interference from pharmaceuticals or plasma samples. In this method, rate of analysis for the determination of hydroxyzine is faster than all the previous reported methods (shown in Table 1). Moreover, the sensitivity of the CL method is comparable or even better than spectrophotometric [9], titrimetric [10, 11], HPLC [22,24,33], capillary electrophoresis [23] and electrochemistry [7,27,29] methods.

MATERIALS AND METHODS

Chemicals and Reagents

All solutions were prepared using reagent grade chemicals and double distilled water.

The reference standards of Hyd dihydrochloride and Cet dihydrochloride were gifted by Tehran Darou Co., Iran. Hyd (1.0×10^{-3} M, 448.0 μg ml^{-1}) and Cet (1.0×10^{-3} M, 462.0 μg ml^{-1}) standard solutions were daily prepared by dissolving 0.0448 g Hyd and 0.0462 g Cet with water in 100.0-ml volumetric flasks. Working solutions of Hyd and Cet were prepared by an appropriate dilution of the stock solution when used. Standard solution of $Ru(phen)_3^{2+}$ (0.01 M) was prepared by dissolving 0.3640 g of dichloro tris(1,10-phenanthroline)ruthenium(II) hydrate (Sigma-Aldrich, Germany) with water in a 50.0-ml volumetric flask. In order to prepare each one of Ce(IV) solutions with concentrations between 1.0×10^{-3} and 2.0×10^{-2} M, the calculated amount of ceric ammonium nitrate (Riedel-de Haën, Germany) was dissolved in a proper volume of H_2SO_4 solution (1.0 M) and then the solution was further diluted with distilled water in a 100.0-ml volumetric flask. Final concentration of H_2SO_4 in the Ce(IV) solutions was between 0.03 and 0.20 M. Methanol was purchased from Dr Mojallaly Co., Iran. The plasma from patients was not exposed to any drug, and at least for 72 h (blank plasma) was kindly supplied by health centre of Gorgan, Iran. Hyd and Cet pharmaceuticals were purchased from local drugstores.

Pharmaceutical and Biological Sample Preparation

Methanol was used as protein precipitant of the plasma samples. 2.0 ml methanol was transferred into a centrifuge tube including 1.0 ml plasma. Then, the mixture centrifuged at 6000 rpm for 10 min. The protein-free supernatant was transferred into a 25.0-ml volumetric flask. Then, calculated volume from standard solution of Hyd was transferred into the flask for the purpose of spiking and then the mixture was diluted to the mark.

Ten tablets of Hyd or Cet (10 mg per tablet) were weighed and powdered separately. An accurately weighed portion of each powder, including active ingredients equivalent to 10.0 mg was transferred into a 100.0-ml calibrated flask containing 50 ml water and the mixture was sonicated for 10 min. Then, the volume was adjusted to 100.0 ml with water and the suspension was centrifuged at 6000 rpm for 10 min. To prepare the samples appropriate for recovery study, each sample including 1.0 ml of the supernatant was spiked with diferent volumes of the standard solution of Hyd or Cet and the mixtures were diluted with water in a 100.0-ml volumetric flask.

One mL Hyd syrup drug or 5.0 ml Cet syrup drug was directly transferred into a 100.0-ml volumetric flask and diluted to the mark. 1.0 ml of this solution and a proper volume of the standard solution of Hyd or Cet were transferred into a 50.0-ml volumetric flask and the mixture was diluted to the mark with water.

Apparatus

A laboratory built CL analyzer with a head on photomultiplier tube (PMT) was used in this study. Operating voltage of PMT (Hamamatsu, model R_{329}) was 1200 V. The light emitted by the CL reaction was detected with no wavelength discrimination. Reaction cell was a 0.50-cm path length quartz cell. The block diagram of the instrument is shown in Fig. 2.

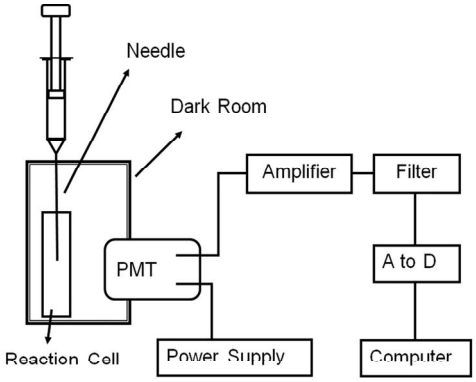

Fig. 2. Schematic block diagram of the CL instrument. PMT: photomultiplier tube; A to D: Analogue-to-digital converter.

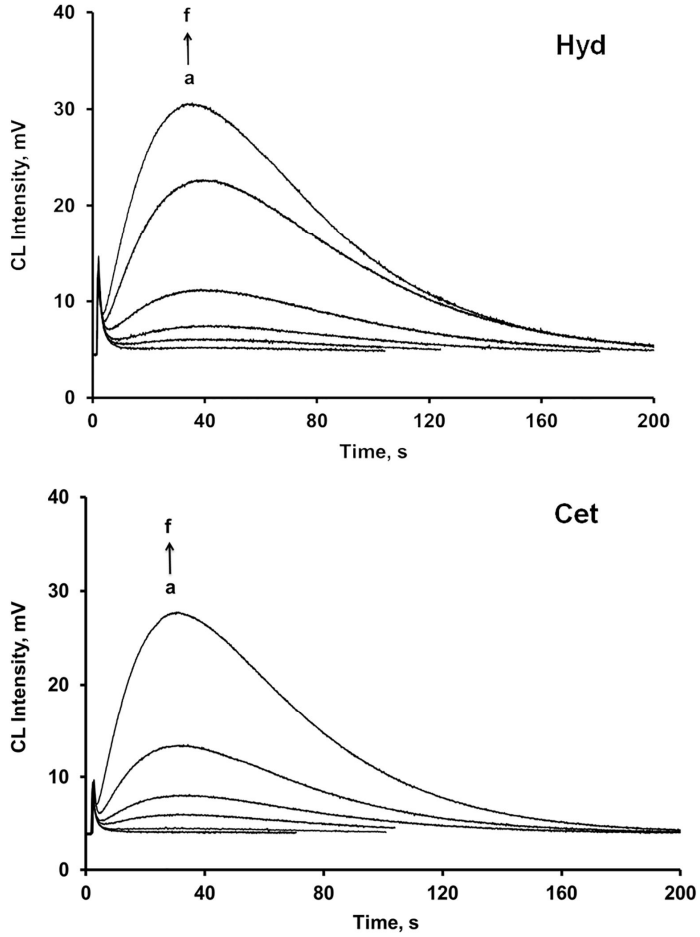

Fig. 3. Typical CL profiles for different concentrations of Hyd and Cet. Concentrations of Hyd were a) blank b) 0.72, c) 1.43, d) 3.58 e) 8.96 and f) 14.33 µg ml^{-1}. Concentrations of Cet were a) blank b) 0.15, c) 0.74, d) 1.48 e) 3.69 and f) 9.24 µg ml^{-1}.

General Procedure

Three different modes of injection were investigated for the quantification of Hyd and Cet. In the first mode, the sample was injected into the mixture of $Ru(phen)_3^{2+}$ and acidic Ce(IV). In the second mode, acidic Ce(IV) solution was injected into the mixture of $Ru(phen)_3^{2+}$ and the sample, and in the third mode, $Ru(phen)_3^{2+}$ solution was injected into the mixture of Ce(IV) and the sample. We found that the highest CL intensity and reproducibility could be attained with the second mode of injection at which acidic solution of Ce(IV) was injected into mixture of $Ru(phen)_3^{2+}$ and the sample. First mode showed a weaker CL signal and the reproducibility in the first and third modes was dependent to the time spent before injection.

In all experiments, the volume of each reagent in the cell was 400 µl and volume of the injected solution was 200 µl. The peak-like CL emissions were recorded in 100 ms time intervals and were automatically collected into an Excel file.

RESULTS AND DISCUSSION

CL Response

CL profiles of Hyd or Cet demonstrated that the CL reaction is slow. It took about 35 s to achieve the maximum peak, compared with 150-200 s for the signal to decline to base. Typical CL profiles for Hyd, Cet and blank samples are shown in Fig. 3. The first maximum at the second 0.9 s is related to the blank (reaction between $Ru(phen)_3^{2+}$ and Ce(IV) solutions without analyte).

Optimization of Chemical Variables

The influences of $Ru(phen)_3^{2+}$, Ce(IV), and H_2SO_4 concentrations on the sensitivity were investigated in presence of Hyd or Cet.

The CL emission depends on the concentration of H_2SO_4. The study was conducted by monitoring the CL time profile for solutions containing variable amounts (0.03 to 0.20 M) of H_2SO_4, 3.0×10^{-3} M Ce(IV), and 2.0×10^{-3} M[1] $Ru(phen)_3^{2+}$. As seen in Fig. 4a, the maximum CL intensity was obtained for 0.1 M H_2SO_4 for both Hyd and Cet.

The CL light intensity also depends on the concentration of Ce(IV) (Fig. 4b). The CL intensity was measured for solutions in which Ce(IV) concentration was varied from $(1.0 \text{ to } 9.0) \times 10^{-3}$ M. Under the conditions noted above, the maximum intensity was obtained at 5.0×10^{-3} M Ce(IV) for both Hyd and Cet.

Under the optimum conditions previously noted, the influence of concentration of $Ru(phen)_3^{2+}$ on the sensitivity was studied in the range of $(0.5\text{-}6.0) \times 10^{-3}$ M of $Ru(phen)_3^{2+}$. The maximum intensity was obtained at 3.0×10^{-3} M of $Ru(phen)_3^{2+}$ for both Hyd and Cet (Fig. 4c).

Analytical Features

CL responses were found to be linear in the concentration ranges of 0.14-14.0 µg ml^{-1} ($r^2 = 0.9966$) and 0.07-10.0 µg ml^{-1} ($r^2 = 0.9984$) for Hyd and Cet, respectively. In the linear ranges, the correlation equations between CL intensity and concentration were $I_{CL} = 1.84C_{Hyd} - 0.09$ and $I_{CL} = 2.75C_{Cet} - 0.17$ for Hyd and Cet, respectively (where I_{CL} is CL intensity (mV) and C_{Hyd} and C_{Cet} are Hyd and Cet concentrations (µg ml^{-1}), respectively). The limit of detection (LOD) was calculated as $3\sigma/m$, where σ is the standard deviation existing in 11 times determination of the blank response and m is slope of the calibration curve. The LODs were 0.06 µg ml^{-1} and 0.03 µg ml^{-1} for Hyd and Cet, respectively. Reproducibility was investigated and the percent of relative standard deviations (n = 11) for 1.0 µg ml^{-1} of Hyd and 0.74 µg ml^{-1} of Cet were 4.5 and 2.3%, respectively. The sampling rate was calculated about 30 samples per hour.

Robustness

The following results obtained in the robustness investigations: i) injection by different users showed that injection rate by the user does not have significant effect on the maximum CL intensity at second 35 (relative standard deviation were between 2 and 5%); ii) according to our investigations [54] the $Ru(phen)_3^{2+}$ complex is stable for at least two months storing in the dark and at 25 ºC; iii) H_2SO_4 and Ce(IV) concentrations are critical parameters and have to be adjusted at 0.1 M and 5.0×10^{-3} M, respectively to obtain maximum sensitivity iv) the highest CL intensity is achieved when acidic Ce(IV) solution is stored for 24 h before being used, v) acidic Ce(IV) solutions are stable for at least five days in the dark and at 25 ºC, and vii) the standard solutions were found to be stable for at least one week (at 10 ºC).

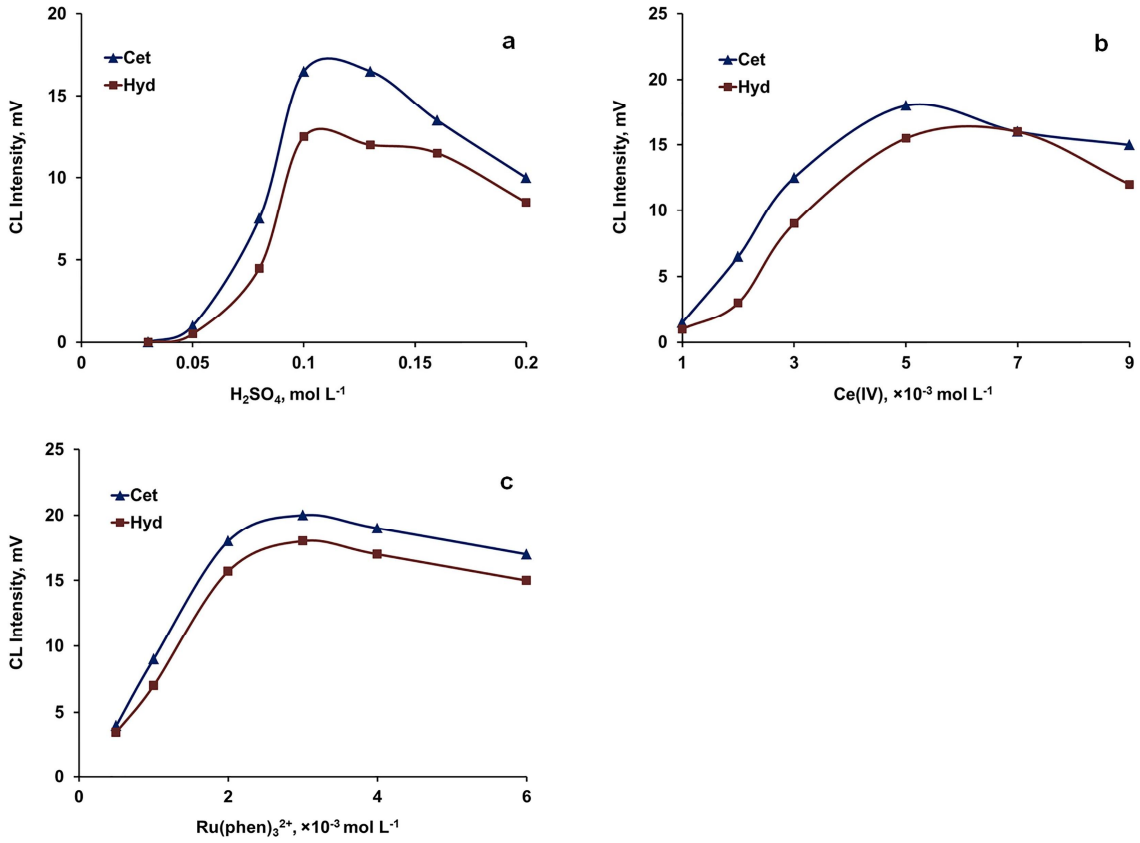

Fig. 4. Optimization of chemical variables; a) H_2SO_4 b) Ce(IV) and c) $Ru(phen)_3^{2+}$. Hyd: 8.96 µg ml^{-1} and Cet: 7.39 µg ml^{-1}.

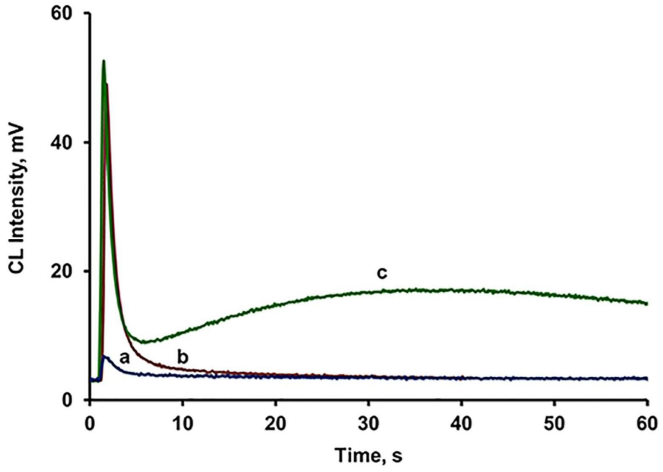

Fig. 5. Typical CL profiles of a) blank (water) b) blank (human plasma) c) Hyd (7.17 µg ml^{-1}) in human plasma.

Influence of Interfering Substances

The selectivity and direct application of the proposed method for analyzing human plasma and formulations were studied by analyzing Hyd in the presence of some ions, excipients in pharmaceutical preparations and amino acids without any prior separation or isolation. The effect of these foreign species under optimized CL conditions was determined by analyzing the standard solution of Hyd (1.4 μg ml^{-1}) in the excess presence of these compounds. The tolerance of each substance was taken equal to the largest amount which yields an error less than 3σ in the analytical signal of 1.4 μg ml^{-1} of Hyd (σ is the standard deviation of response obtained from 11 repeating determination of 1.4 μg ml^{-1} Hyd). The results are shown in Table 2.

Compounds with a tertiary amine or carboxylic acid group, and also some ions can enhance the CL intensity in the reaction between $Ru(phen)_3^{2+}$ and an oxidizing agent [55-59]. The CL kinetic profile of some of investigated compounds presented in Table 2 is very or relatively sharp so that their CL intensity is little or approximately equal to zero at 35 s after starting CL reaction. Therefore, they have little zero blank interference for the determination of Hyd or Cet with a maximum at 35 s after injection of Ce(IV) solution. However, some of the investigated substances in the interference study section such as morphine, tyrosine, and I^- had an inhibition on the CL intensity and their interference remained until 35 s after starting the CL reaction.

Analysis of Pharmaceutical and Biological Samples

The proposed method was applied for the quantification of Hyd and Cet in tablet, syrup and human plasma samples. All experiments involving human subjects were approved by the Golestan University's committee, and they were performed in compliance with the relevant laws and institutional guidelines. Moreover, written consent was obtained from all human subjects prior to the experiment.

The time resolved CL approach was used to reduce the effect of some blank interferences in plasma samples. The broad time profile of Hyd and Cet allowed us to reduce the effect of some interfering substances such as ascorbic acid and cysteine in plasma samples. We used this strategy successfully for determination of carminic acid [60], ketotifen [61] and recently aspirin [62] in real samples. We found that, a plasma sample prepared as mentioned in experimental section, has a sharp peak which its maximum appears at about 1.0 s after injection of Ce(IV) solution and its CL intensity decreases rapidly to baseline after about 10 to 15 s. The CL raised from the plasma sample could be due to its cysteine and ascorbic acid contents which they have a sharp peak in this CL system [61,62]. In the CL system proposed in this study, Hyd or Cet had a broad time profile with a maximum at about 35 s after injection of Ce(IV) solution. Therefore, analytical signal (maximum CL intensity at second 35) could be free of blank interferences from some substances such as ascorbic acid and cysteine in human plasma. As can be seen in Fig. 5, CL kinetic profile of plasma does not have detectable response at 10 s after injection of Ce(IV) solution.

Table 3 shows the analytical recoveries from real samples ranged from 97.6-104.0%.

The results from analyzing of tablets were also certified using the reference method described in British Pharmacopoeia (BP) [63]. Accuracy and precision of the method was revealed by the Student's *t*-value and variance ratio *F*-value. Statistical analysis did not detect any significant difference between the performance of the proposed methods and the reference method. The results of the assay are given in Table 4.

Possible CL Mechanism

The CL mechanism was investigated according to our recently published work [64]. In this CL system, the detection chemistry generally relies upon two key steps. Firstly, chemical or electrochemical oxidation of $Ru(phen)_3^{2+}$ yields $Ru(phen)_3^{3+}$. Secondly, this complex is reduced by an appropriate analyte (or analyte oxidation product) to produce an electronically excited $[Ru(phen)_3^{2+}]^*$, which can return to the ground state by the emission of light [65]. This CL matches the characteristic photoluminescence of $Ru(phen)_3^{2+}$, where a short-lived triplet state is generated by the promotion of an electron from the $t_{2g}d^6$ orbital on the metal to the π* antibonding orbital (a metal-ligand charge transfer; MLCT) [65].

Solution of $Ru(phen)_3^{2+}$ is orange and its color changes

Table 2. Effect of Foreign Substances on the Determination of 1.4 μg ml^{-1} Hyd

Substance	Substance to Hyd[a]
Lactose, Sucrose, Glucose, Fructose, Saccharin, Starch, Valine, Leucine, Serine,	1000
Threonine, Cystine, Benzoate, Methanol, K^+, Na^+, Cl^-, NH_4^+, SO_4^{2-}, Urea, Thiourea	
HCO_3^-, CO_3^{2-}, PO_4^{3-}, Ni^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Proline, Alanine, Glycine, Mannitol	100
Fe^{2+}, Cysteine, Ascorbic acid, Citric acid	25
Tyrosine, Morphine, I^-	1

[a]Molar Ratio of Substance to Hyd.

Table 3. Determination of Hyd and Cet in Real Samples

Sample	Added (μg ml^{-1})	Found (μg ml^{-1})	Recovery (%)
Hydroxizine syrup 10 mg/5 ml	0.000	0.399 ± 0.027	-
	0.448	0.839 ± 0.028	98.2
	1.791	2.262 ± 0.147	104.0
	3.583	4.042 ± 0.175	101.7
Hydroxyzine Tablet 10 mg	0.000	1.017 ± 0.082	-
	0.448	1.460 ± 0.074	98.9
	1.791	2.765 ± 0.155	97.6
	3.583	4.614 ± 0.348	100.4
Cetirizine Tablet 10 mg	0.000	0.993 ± 0.091	-
	0.462	1.460 ± 0.054	101.2
	1.847	2.873 ± 0.169	101.8
	3.694	4.817 ± 0.337	103.5
Cetirizine Syrup 5 mg/5 ml	0.000	0.956 ± 0.075	-
	0.462	1.413 ± 0.065	98.9
	1.847	2.803 ± 0.171	100.0
	3.694	4.746 ± 0.102	102.6
Plasma spiked with Hyd	0.448	0.455 ± 0.071	101.6
	1.791	1.905 ± 0.164	106.4
	3.583	3.377 ± 0.243	94.3

Table 4. Results of Analysis of Tablets by the Proposed Methods and Statistical Comparison of the Results with the Reference Method

Tablet brand name	Nominal value (mg/tablet)	Analytical results[a]		*t*-test[d]	*F*-test[e]
		CL method (mg)	BP method (mg)		
Tedazine 10	10 mg Hyd	10.17 ± 0.82	10.25 ± 0.34[b]	0.25	6.10
Razo Cetirizine 10	10 mg Cet	9.93 ± 0.91	10.32 ± 0.57[c]	1.03	2.55

[a]Mean values of four replications. [b]The method was based on potentiometric titration using 0.1 M perchloric acid as titrant. [c]The method was based on potentiometric titration using 0.1 M sodium hydroxide as titrant. [d]Tabulated *t*-value at the 95% confidence level and for three degrees of freedom is 3.182. [e]Tabulated *F*-value at the 95% confidence level and for three degrees of freedom is 9.28.

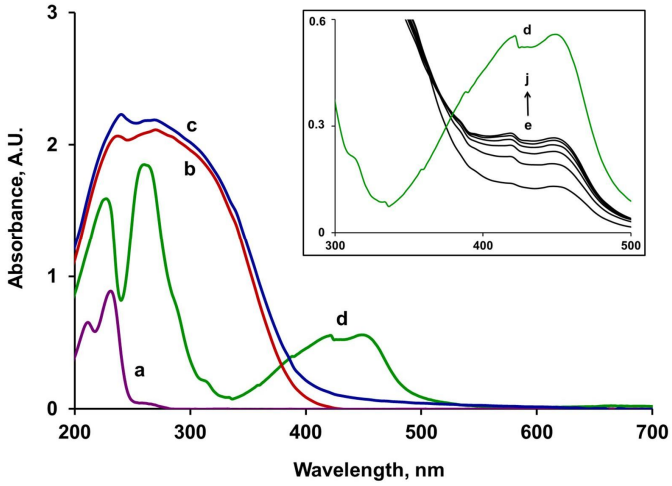

Fig. 6. UV-Vis spectrum of a) Hyd b) Ce(IV) c) Ce(IV)-Hyd d) $Ru(phen)_3^{2+}$, inset: e to j) mixture of $Ru(phen)_3^{2+}$-Ce(IV) with 1 minute intervals. Conditions: a) Hyd (14.3 µg ml^{-1}) b) Ce(IV) (7.0×10^{-4} M in 0.014 M of H_2SO_4) c) same as a and b d) $Ru(phen)_3^{2+}$ (5.0×10^{-5} M) e to j) 2 ml $Ru(phen)_3^{2+}$ (5.0×10^{-5} M) and 1.0 ml Ce(IV) (7.0×10^{-4} M in 0.014 M of H_2SO_4).

to green immediately after mixing with oxidizing agent, Ce(IV) solution, and production of $Ru(phen)_3^{3+}$ [66,67]. During about 3-6 min after mixing of $Ru(phen)_3^{2+}$ with Ce(IV), the color of the mixture changes slowly from green to orange. UV-Vis spectrum of Hyd (spectrum a), Ce(IV) solution (spectrum b), Ce(IV)-Hyd mixture (spectrum c), $Ru(phen)_3^{2+}$ (spectrum d) and the mixture of $Ru(phen)_3^{2+}$-Ce(IV) with one minute interval times (spectrum e to j) are shown in Fig. 6.

As seen in Fig. 6, absorbance in the range 400-500 nm related to the $Ru(phen)_3^{2+}$ complex decreases immediately after mixing the $Ru(phen)_3^{2+}$ solution with Ce(IV) solution (spectrum e) and it increases slowly to its equilibrium value (spectrum f to j). The reason is that, the resulting $Ru(phen)_3^{3+}$ produced in the reaction of $Ru(phen)_3^{2+}$ with acidic Ce(IV), is a powerful oxidant and oxidizes water into

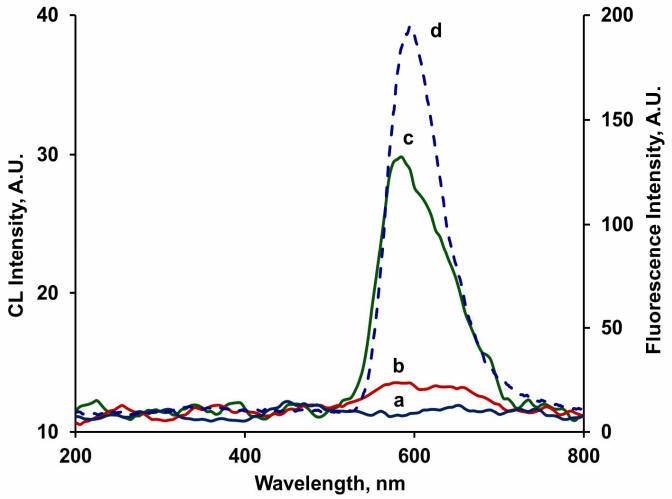

Fig. 7. CL spectra of (a) Ce(IV)-Hyd (b) $Ru(phen)_3^{2+}$-Ce(IV) (c) $Ru(phen)_3^{2+}$-Ce(IV)-Hyd mixtures d) fluorescence emission (λ_{ex} = 325 nm) of $Ru(phen)_3^{2+}$ at 595 nm. Conditions: Hyd: 1.4 µg ml^{-1}, Ce(IV): 5.0×10^{-3} M in 0.1 M of H_2SO_4, $Ru(phen)_3^{2+}$: 2.0×10^{-3} M. Inset.

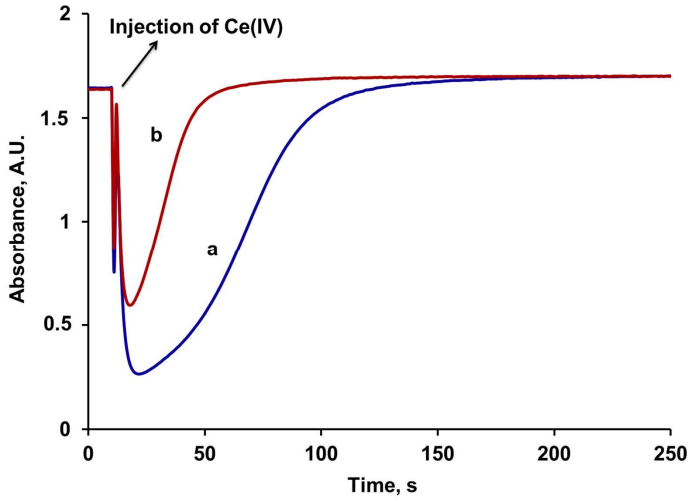

Fig. 8. Time course curve of $Ru(phen)_3^{2+}$ after mixing with Ce(IV) solution at 450 nm. a) in the absence and b) in the presence of Hyd. Conditions: 0.5 ml $Ru(phen)_3^{2+}$ (2.0×10^{-3} M), 0.5 ml Ce(IV) (5.0×10^{-3} M in 0.1 M of H_2SO_4) along with a) 2 ml H_2O b) 2 ml Hyd (23.3 µg ml^{-1}).

O_2 and protons [68]. Therefore, it returns slowly to its reduced state. If there was a reducing agent in the reaction media, it can reduce $Ru(phen)_3^{3+}$ very fast. The electrons from reducing agent transfer to the π*-orbital of phenanthroline ligand and the $Ru(phen)_3^{2+}$ π* metal-to-ligand charge transfer (MLCT) excited state can be produced [69]. The excited electron then undergoes intersystem crossing to the lowest triplet state of

$Ru(phen)_3^{2+}$, from where emission occurs [70].

In order to confirm the mechanism proposed above, some CL pathways might be investigated for the $Ru(phen)_3^{2+}$-Ce(IV)-Hyd CL system, involving the formation of Ce(III)* [71], oxidation products in excited state and $[Ru(phen)_3^{2+}]^*$ [72].

No detectable CL intensity obtained for the mixture of Ce(IV)-Hyd. This suggests that oxidation products and Ce(III)* are not the main emitters. The CL spectrum was scanned with a spectrofluorometer (Spectrolab, model Spectro-96) using batch mode, a fast scan (15000 nm min^{-1}) and with turned off excitation lamp. The CL spectra were acquired as shown in Fig. 7 for Ce(IV)-Hyd (spectrum a), $Ru(phen)_3^{2+}$-Ce(IV) (spectrum b), and $Ru(phen)_3^{2+}$-Ce(IV)-Hyd (spectrum c).

It was clearly indicated that the maximum emission for both mixtures (b and c) was ~595 nm which is the same as maximum fluorescence emission (λ_{ex} = 325 nm) of $Ru(phen)_3^{2+}$ at 595 nm (inset in the Fig. 7). This indicated that the CL spectra were independent of Hyd and the emitter is $Ru(phen)_3^{2+}$.

Hyd is a tertiary amine and from previous studies, the oxidation of tertiary amines is understood to produce a short-lived radical cation. The α-carbon is then deprotonated, yielding a strongly reducing intermediate. This reduces $Ru(phen)_3^{3+}$ (produced by oxidant) to the excited state that subsequently emits light [59,73-75]. To establish this phenomenon, the changes in the concentration of $Ru(phen)_3^{2+}$ were also obtained after mixing with Ce(IV) solution at 450 nm, according to our prevous work [64]. In this way, time course curve for $Ru(phen)_3^{2+}$ was obtained in the presence (Fig. 8b) and absence (Fig. 8a) of Hyd as a reducing agent. Because there is no an interference from Ce(IV), Hyd and Ce(III) at 450 nm, the absorbance at 450 nm is proportional to $Ru(phen)_3^{2+}$ concentration. As seen in Fig. 8, for solution including Hyd (Fig. 8b), smaller decrease in absorbance and faster equilibrium occurre. After injection of the Ce(IV) solution and in the presence of Hyd, $Ru(phen)_3^{2+}$ received to the highest concentration after about 50 seconds but in the absence of Hyd it lasts about 120 s. This phenomenon shows that Hyd can reduce $Ru(phen)_3^{3+}$ and it can speedup production of $Ru(phen)_3^{2+}$ from $Ru(phen)_3^{3+}$.

According to the above discussion, the following mechanism is proposed for the CL reaction of Hyd or Cet.

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

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

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

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

$$[Ru(phen)_3^{2+}]^* \rightarrow Ru(phen)_3^{2+} + h\upsilon\ (595\ nm)$$

CONCLUSIONS

The proposed method is the first reported use of a CL reaction for the determination of Hyd and the second CL method for the determination of Cet. The method is based on the enhancement effect of Hyd and Cet on the CL reaction of $Ru(phen)_3^{2+}$ with acidic Ce(IV). The broad time profile of Hyd and Cet allowed us to reduce the effect of some interfering substances such as ascorbic acid and cysteine in plasma samples. Therefore, the method is simple for the determination of Hyd and Cet in pharmaceuticals and human plasma.

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