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In vitro detecting ultra-trace novalgin in medicine and human urine by chemiluminescence

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Abstract

A sensitive chemilumimetric method for the determination of novalgin at the sub-nanogram level is presented. The method is based on immobilized luminol and dichromate chemiluminescence detection coupled with a flow injection system. The intensity of the chemiluminescence can be strongly inhibited by novalgin and the decrement of CL intensity was linear with the logarithm of novalgin concentration in the range of 5.0×10^{-11} to 5.0×10^{-8} g ml^{-1}. The detection limit is 2.0×10^{-11} g ml^{-1} (3σ) and the relative standard deviation is 2.57% ($n = 5$) for a 1.0×10^{-10} g ml^{-1} novalgin sample. A typical analytical procedure, including sampling and washing, could be performed in 1 min at a flow rate of 2.0 ml min^{-1}, giving a throughput of 60 h^{-1}. The proposed procedure was applied successfully in pharmaceutical preparations and furthermore the monitoring of novalgin in human urine without any pre-treatment process during 10 h. It was found that the novalgin concentration reached its maximum after orally administrated for about 4 h, and the novalgin metabolism ratio in 10 h was 10.83% in the body of volunteers. The flow system offered reagentless procedures and remarkable stability in determination of novalgin, and could be easily reused over 600 times.

Keywords: Novalgin; Flow injection; Chemiluminescence; Urine

1. Introduction

Novalgin (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. It is present in many commercial analgesic preparations. While long-term application of novalgin was associated with cases of the allergic reactions, therefore the analysis of novalgin in biofluids was necessary. So various methods have been established for the quantitative determination of novalgin in pharmaceutical preparations and biological fluids, including 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].

In recent years more and more strict regulations related to the quality control of pharmaceuticals led to increasing demands on the simplicity and rapidity of analytical assay [19–21]. We have

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currently reported on the luminol–$K_3Fe(CN)_3$ CL system for determination of isoniazid [22], riboflavin [23] and berberine [24] and the luminol–KIO_4 CL system determination of rutin [25] using flow injection (FI) CL system. Huang et al. has reported the CL methods for determination of novalgin in tablets on the basis of Rhodamine 6G, Ce^{4+} and hydrogen peroxide oxidation CL with the linear rang of concentration from 50 to 10 μg ml^{-1} [26–28]. However, there was no report dealing with a CL monitoring novalgin in human urine during the metabolism so far.

In this work, a method based on luminol–$K_2Cr_2O_7$ CL system was proposed to quantify the content of novalgin in pharmaceutical preparations and human urine. Through injection of 100 μl eluant, the reagents on the anion-exchange resin column were eluted and in the presence of novalgin, the CL intensity was decreased, by which novalgin could be detected. It was found that the decrement of CL was linear over the logarithm of the novalgin concentration range of 0.05–50.0 ng ml^{-1} with a relative standard deviation of less than 3.0%. The proposed procedure was applied directly in the assay of some pharmaceutical preparations and body fluids without any pretreatment.

2. Experimental

2.1. Reagents

All chemicals used were of analytical reagent grade. Double-distilled water was used throughout. Luminol (Fluka, Biochemika) was obtained from Xi'an Medicine Purchasing and Supply Station, China. Potassium dichromate was purchased from Xi'an Chemical Reagent Plant. Novalgin was obtained from Shaanxi Institute for Drug Control. Novalgin for calibration was prepared from novalgin stored in a brown calibrated flask at 4 °C. Luminol was used as supplied to prepare a 0.025 mol l^{-1} stock standard solution in 0.5 mol l^{-1} NaOH in a 1000-ml calibrated flask.

2.2. Preparation of resin with immobilized reagents

Amberlyst (from Rohm and Haas Co.) A-27 anion-exchange resin (2.0 g) was shaken with 50 ml 0.025 mol l^{-1} luminol or 0.01 mol l^{-1} potassium dichromate for 12 h, and then the resin was filtered, washed with double-distilled water and dry-stored. The most convenient method to determine the amounts of luminol and potassium dichromate immobilized was to measure the losses of these reagents from the immobilization solutions. The concentration was detected at 360 nm for luminol and at 352 nm for dichromate by UV–vis. In the proposed method, the amounts of luminol and potassium dichromate immobilized were 1.99 ± 0.02 mmol g^{-1} ($n = 3$) and 2.40 ± 0.01 mmol g^{-1} ($n = 3$) resin, respectively. (The amounts of periodate and permanganate immobilized were 1.01 ± 0.01 mmol g^{-1} ($n = 3$) and 1.23 ± 0.02 mmol g^{-1} ($n = 3$), respectively.)

2.3. Apparatus of flow injection system

The FI system used in this work is shown in Fig. 1. A peristaltic pump (Shanghai meter electromotor plant, model ND-15, 15 rpm) was used to generate the flows. PTFE tubing (1 mm i.d.) was utilized in the flow system. The anion-exchange resins containing immobilized with luminol (0.05 g) and dichromate (0.10 g) were mixed together and packed into a glass column (3 mm i.d. and total volume of about 0.5 ml), which was plugged with glass wool at both ends to prevent the resins from leaking. A six-way valve was employed to inject 100 μl of eluant for elution of immobilized reagents. Before reaching the flow cell, the streams

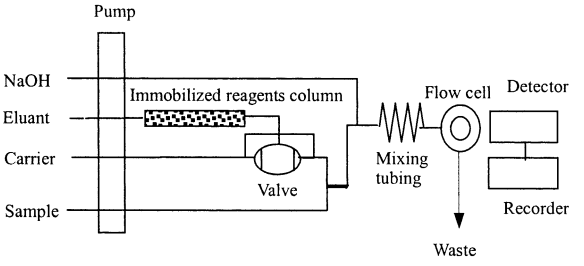

Fig. 1. Schematic diagram of the flow-injection system for novalgin determination.

of luminol, potassium dichromate, sodium hydroxide and analyte were merged in a mixing tube (50 mm in length). The CL emission cell is a spiral glass tube (1 mm i.d., 15 cm length), producing 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 the CL emission cell 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).

2.4. 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 of eluant solution was injected into the carrier stream, luminol and dichromate 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 decrease of 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 novalgin, respectively.

3. Results and discussion

3.1. The CL intensity–time profile

Before the FI method was carried out, the batch method for the CL profiles was employed. Without any special eluant, the mixture of luminol and dichromate rinsed by water gave an evident CL signal. As Fig. 2 shows, the CL intensity reached a maximum 12 s after injection, and then died within 50 s. When the sample was added into the above mixing solution, a decreased CL signal was recorded. The decrements of peak heights of the CL emission were proportional to the logarithm of novalgin concentration.

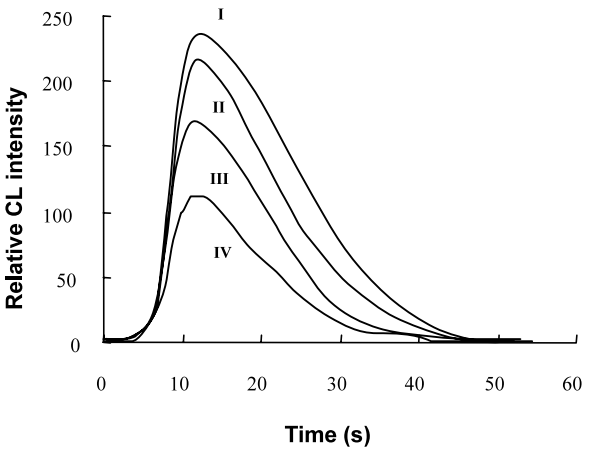

Fig. 2. CL time profile in the batch system. I, CL intensity in the absence of novalgin; II, CL intensity in the presence of novalgin (0.3 ng ml^{-1}); III, CL intensity in the presence of novalgin (3.0 ng ml^{-1}); IV, CL intensity in the presence of novalgin (30.0 ng ml^{-1}).

3.2. Selection of oxidants

Commonly used oxidants, including $KMnO_4$, $K_3Fe(CN)_6$, $K_2Cr_2O_7$, KIO_4 and H_2O_2, were injected into luminol solution in a static system, and the results of producing the CL emission are shown in Table 1. It was found that luminol-dichromate–novalgin gives a maximum decrement of CL intensity (ΔI), which suggested the luminol-dichromate CL system was more sensitive and selective for determination of novalgin.

3.3. Selection of immobilized reagents columns

To test the influence of different columns with immobilized reagents, resins (0.15 g) with different immobilized reagents were packed into a glass column with an internal diameter of 5 mm and total volume of about 0.6 ml. The experiments lasted for 10 h and the CL intensity was measured in average of five determinations every 1 h. While injecting 100 μl water, different amounts of reagents were eluted from the resins and CL intensity proceeded was shown in Fig. 3. The

Table 1
Character of oxidants for novalgin determination

Types of CL intensity	Relative CL intensity[a] ($n = 5$)				
	$KMnO_4$	$K_3Fe(CN)_6$	$K_2Cr_2O_7$	KIO_4	H_2O_2
I_o	10	29	25	176	21
I_s	7	29	8	169	20
$I_o - I_s$	3	0	17	7	1

The concentration for novalgin, luminol and oxidant were 5.0 ng ml^{-1}, 5×10^{-7} mol l^{-1}, 5×10^{-5} mol l^{-1}, respectively. I_o, the CL intensity of luminol-oxidant system. I_s, the CL intensity of luminol-oxidant–novalgin system. $I_o - I_s$, the decrement of CL intensity (ΔI).

[a] HV = −650 V.

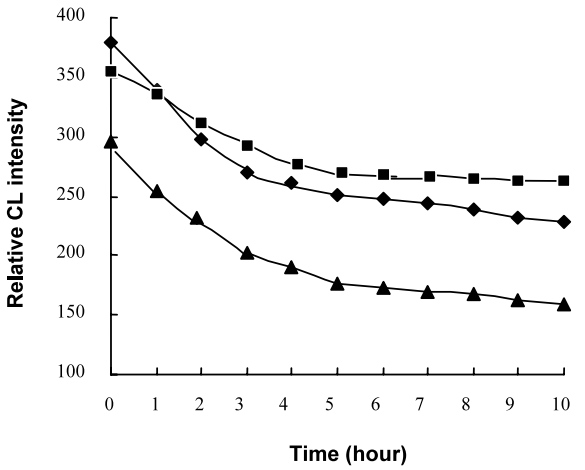

Fig. 3. Character of columns with different reagents. The column with immobilized luminol-periodate (—◆—); the column with immobilized luminol-dichromate (—■—); the column with immobilized luminol-permanganate (—▲—).

good stability and signal-to-noise ratio was found with immobilized luminol-dichromate column.

3.4. 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 novalgin, the CL intensity 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 Fig. 4, the whole analysis process, including sampling and washing, could be accomplished in 1.0 min if the column was put in front of the valve manifold, that is, as shown in Fig. 1, whereas the process took more than 2.0 min if the column was put behind the valve manifold. Fig. 1 gave the better precision; therefore, the manifold depicted in Fig. 1 was chosen for subsequent work.

3.5. Selection of eluant

One hundred microliter of different kinds of eluant was injected through the resin column, releasing different amounts of luminol and dichromate, thus producing the CL emission. The results are shown in Table 2. It was found that sodium sulfate gives a maximum CL emission on the CL

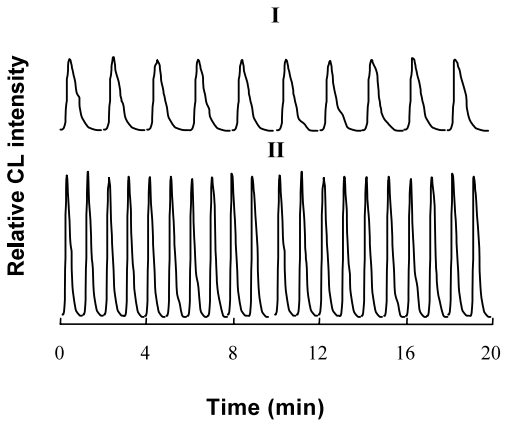

Fig. 4. CL signals in two manifolds. I, The column set behind the injector; II, the column set in front of the injector.

Table 2
Character of eluants for novalgin determination

Type of CL intensity	Relative CL intensity				
	H_2O	NaCl	Na_2CO_3	Na_2SO_4	Na_3PO_4
I	77	104	96	150	85
II	67	98	83	96	75
III	10	6	13	54	10

The concentration of each solution was 1.0×10^{-4} mol l^{-1}. I, CL intensity in the absence of novalgin; II, CL intensity in the presence of 10.0 ng ml^{-1} novalgin; III, the decrease of CL intensity.

reaction. Nevertheless, it was observed that a continuous flow of eluant through the column results in a rather short lifetime of the column down to only a few hours. It was shown that the immobilized luminol and dichromate anions on the anion exchange resin undergo dissociation with water, thus release trace amounts of luminol and dichromate from the column, and the decrease of CL signal could be easily observed. In this case, the column could be used over 15 days. As a compromise between higher CL intensity and longer lifetime of the column (discussed in Section 4), water was used as eluant in subsequent work.

3.6. Effect of pH on CL and column 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 increased while the lifetime of the column decreased considerably (Fig. 5). This phenomenon is probably due to the increasing quantities of hydroxide ions in eluant. A pH of 6.5 was then chosen as a compromise between column lifetime and sufficient CL intensity. In this case, the column with immobilized CL reagents could be used more than 120 h in continuous-injection system.

3.7. Effect of molar ratio of immobilized luminol and dichromate

To examine the influence of the mixing ratio, resins (0.15 g) with different mixing ratios were packed into a column with the same internal diameter and volume. By the injection of water

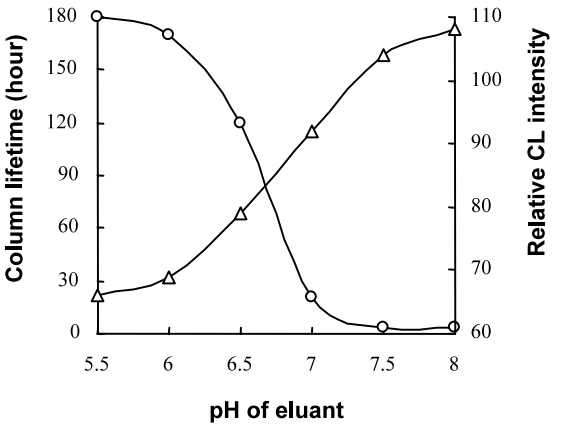

Fig. 5. Effect of eluant pH on column lifetime (—○—); effect of eluant pH on CL intensity (—△—).

at a fixed volume of 100 μl, different amounts of luminol and dichromate were eluted from the resins and different CL intensities were emitted. As Fig. 6 shows, the CL intensity dropped

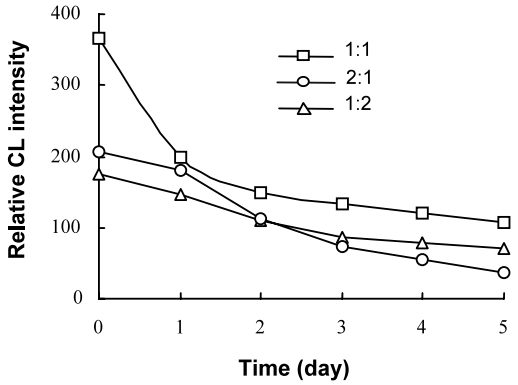

Fig. 6. Effect of molar ratio of luminol and dichromate on CL intensity and column lifetime.

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 vs dichromate), which was then used for the following determination.

3.8. Effect of sodium hydroxide concentration

It was found that luminol reacts with dichromate and emits CL signal favourably in an alkaline medium. The concentration of NaOH less than 0.05 mol l^{-1} leads to an apparent decrease in ΔI. The maximum intensity was found with 0.05 mol l^{-1} NaOH. While concentration of NaOH is higher than 0.1 mol l^{-1}, there is a scattering effect in the flow cell due to the discrepancy between refractive indices of various components. Thus, 0.05 mol l^{-1} NaOH was selected as an optimal condition.

3.9. 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 ratio decreased at a higher flow rate because the higher flow rate would influence the rate of contact of sample molecules with the ion-exchange resin. The lower flow rate caused broadening of the peak and slowing of the sampling efficiency. Nevertheless, the high flow rate could lead to an unstable baseline and shortening of the column lifetime. A rate of 2.0 ml min^{-1} was then chosen as a suitable condition with 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 5.0 cm of mixing tubing afforded the best results with regard to sensitivity and reproducibility.

3.10. Performance of the flow system for novalgin measurements

A series of novalgin standard solutions were injected into the manifold depicted in Fig. 1 under the above optimum conditions to test the linearity of novalgin. The decrement of CL intensity was found to be proportional with the logarithm of novalgin concentration. The linear range is from 0.05 to 50 ng ml^{-1} and the regression equation is

$$\Delta I = 40.147 \ln C_{\text{novalgin}} + 48.005 \quad R^2 = 0.9973.$$

The relative standard deviations of five determinations were 2.86, 1.78, 1.22% with novalgin concentrations of 0.1, 1, 10 ng ml^{-1}, and the limit of detection was 20 pg ml^{-1}. At a flow rate of 2.0 ml min^{-1}, the determination of analyte could be performed in 1 min, including sampling and washing, giving a throughput of about 60 times per hour with a relative standard deviation of less than 3.0%.

3.11. Interference studies

The effect of foreign ions was tested by analyzing a standard solution of novalgin, to which increasing amounts of interfering ions were added. The tolerable concentration ratios with respect to 1.0 ng ml^{-1} novalgin for interference at 5.0% level were over 3000 for Ca^{2+}, Cl^-, NO_3^-, I^-, SO_4^{2-}, PO_4^{3-}, methanol, ethanol, and oxalate, 1600 for Li^+, Mg^{2+}, Zn^{2+}, Pb^{2+}, Ba^{2+} and borate, 300 for myoglobin and globulin, 100 for Mn^{2+}, and Fe^{3+}, 20 for uric acid. Common excipients such as starch and sugar in tablets do not interfere in the determination. Compounds abundant in human

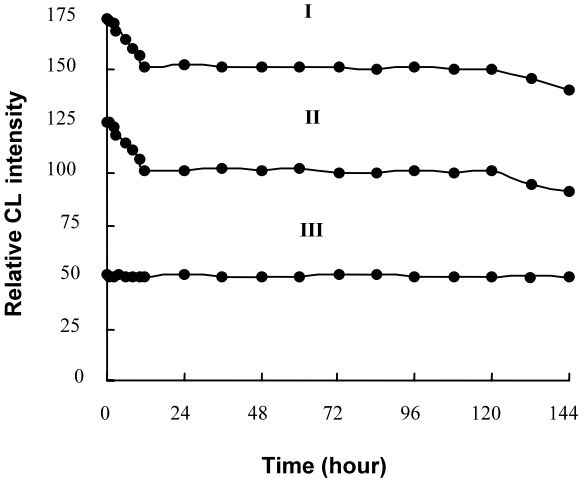

Fig. 7. Stability of the flow system. I, CL intensity in absence of novalgin (I_o); II, CL intensity in presence of 1.0 ng ml^{-1} novalgin (I_s); III, the decrease of CL intensity ($\Delta I = I_o - I_s$).

Table 3
Results of novalgin 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 (g tab^{-1})	Content (g tab^{-1})
00908	18.3	20	37.5	96.3	0.438	0.435
00837	18.6	20	37.8	92.9	0.461	0.459
63583	17.6	20	38.0	102.2	0.451	0.449

[a] The average of five determinations.

urine such as urea, uric acid, salt, glucose and albumin have almost no effect on the determination of novalgin at sub-nanogram level.

3.12. Operational stability of the flow system

One hundred microliters of eluant (water) was flow-injected through the system in the presence of 1.0 ng ml^{-1} novalgin solution and the ΔI ($I_o - I_s$) was recorded to test the operational stability of the immobilized reagents column. The experiment lasted for 15 days and the flow system was regularly used for more than 8 h per day. Fig. 7 shows the stability of the immobilized reagents column, and the average ΔI was calculated in 10 spot check determinations with RSDs of less than 3.0%. The flow system showed remarkable stability and could be easily reused for more than 120 h.

4. Applications

4.1. Determination of novalgin in pharmaceutical preparations

The proposed method was also applied to the determination of novalgin in pharmaceutical preparations. Three different preparations were pur-

Table 4
Determination of the studied drugs in spiked urine

Conc. spiked (ng ml^{-1})	Results by the proposed method[a]					Results by HPLC
	Novalgin supplement (ng ml^{-1})	Mean (ng ml^{-1})	Recovery (%)	RSD (%)	Novalgin in urine (μg ml^{-1})	Novalgin in urine (μg ml^{-1})
10	0	9.7	111.7	0.73	0.97	0.98
	10	20.9		1.1		
15	0	15.1	108.3	2.1	1.51	1.49
	10	25.9		0.71		
20	0	19.4	90.0	1.3	1.94	1.94
	10	28.4		1.4		
25	0	25.2	106.4	0.44	2.52	2.49
	10	35.8		1.5		
30	0	29.8	92.8	2.2	2.98	3.00
	10	39.1		2.1		
35	0	34.3	91.3	0.55	3.43	3.41
	10	43.4		1.7		
40	0	39.1	92.5	0.89	3.91	3.87
	10	48.4		2.4		

[a] The average of five determinations.

Table 5
Results of novalgin in human urine samples

Time (h)	Novalgin supplement (ng ml^{-1})	Mean (ng ml^{-1})	Recovery (%)	RSD (%)	Novalgin in urine $M_{(mg)}/V_{(ml)}$	Novalgin metabolism ratio in urine (%)	t-test ($t_{0.05,4} = 2.78$)
1	0	0.10	103.0	2.6	4.30/220	0.43	1.08
	1.0	1.13		3.5			
2	0	0.31	111.0	2.0	11.20/180	1.12	1.96
	1.0	1.42		1.7			
3	0	0.84	86.0	0.9	23.50/140	2.35	0.86
	1.0	1.70		1.8			
4	0	1.04	108	2.2	52.10/250	5.21	1.23
	1.0	1.12		1.8			
5	0	0.36	112	0.5	10.90/150	1.09	0.74
	1.0	1.48		1.3			
6	0	0.11	90.0	2.4	3.90/180	0.39	2.29
	1.0	1.01		0.8			
7	0	0.06	88.0	2.5	1.80/150	0.18	1.51
	1.0	0.94		1.2			
8	0	0.03	110.0	1.8	0.60/90	0.06	2.98
	1.0	1.13		2.3			
9	0	0.0	95.0	2.5	0.0/150	0.0	1.34
	1.0	0.95		1.4			
10	0	0.0	92.0	0.8	0.0/110	0.0	0.65
	1.0	0.92		1.2			
Total					10.83/1520	10.83%	

The average of five determinations.

chased from the local market. The measured novalgin contents (an average of five determinations) were listed in Table 3. The results obtained by the proposed method were 438 mg per tablet, 461 mg per tablet and 451 mg per tablet, which were in well agreement with results obtained by HPLC. The recovery studies were performed on each of the analyzed samples by adding a known amount of novalgin to the sample before the recommended treatment.

4.2. Determination of novalgin in spiked urine

In order to demonstrate the selectivity of the proposed method toward novalgin, the values of the novalgin in spiked urine obtained by the method have been compared with HPLC method, and the results of trial determination were summarized in Table 4. The regression equation which shows the relation of results between the proposed method and HPLC is $Y_{CL} = 0.990X_{HPLC} + 0.012$, $R^2 = 0.9997$. It is shown that the results obtained by the proposed method agreed well with those by HPLC.

4.3. Determination of metabolic novalgin in human urine

The proposed method was also applied to the determination of novalgin in human urine samples. Two apparently healthy male volunteers took novalgin tablets (1000 mg) in morning with empty stomach. From then on, first-voided urine samples were collected in dark glass bottles after 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 h, respectively. After urinary novalgin was diluted with distilled water directly, it could be determined relatively simply by FI–CL without any pre-treatment procedures. The results of trial determinations are summarized in Table 5 and the metabolic profile of novalgin is shown as in Fig. 8. From the curve it could be seen that novalgin was metabolized rapidly after taking novalgin tablets. The total novalgin excreted through urine was 108.3 mg in a total volume of 1.52 liter in 10 h. The concentration of novalgin reached its maximum after orally administrated for 4 h and dropped sharply within a few hours, and the novalgin metabolism ratio in 10 h was 10.83% in the body of volunteers.

4.4. The possible mechanism

A possible CL mechanism of luminol-dichromate–novalgin was proposed by studying chemiluminescence kinetic characteristics of the CL reaction in detail. It was found that the rate of the reaction of dichromate with novalgin 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 6. It was obvious that the absorption intensity of novalgin increased quickly in the presence of dichromate. It was also found that the product of reaction between dichromate and novalgin could not oxidize luminol chemiluminescently. Hence, the mechanism of the inhibitory effect of novalgin on luminol-dichromate CL system could be presented as below:

$$\text{novalgin (Red state, colorless)} + \text{dichromate} \rightarrow \text{novalgin (Ox state, yellow)}$$

$$\text{dichromate} + \text{luminol} \xrightarrow{OH^-} \text{chromium (III)} + h\nu\ (\lambda_{max}\ 425\ \text{nm})$$

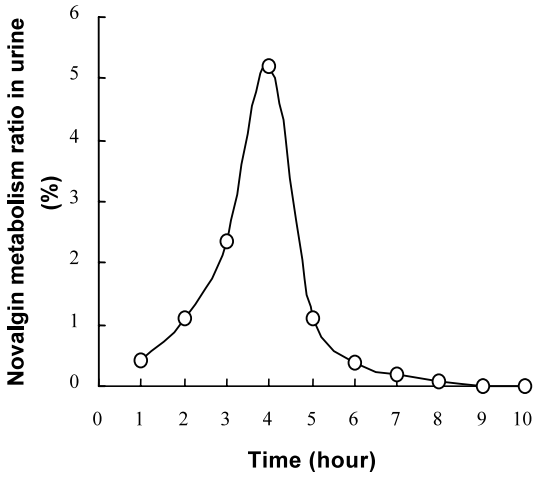

Fig. 8. Metabolism of novalgin in human urine.

Table 6
The results of detecting dichromate–novalgin by UV at 254 nm

Species[a]	A[b]
Dichromate	0.172
Novalgin	0.288
Dichromate+novalgin	0.616

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

5. Conclusions

The proposed method is rapid, simple and sensitive than the existing manual and automated methods. The method makes possible rapid and direct determination of novalgin in body fluids without tedious pretreatment.

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