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In Vitro Monitoring Sub-Nanogram Amounts Analgin in Human Urine by Its Inhibitory of the Luminol-Periodate Chemiluminescence Reaction Using Reagent Immobilization Release Technique

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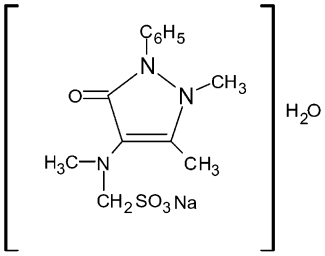
Abstract—A selective and sensitive as well as rapid chemiluminescence (CL) flow sensor for the determination of analgin is described. The analytical reagents involved in chemiluminescence reaction, luminol and periodate, were both immobilized on an anion-exchange column. The CL signals produced by the reaction between luminol and periodate, which were eluted from the column through water injection, were decreased in the presence of analgin. Analgin was sensed by measuring the decrement of CL intensity, and which was observed linear over the logarithm of analgin concentration range of 0.1 to 50.0 ng mL^{-1}, and the limit of detection was 0.04 ng mL^{-1} (3ó). At a flow rate of 2.0 mL min^{-1}, including sampling and washing, the detection could be performed in 0.5 min with a relative standard deviation of less than 3.0%. The proposed procedure was applied successfully in the monitoring of analgin in human urine samples without any pre-treatment process. It was found that the analgin concentration reached its maximum after being orally administrated for 4 h, and the analgin metabolism ratio in 10 h was 9.28% in the body of volunteers. The flow sensor offered reagentless procedures and remarkable stability in determination of analgin, and could be easily reused over 80 h.

Introduction

Analgin (novalgin, metamizol, dipyrone), which is the sodium salt of [(2,3-dihydro-1,5-dimethyl-3-oxo-2-phenyl-*1*H-pyrazol-4-yl) methylamino] methanesulfonic acid, is a pyrazolone derivative with a strong analgesic, antipyretic and spasmolytic activity.



Analgin forms the active constituent of several pharmaceutical preparations and its determination in these formulations is therefore very important. This compound has been determined in tablets and injections by spectrophotometry,[1–9] near-infrared spectroscopy,[10] fluorometry,[11] electrometric,[12,13] chromatography,[14,15] titrimetry,[16] densitometry,[17] as well as spot test.[18]

Chemiluminescence (CL) has been successfully extended to reactions involving organic compounds of pharmaceutical or biological interests.[19–21] It proved to be useful for analytical applications and increasing investigations resulted in highly sensitive and selective detection methods. We have currently reported on the luminol-$Fe(CN)_3^{3-}$ CL system determination VB_2, rutin and berberine using flow injection technology.[19–21] Huang et al.[22,23] have described a few CL methods for determination of analgin in tablets on the basis of Rhodamine 6G or Ce^{4+} oxidation CL system. It is well known that methods for the determination of urinary analyte generally include a sample preparation step and isolation of the drug from the sample matrix prior to analysis. Huang et al.[24] also reported a method on the Rhodamine 6G CL system in spiked urine samples with four steps treated urine samples. However, this method has

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relatively complex devices and time consuming procedure. This is undesirable not only for operational convenience, but also for the cost, environment and resource considerations.[25] However, no reports have been found on the CL monitoring of anaglin in human urine during the metabolism so far.

It is well known that the fast oxidation reaction between luminol and periodate in alkaline medium produces a strong CL signal.[26,27] We found that the CL intensity from the oxidation between luminol and periodate could be inhibited in the presence of analgin. To achieve a homogeneous mixing of CL reagents, which will result in a more stable background and reproducible results, both CL reagents were immobilized on anion-exchange resin. Through injection of 100 μL water, the CL reagents on the resin were eluted and the CL intensity was decreased in the presence of analgin, by which analgin could be detected.

The system responded linearly in a concentration range from 0.1 to 50 ng mL^{-1} with a relative standard deviation of less than 3.0%. At a flow rate of 2.0 mL min^{-1}, the procedure could be performed within 0.5 min, including sampling and washing, giving a throughput of about 120 times per hour. The method has an extremely low limit of detection down to 40.0 pg mL^{-1}, thus it can be applied directly in the assay of human urine and some pharmaceutical preparations without any pretreatment.

Experimental

Reagents

All chemicals used were of analytical-reagent grade. Doubly distilled water was used throughout. Luminol (Fluka, biochemika) was obtained from Xi'an Medicine Purchasing and Supply Station, China. Potassium periodate was purchased from Xi'an Chemical Reagent Plant. Analgin was obtained from Shaanxi Institute for Drug Control.

Analgin for calibration was prepared from analgin stored at 4 °C. Luminol was used as supplied to prepare a 0.25 mol stock standard solution in 0.5 mol L^{-1} NaOH in a 1000 mL calibrated flask. A 0.04 mol L^{-1} stock standard solution of potassium periodate was made by dissolving the solid in distilled water and diluting to 250 mL in a calibrated flask.

Preparation of resin with immobilized reagents

Amberlyst (from Rohm and Haas Co.) A-27 (2.0 g) was shaken with 50 mL 0.25 mol L^{-1} luminol or 0.04 mol L^{-1} potassium periodate for 12 h, and then the resin was filtered, washed with doubly distilled water and dry-stored. The most convenient method to determine the amounts of luminol and potassium periodate immobilized was to measure the losses of these reagents from the immobilization solutions. The concentration was detected at 360 nm for luminol and at 225 nm for potassium periodate by UV–vis. In the proposed method, the amounts of luminol and potassium periodate immobilized were 1.99 ($\pm$0.02, $n=3$) mmol g^{-1} and 1.01 ($\pm$0.01, $n=3$) mmol g^{-1} resin, respectively.

Apparatus of flow injection system

The flow injection (FI) system used in this work is shown in Figure 1. A peristaltic pump (Shanghai meter electromotor plant, Model ND-15, 15 r/min) was used to generate the flows. PTFE tubing (1 mm i.d.) was used in the flow system. The anion-exchange resins containing immobilized luminol (0.05 g) and potassium periodate (0.10 g) were mixed together and packed into a glass column (3 mm i.d. and total volume of about 0.5 mL) and plugged with glass wool at both ends to prevent the resins from leaking. A six-way valve injected 100 μL of eluant. Before reaching the flow cell, the streams of luminol, potassium periodate, sodium hydroxide and analyte were combined in a mixing tube (50 mm in length). The CL emission cell is a twisty glass tube (1 mm i.d., 15 cm length) in order to produce a large surface area exposed to the adjacent photomultiplier tube (PMT) (Hamamatsu, Model IP28). Extreme precautions were taken to ensure that the sample compartment and PMT were light tight. The CL signal produced in flow was detected without wavelength discrimination, and the PMT output was amplified and quantified by a luminosity meter (Xi'an Remax Electronic Science-Tech. Co. Ltd. Model GD-1) connected to a recorder (Shanghai Dahua Instrument and Meter Plant, Model XWT-206).

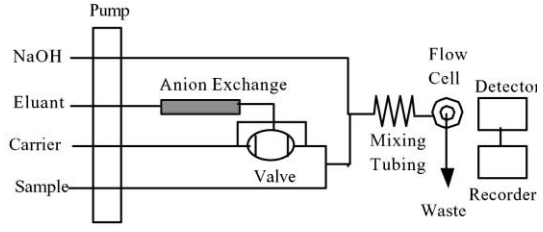

Figure 1. Schematic diagram of the flow-injection system for analgin determination.

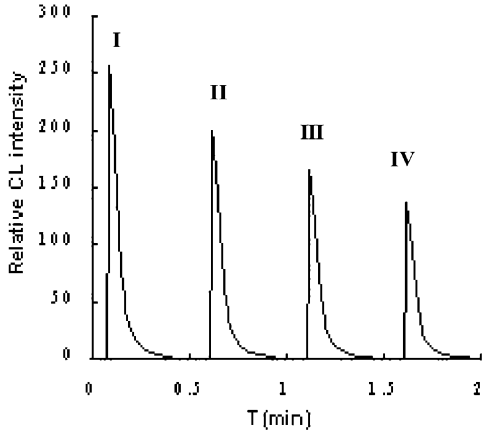

Figure 2. CL time profile in the batch system: **I**: CL intensity in the absence of analgin; **II**: CL intensity in the presence of analgin (0.5 ng mL^{-1}); **III**: CL intensity in the presence of analgin (1.0 ng mL^{-1}); **IV**: CL intensity in the presence of analgin (3 ng mL^{-1}).

Procedures

The carrier water and the solutions (NaOH, sample and eluant) were propelled at a constant flow rate on each flow line. The pump was started to wash the whole flow system until a stable baseline was recorded. Then 100 μL eluant solution was injected into the carrier stream, luminol and periodate were eluted quantitatively, which was then mixed with the sample stream, the mixed solution was delivered to the CL cell, and the peak height of the CL signal was detected with the PMT and the luminometer. The concentration of sample was quantified by decreased CL intensity, $\Delta I = I_o - I_s$, where I_o and I_s were CL signals in the absence and in the presence of analgin, respectively.

Results and Discussion

The CL intensity–time profile

Before carrying out the flow injection method, the batch method for the CL profiles was used. Without any special eluant, the mixture of luminol and periodate rinsed by water gave out an evident CL signal. As Figure 2 shows, the CL intensity reached a maximum 10s after injection, and then died within 25 s. On joining of the sample into the above mixing solution, a decreased CL signal was recorded. The peak heights of the CL emission were proportional to the logarithm of analgin concentration.

Designation for the FI–CL system

The assay could be carried out by a continuous-flow mode in two different manifolds. Through injection of 100 μL eluant (5.0×10^{-5} mol L^{-1} of Na_3PO_4), the reagents on the anion-exchange resin column were eluted and in the presence of analgin, the CL intensity was decreased, and the decrease of CL intensity was recorded. It was found that while the column with immobilized reagents was put in front of or behind the valve, two significantly different results were observed. As illustrated by results in Figure 3, the whole analysis process, including sampling and washing, could be accomplished in 0.5 min when the column was put in front of the valve *viz* Figure 1 manifold, whereas it must take more than 2.0 min when the column was put behind the valve and also manifold Figure 1 gave the better precision. Therefore, the manifold depicted in Figure 1 was chosen for subsequent work.

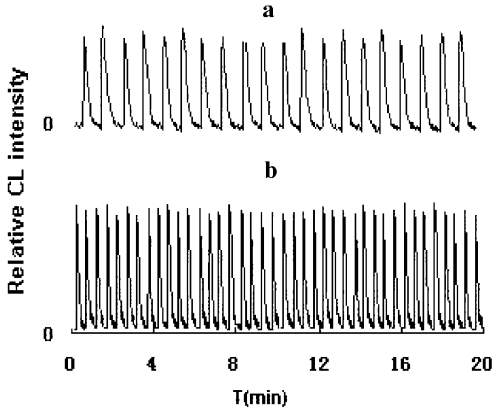

Figure 3. CL signals in two manifolds. a: The column set behind the injector; b: the column set in front of the injector.

Selection of eluant

One-hundred microliters different eluant were injected through the resin column and releasing different amounts of luminol and periodate, thus producing the CL emission. The results are shown in Table 1. It was found that sodium sulfate gives a maximum CL emission while sodium carbonate shows some inhibitive effects on the CL reaction. Nevertheless, it was observed that a continuous flow of eluant through the column results in a rather short lifetime of sensor down to only a few hours. It was shown that the immobilized luminol and periodate anions on the anion-exchange resin undergo dissociation with water, thus release trace amounts of luminol and periodate from the column, and the decrease of analgin CL signal could be easily observed. In this case, the column could be used over 80 h. As a compromise between higher CL intensity and longer lifetime of the column (discussed in Applications), water was used as eluant in subsequent work.

Effect of pH on CL and sensor lifetime

The best pH of eluant (water) on the performance of the system was evaluated. It was found that along with the increase of pH in eluant, the CL intensity decreased while the lifetime of sensor decreased considerably (Fig. 4). This phenomenon is probably due to the quantities

Table 1. Character of eluants for analgin determination[a]

Type of CL intensity	Relative CL intensity				
	H_2O	NaCl	Na_2CO_3	Na_2SO_4	Na_3PO_4
I	758	1038	370	1362	1218
II	435	599	252	764	699
III	323	449	118	598	519

[a]The concentration of each solution was 1.0×10^{-4} mol L^{-1}.
I: CL intensity in the absence of analgin.
II: CL intensity in the presence of 1.0 ng mL^{-1} analgin.
III: The decrease of CL intensity.

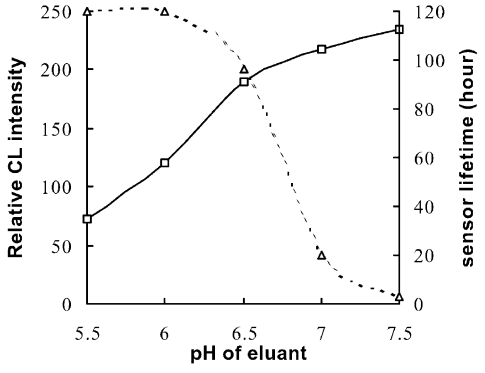

Figure 4. —□— Effect of eluant pH on CL intensity. —△— Effect of eluant pH on sensor lifetime.

of hydroxide ions in eluant were increasing. A pH of 6.0 was then chosen as a compromise between lifetime and a sufficient CL intensity. In this case, the column with immobilized CL reagents could be used more than 80 h in continuous-injection system.

Effect of molar ratio of immobilized luminol and periodate

To examine the influence of the mixing ratio, resins (0.15 g) with different mixing ratios were packed into column with same internal diameter and volume. By the injection of water at a fixed volume of 100 μL, different amounts of luminol and periodate were eluted from the resins and emitted CL signals with different intensity. As Figure 5 shows, the CL intensity dropped drastically from beginning to next day, then it went down slowly. The most stable CL signal was found with a molar ratio of 1:2 (luminol to periodate) and a middling CL intensity is in favor of measuring an enhancive effect of analgin on CL reaction.

Effect of NaOH concentration

It was found that luminol reacts with periodate and emits CL signal only in an alkaline medium. As Figure 6 shows, a NaOH concentration less than 0.05 M lead to an apparent decrease in ΔI. The maximum intensity was found with 0.1 M NaOH. While concentration of NaOH is higher than 0.2 M, there is a scattering effect in flow cell due to the discrepancy between refractive index of various components. Thus 0.1 M NaOH was selected as an optimal condition.

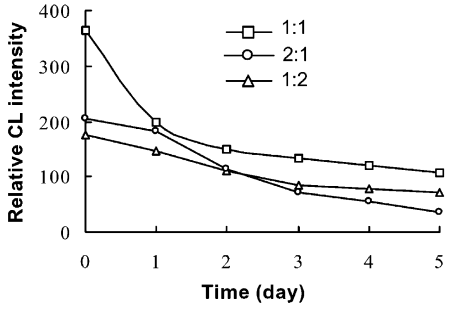

Figure 5. Effect of molar ratio of luminol and periodate on CL intensity and sensor lifetime.

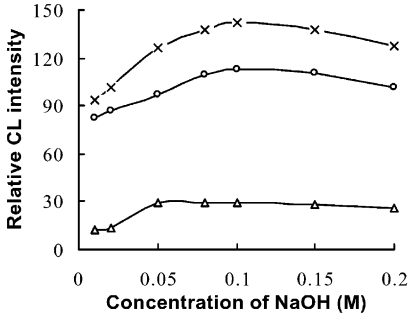

Figure 6. Effect of concentration of NaOH on CL intensity. —○— CL intensity in the presence of analgin (I_s); —×— CL intensity in the absence of analgin (I_o); —△— The decreased of CL intensity (ΔI).

Effect of flow rate and the length of mixing tubing

The CL signal was also dependent on the flow rate of carrier and eluant. The signal-to-noise rate decreased at a higher flow rate because the higher flow rate would impact the rate of contact of sample molecules with the ion-exchange resin. The lower flow rate caused broadening of the peak and slowing down of the sampling rates. Nevertheless, the high flow rate could lead to an unstable baseline and shortening of the sensor lifetime. A rate of 2.0 mL min^{-1} was then chosen as a compromise between good precision and lower reagent consumption.

The length of the mixing tubing was also adjusted to yield maximum light emission in the cell. It was found that a 5.0 cm of mixing tubing afforded the best results as regards sensitivity and reproducibility.

Performance of the flow sensor for analgin measurements

Under the above optimum conditions, the linearity of analgin was tested by determining a series of standard solutions with the flow sensor. The inhibited CL intensity was found to be proportional with the logarithm of analgin concentration. As Figure 7 shows, the linear range is from 0.1 ng mL^{-1} to 50.0 ng mL^{-1} and the regression equation is

$$\Delta I = 13.952 \mathrm{LnC}_{\mathrm{analgin}} + 47.863 \qquad R^2 = 0.9995.$$

The relative standard deviation of five determinations was 1.52% with analgin concentration of 0.5 ng mL^{-1}, and the limit of detection was 0.04 ng mL^{-1}. At a flow rate of 2.0 mL min^{-1}, the determination of analyte could be performed in 0.5 min, including sampling and washing, giving a throughput of about 120 times per hour with a relative standard deviation of less than 3.0%.

Interference studies

The effect of foreign ions was tested by analyzing a standard solution of analgin, to which increasing amounts of interfering ions were added. The tolerable

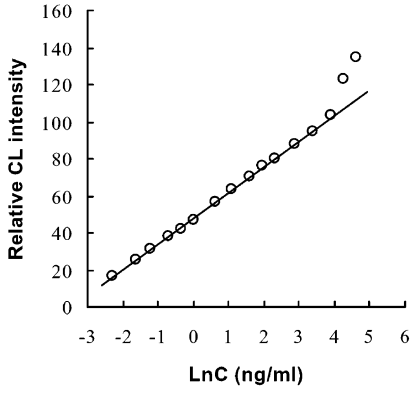

Figure 7. ΔI versus natural logarithm of analgin concentration. The concentration range of analgin is from 0.1 to 50.0 ng mL^{-1}.

concentration ratios with respect to 1.0 ng mL^{-1} analgin for interference at 3.0% level were over 1500 for Cl^-, NO_3^-, Ac^-, I^-, SO_4^{2-}, PO_4^{3-}, $Cr_2O_7^{2-}$, borate, oxalate, urea, and 1200 for NH_4^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+}, Cr^{3+}, and 800 for methanol, ethanol, and 20 for Cu^{2+}, Fe^{3+}, and 5 for uric acid, respectively. Common excipients such as starch and sugar in tablets do not interfere in the determination. Compounds abundant in human urine such as urea, uric acid, salt and glucose have almost no effect on the determination of analgin at sub-nanogram level.

Operational stability of the sensor

One-hundred microliters of eluant (water) was flow-injected through the system in the presence of 5.0 ng mL^{-1} analgin solutions and the ΔI ($I_o - I_s$) was recorded to test the operational stability of the sensor. The experiment lasted for 10 days and the flow system was regularly used over 8 h per day. Figure 8 shows the stability of the flow sensor, and the average of ΔI was calculated in 10 spot check determinations with RSD less than 3.0%. The flow sensor showed remarkable stability and could be easily reused over 80 h.

Applications

Determination of metabolic analgin in human urine

Following the procedure described in Experimental, the proposed method was applied to the determination of analgin in human urine samples. Two apparently healthy male volunteers took analgin tablets orally in the morning with empty stomach. According to the marked content, the net dosage of analgin they took is 1000 mg. From then on, first-voided urine samples were collected in dark glass bottles after 1, 2, 3, 4, 5, 6, 7, 8 and 10 h, respectively.

Urine samples were collected from volunteers, diluted with distilled water directly and sometimes supplemented with analgin to test the recovery of the method. Thus, urinary analgin could be determined relatively simply by FI–CL without any pre-treatment procedures. To eliminate the interference of uric acid, the urinary analgin concentration has to be diluted to pg mL^{-1} level with a uric acid concentration lower than 5.0 ng mL^{-1}. The results of trial determinations are summarized in Table 2. The metabolic profile of analgin is shown as in Figure 9. From the curve it could be seen that analgin was metabolized rapidly after taking analgin tablets. The total analgin excreted through urine was 92.8 mg in a total volume of 1.37 L in 10 h. The concentration of analgin reached its maximum after orally administrated for 4 h and dropped sharply within a few hours, and the analgin metabolism ratio in 10 h was 9.28% in the body of volunteers.

Determination of analgin in spiked urine

In order to demonstrate the selectivity of the proposed method toward analgin, the values of the analgin in spiked urine obtained by the method has been compared with HPLC method, and the results of trial determination are summarized in Table 3. The relation of results between the proposed method and HPLC is shown in Figure 10, and the regression equation is

$$Y_{CL} = 0.996X_{HPLC} - 0.012 \quad R^2 = 0.9994$$

It is shown that the results obtained by the proposed method agreed well with those by HPLC.

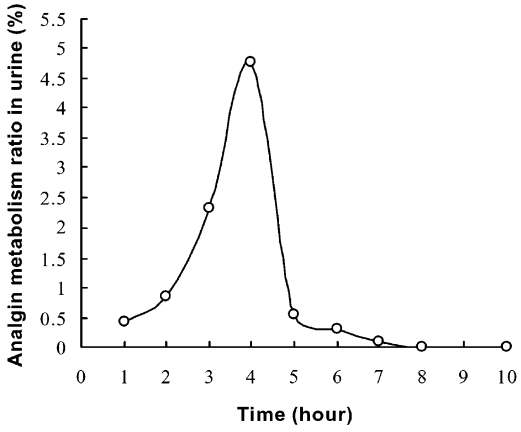

Figure 9. Metabolism of analgin in human urine.

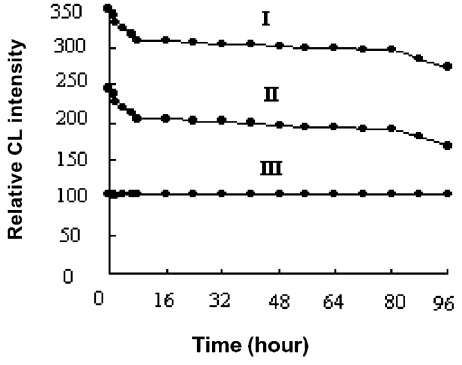

Figure 8. Stability of the flow sensor. **I**: CL intensity in absence of analgin (I_o); **II**: CL intensity in presence of 1.0 ng mL^{-1} analgin (I_s); **III**: the decreased of CL intensity ($\Delta I = I_o - I_s$).

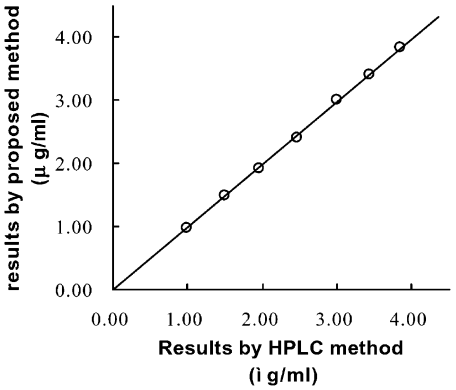

Figure 10. Comparison between the analytical results obtained by the proposed method and by the HPLC method.

Determination of analgin in pharmaceutical preparations

The proposed method also was applied to the determination of analgin in pharmaceutical preparations. Three different preparations were purchased from the local market. The measured analgin contents (an average of five determinations) are listed in Table 4. The results obtained by the proposed method were 470 mg per tablet, 435 mg per tablet and 453 mg per tablet, which were well in agreement with results obtained by HPLC. The recovery studies were performed on each of the analyzed samples by adding a known amount of analgin to the sample before the recommended treatment and the experimental results were also verified by *t*-test.

Table 2. Results of analgin in human urine samples[a]

Time (h)	Analgin supplement ($ng\,mL^{-1}$)	Mean* ($ng\,mL^{-1}$)	Recovery (%)	RSD (%)	Analgin in urine $M_{(mg)}/V_{(mL)}$	Analgin metabolism ratio in urine (%)	*t*-test ($t_{0.05,4}=2.78$)
	0	0.11		3.6			
1			103.0		4.40/200	0.44	0.15
	1.0	1.14		2.3			
	0	0.14		1.0			
2			111.0		8.40/300	0.84	3.02
	1.0	1.25		0.7			
	0	0.77		0.8			
3			86.0		23.10/150	2.31	0.69
	1.0	1.63		0.7			
	0	0.85		0.2			
4			108		47.60/280	4.76	0.25
	1.0	1.93		0.8			
	0	0.55		1.5			
5			112		5.50/50	0.55	0.24
	1.0	1.67		0.6			
	0	0.19		1.2			
6			90.2		3.01/80	0.30	1.43
	1.0	1.09		1.2			
	0	0.08		2.5			
7			88.0		0.80/50	0.08	0.41
	1.0	0.96		1.3			
	0	0.0		1.6			
8			110.0		0.0/40	0.0	0.08
	1.0	1.10		3.1			
	0	0.0		0.2			
10			98.0		0.0/220	0.0	1.29
	1.0	0.98		0.2			
					Total: 9.28/1370	Total: 9.28%	

[a]The average of five determinations.

Table 3. Determination of the studied drugs in spiked urine

Concn taken ($\mu g\,mL^{-1}$)	Results by the proposed method[a]						Results by HPLC
	Analgin supplement ($ng\,mL^{-1}$)	Mean* ($ng\,mL^{-1}$)	Recovery (%)	RSD (%)	Analgin in urine ($mg\,mL^{-1}$)	*t*-test ($t_{0.05,\,4}=2.78$)	Analgin in urine ($\mu g\,mL^{-1}$)
	0	9.80		0.73			
10			108.0		0.98	1.42	0.99
	5	15.2		1.1			
	0	15.0		2.1			
15			109.1		1.50	0.28	1.48
	5	20.5		0.71			
	0	19.6		1.3			
20			92.3		1.96	0.45	1.92
	5	24.2		1.4			
	0	24.6		0.44			
25			91.7		2.46	13.7	2.41
	5	29.2		1.5			
	0	30.0		2.2			
30			112.5		3.00	0.28	3.01
	5	35.6		2.1			
	0	34.4		0.55			
35			105.9		3.44	0.59	3.40
	5	39.7		1.7			
	0	38.5		0.89			
40			90.0		3.85	0.78	3.84
	5	43.0		2.4			

[a]The average of five determinations.

Table 4. Results of analgin in different pharmaceutical preparations

Sample batch number	Results by the proposed method[a]						Results by HPLC
	Found (ng mL^{-1})	Added (ng mL^{-1})	Total (ng mL^{-1})	Recovery (%)	Content (mg tablet^{-1})	t-test ($t_{0.05,4}=2.78$)	Content (mg tablet^{-1})
00904	4.7	5.0	9.6	98.2	0.470	0.00	0.452
00637	4.5	5.0	9.5	98.7	0.453	0.12	0.436
93758	4.4	5.0	9.5	103.8	0.435	1.04	0.431

[a]The average of five determinations.

Table 5. The results of detecting periodate–analgin by UV at 254 nm

Species[a]	A[b]
Periodate	0.061
Analgin	>2
Periodate + Analgin	0.293

[a]The same concentration and injection volume (10^{-4} mol L^{-1}, 25 mL).
[b]The average of five determinations.

The possible mechanism

In the present work, chemiluminescence kinetic characteristics of the CL reaction of luminol–periodate–analgin were studied in detail. It was found that the rate of the reaction of periodate with analgin in solution was very fast. The reaction process was followed by UV at 254 nm in flow system and the results are listed in Table 5. It was obvious that the absorption intensity of analgin decreased quickly in the presence of periodate. It was also found that the product of reaction between periodate and analgin could not oxidize luminol chemiluminescently. Hence, the mechanism of the inhibit effect of analgin on luminol–periodate CL system could be presented as below:

$$\text{analgin (Red, colorless)} \xrightarrow{IO_4^-} \text{analgin (Ox, yellow)}$$

$$\text{periodate} + \text{luminol} \xrightarrow{OH^-} \text{aminophthalate} + h\nu\ (\lambda_{max}425\ \text{nm})$$

Conclusions

By combination with flow injection system, a novel chemiluminescence sensor was constructed by using reagent immobilization release technology for quantitative analysis of analgin in urine and medicine in this paper. The method was applied successfully to the determination of thiamine in pharmaceutical preparation and human urine samples without any pre-treatment. Compared with other methods for the determination of analgin, the proposed continuous flow sensor offers advantages in instrumental simplification, high sensitivity and reducing reagent consumption. Being conformed by its low detection limit and operational stability, the continuous flow sensor developed possessed good reproducibility, selection, precision and recovery in assay of analgin in bio-fluids.

Acknowledgements

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