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Bioorganic & Medicinal Chemistry Letters 14 (2004) 4127–4130
Bioorganic & Medicinal Chemistry Letters

In vitro monitoring of nanogram levels of puerarin in human urine using flow injection chemiluminescence

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Received 20 May 2004; revised 10 June 2004; accepted 11 June 2004

Abstract—A rapid and sensitive chemiluminescence (CL) determination of puerarin with the flow injection technique was presented. It was found that puerarin could enhance the CL generated from luminol–KIO_4 system significantly. The increment of CL intensity was linear over the concentration of puerarin ranging from 0.3 to 100.0 ng mL^{-1} ($r^2 = 0.9963$), offering a detection limit as low as 0.1 ng mL^{-1} (3σ). At a flow rate of 2.0 mL min^{-1}, one analysis cycle, including sampling and washing, could be accomplished in 20 s with a relative standard deviation of less than 5.0%. The experimental results demonstrated that the excretive amounts of puerarin reached its maximum in 3 h after taking 250 mL green drink containing 275 mg puerarin, and the puerarin excretive ratio during 24 h was 9.70% in the body of volunteers.

1. *Pueraria lobata (Willd.) Ohwi*

Pueraria lobata (Willd.) Ohwi is a climbing, semi-woody, perennial vine in the pea family, which is originally cultivated in Asia as a semi-domesticated crop, grown mainly for its large starchy root that is used medicinally and for food.[1] The active components in *Pueraria* root have been separated and identified as isoflavone, in which puerarin is present in the greatest amount. The biomedical effects of puerarin include antiproliferactive effects on human cancer cell lines,[2] inhibiting alcohol dehydrogenase[3] and xanthine oxidase.[4] Recent investigations demonstrate that puerarin is an effective antioxidant and shows effects against glutamate excitotoxicity on cultured mouse cerebral corticalneurous.[5] From the literature, determination of puerarin in *Pueraria* root and its medicinal preparations can be accomplished using several techniques, including high performance liquid chromatography[6–8] and capillary electrophoresis.[9–11] However, no report has been found on a chemiluminescence (CL) method for the determination of puerarin to date.

In this paper, it was found that puerarin could enhance the CL intensity from luminol and KIO_4 oxidation reaction in alkaline medium. The enhancement of CL intensity was proportional to the concentration of puerarin ranging from 0.3 to 100.0 ng mL^{-1} with a relative standard deviation of less than 5.0% and the detection limit was 0.1 ng mL^{-1}. At a flow rate of 2.0 mL min^{-1}, the procedure could be performed within 20 s, including sampling and washing, giving a throughput of about 180 times per hour. The proposed method was applied directly in the determination of puerarin in pharmaceutical injections and monitoring puerarin in human urine samples without pre-treatment procedures. It was found that the excretive amounts of puerarin reached its maximum after taking 250 mL green drink containing 275 mg puerarin for 3 h, with a total excretive ratio of 9.70% in 24 h.

2. Reagents

All reagents were of analytical grade and double-distilled water (Milli-Q, Millipore, MA, USA) was used throughout. Puerarin was obtained from Xi'an Modern Chemistry Research Institute (Xi'an, China). Luminol (Fluka, Biochemika) was obtained from Xi'an Medicine Purchasing and Supply Station, China. Potassium periodate was purchased from Xi'an Chemical Reagent Plant.

Keywords: Puerarin; Chemiluminescence; Flow injection; Pharmaceutical injection; Human urine.
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doi:10.1016/j.bmcl.2004.06.036

3. Instrument and procedures

The flow injection system used in this work is shown in Figure 1. A peristaltic pump was used to deliver all flow streams. PTFE tubing (2.0 mm i.d.) was used as connection material in the flow system. The carrier water and the solutions (sample, KIO_4, luminol, and NaOH) were propelled at a constant flow rate of 2.0 mL min^{-1}. Luminol (100 μL) was injected into the carrier stream by a six-way valve quantitatively, which was then merged with the puerarin and KIO_4 streams. The mixed solution was delivered to the CL cell in an alkaline medium, and the CL signal produced was therefore detected with a photomultiplier tube (PMT) (HAMAMATSU, model IP28) and a luminometer (Xi'an Remax Electronic Instrument Co. Ltd, model GD-1). The concentration of sample was quantified by measuring the increased CL intensity, $\Delta I = I_s - I_o$, where I_o and I_s are CL signals in the absence and in the presence of puerarin, respectively.

4. The CL intensity–time profile

The CL intensity–time profile was examined by a static method with 5.0×10^{-6} mol L^{-1} luminol and 5.0×10^{-5} mol L^{-1} KIO_4. The luminol and KIO_4 gave an evident CL signal in alkaline medium, which reached a maximum at 2 s after initiating the reaction, and tended to be vanishing within 18 s thereafter; while in the presence of puerarin the CL intensity increased and reached a maximum at 1 s and then died within 12 s, which demonstrated that puerarin accelerates the CL reaction and enhanced CL intensity.

5. Selection of oxidant

The characteristics of several oxidants, including $KMnO_4$, H_2O_2, $K_3Fe(CN)_6$, $K_2Cr_2O_7$, KIO_4 of the same concentration reacting with luminol in the presence of puerarin were evaluated. It was concluded that puerarin as an enhancer exhibiting a large enhancement only for luminol–KIO_4 CL system.

6. Effect of luminol, KIO_4, and NaOH concentration

The maximum CL signal was found with luminol at 5.0×10^{-6} mol L^{-1}, with KIO_4 at 5.0×10^{-5} mol L^{-1}, and 0.025 mol L^{-1} for NaOH, which were selected as optimum conditions.

7. Effect of flow rate and the length of reaction tube

The flow rate of 2.0 mL min^{-1} was selected as an appropriate condition considering both good analytical precision and lower solution consumption, and it was found that a 5.0 cm mixing tube afforded the best results with good sensitivity and reproducibility.

8. Performance of proposed method for puerarin measurements

Under the optimum conditions as above, the calibration graph of CL increment (ΔI) versus puerarin concentration was linear over the range of 0.3–100 ng mL^{-1}, and the regression equation was $\Delta I = 0.62C_{puerarin} + 68.82$ ($r^2 = 0.9963$) with the detection limit of 0.1 ng mL^{-1}. The relative standard deviations (RSDs) were 3.47%, 2.14%, and 1.08% for 0.3, 3.0, and 30 ng mL^{-1} puerarin, respectively.

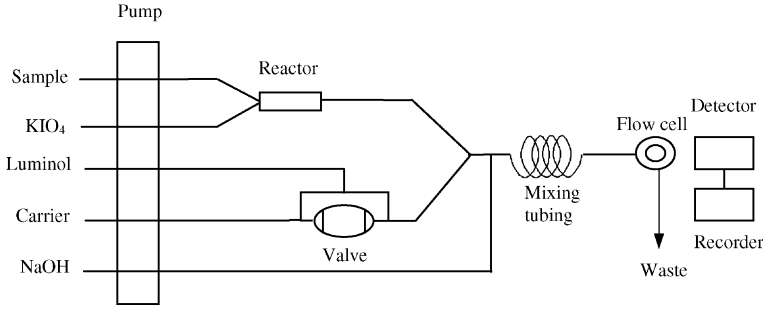

Figure 1. Schematic diagram of the present FI–CL system for puerarin.

Table 1. Results of puerarin in Shuren™ pueraria powder[a]

Sample No.	Results by proposed method						Results by HPLC
	Found (ng mL^{-1})	Added (ng mL^{-1})	Total (ng mL^{-1})	Recovery (%)	RSD (%)	Content (%)	Content (%)
1	1.76	1.00	2.73	97.0	1.96	0.57	0.53
2	1.71	2.00	3.63	95.8	2.14	0.55	0.56
3	1.63	3.00	4.74	103.6	1.34	0.53	0.51

[a] The average of five determinations.

9. Interference studies

The effect of interferents was tested by analyzing a standard solution of puerarin ($10\,ng\,mL^{-1}$) to which increasing amounts of interferents were added. The tolerable limit of interferents was taken if it caused a relative error of less than 5%. The tolerable concentrations were over $50\,\mu g\,mL^{-1}$ for K^+, Na^+, Cl^-, I^-, NO_3^-, Ac^-, HCO_3^-, PO_4^{3-}, $Cr_2O_7^{2-}$, SO_3^{2-}, Br^-, malic acid, citrate, oxalate, and tartrate, $20\,\mu g\,mL^{-1}$ for NH_4^+, Mg^{2+}, $Ba^{2+}Zn^+$, Ni^{2+}, Mn^{2+} and Ca^{2+}, $15\,\mu g\,mL^{-1}$ for methanol, ethanol, sucrose, urea, gelatin, globulin, starch, and dextrin. $10\,\mu g\,mL^{-1}$ for Ni^{2+}, $5\,\mu g\,mL^{-1}$ for glucose and polyvinyl alcohol, $3\,\mu g\,mL^{-1}$ for Cr^{3+}, $0.5\,\mu g\,mL^{-1}$ for Fe^{3+} and Fe^{2+}, $0.1\,\mu g\,mL^{-1}$ for baicalin, $50\,ng\,mL^{-1}$ for uric acid, $30\,ng\,mL^{-1}$ for Cu^{2+}, and $0.3\,ng\,mL^{-1}$ for rutin. The total urinary protein in a healthy adult is $100\,\mu g\,mL^{-1}$; therefore, these proteins do not interfere with the determination of puerarin after urine is diluted at 10^5 with water.

10. Determination of puerarin in nourishment

The proposed method was applied to the determination of puerarin in Shuren™ *pueraria* powder that is a kind of drink, purchased from the local market. The *pueraria* powder was ground into fine powder, dissolved with 30% (v/v) methanol solution, digested ultrasonically for 30 min, then filtered. The samples were determined directly and the results are listed in Table 1. The measured puerarin contents were 0.57%, 0.55%, and 0.53%, which were well in agreement with the results obtained by HPLC (Waters, μBondapak C_{18}).

Table 2. Results of determination of puerarin in pharmaceutical injections

Sample No.	Found ($ng\,mL^{-1}$)	Added ($ng\,mL^{-1}$)	Total ($ng\,mL^{-1}$)	RSD (%)	*t*-Test $t_{0.05,4} = 2.78$	Recovery (%)	Content ($mg\,mL^{-1}$)
1	1.02	1.00	1.68	1.75	2.70	94.0	81.65
2	2.96	5.00	8.25	1.25	2.20	105.7	79.03
3	4.98	5.00	9.61	1.15	0.69	92.6	79.72
4	2.98	1.00	4.06	1.57	0.86	108.1	79.52
5	1.02	3.00	4.08	1.35	2.46	102.2	81.21
6	4.94	3.00	7.80	1.31	2.20	95.4	78.98

Table 3. Monitoring excretive puerarin in human urine after taking *pueraria* powder

Time (h)	Added ($ng\,mL^{-1}$)	Found ($ng\,mL^{-1}$)	RSD (%)	Recovery (%)	*t*-Test $t_{0.05,4} = 2.78$	In urine M_{mg}/V_{mL}	Excretive ratio (%)
0.5	0	0.30	2.84	93.3	1.53	0.23/75	0.08
	0.5	0.77	2.14				
1	0	0.18	2.72	107.1	3.06	0.71/80	0.25
	1.0	1.25	2.23				
1.5	0	0.20	2.35	86.7	2.75	1.10/110	0.40
	0.5	0.63	1.79				
2	0	0.43	3.33	114.3	2.07	1.71/80	0.62
	1.0	1.57	1.89				
2.5	0	0.66	1.78	96.9	1.08	4.27/130	1.55
	1.0	1.63	1.25				
3	0	1.32	1.73	112.0	0.70	6.60/100	2.40
	2.0	3.56	1.33				
4	0	0.77	2.01	103.9	1.96	4.62/120	1.68
	1.0	1.81	1.67				
5	0	0.45	3.41	106.9	2.58	2.47/110	0.90
	1.0	1.52	1.71				
6	0	0.38	1.83	96.6	1.95	1.61/85	0.64
	1.0	1.34	1.70				
7	0	0.23	1.63	86.7	2.41	1.17/100	0.42
	0.5	0.67	1.35				
8	0	0.58	3.02	109.1	0.69	0.92/160	0.33
	1.0	1.67	1.64				
10	0	0.46	2.37	92.9	1.28	0.46/100	0.18
	0.5	0.93	2.23				
12	0	0.30	2.78	97.0	1.41	0.38/125	0.14
	1.0	1.27	2.56				
17	0	0.18	1.98	103.0	2.62	0.24/130	0.08
	1.0	1.21	1.91				
24	0	0.27	2.22	97.0	2.05	0.19/75	0.06
	1.0	1.24	2.09				
						Total: 26.67	Total: 9.70%

11. Determination of puerarin in pharmaceutical injections

The six pharmaceutical injections purchased from the local market were determined by the standard addition method. The results of the determination are listed in Table 2. Also the recovery and *t*-test were carried out to verify the proposed method.

12. In vitro monitoring of excretive puerarin in human urine

Two healthy volunteers took the 250 mL drink containing 275 mg puerarin in the morning, respectively. Urine samples were periodically collected from volunteers, diluted with water directly and sometimes supplemented with puerarin to test the recovery of the method. The results of the trial determinations were summarized in Table 3. It was found that excretive puerarin reached a maximum in 3.0 h after taking the drink; the puerarin excreted through urine was 26.67 mg in a total volume of 1.575 L with an excretive ratio of 9.70% in 24 h.

Acknowledgements

The authors gratefully acknowledge the financial support from Shaanxi Province Nature Science Foundation and Ministry of Education, China, Grant No. 2003B15 and No. 04Jk103.

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