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Research Article

Determination of Hesperetin in Pericarpium Citri Reticulatae and Human Serum Using Flow Injection Chemiluminescence

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A sensitive chemiluminescence (CL) method for the determination of hesperetin at nanogram levels was first presented. It was based on the inhibitory effect of hesperetin on luminol-dissolved oxygen CL reaction in a flow injection system. The decrements of CL intensity were logarithm over the concentrations of hesperetin in a range of 0.5 to 1000 ng mL^{-1}, with a detection limit of 0.2 ng mL^{-1} (3σ) and the relative standard deviations lower than 2.1%. At a flow rate of 2.0 mL min^{-1}, the whole determining performance including sampling and washing could be accomplished in 30 s, giving a sampling efficiency of 120 h^{-1}. The proposed method was applied successfully to the direct hesperetin determination in human serum with recoveries from 97.0 to 106.6%, and indirect hesperidin quantification in 2.5 g pericarpium citri reticulatae showing that the content is 8.1 ± 0.2%. The possible CL mechanism of luminol-dissolved oxygen-hesperetin reaction was also discussed in detail.

1. Introduction

Hesperetin (5, 7, 3′-trihydroxy-4′-methoxy-flavanone, Figure 1), a bioflavonoid compound with glycosidic hesperidin as its natural existing form which is abundant in citrus fruits including lemon, lime (*Citrus aurantifolia*), and mandarin (*Citrusreticulata*) [1], possesses a wide spectrum of biological activities such as antiplatelet, anti-inflammatory, antioxidant and neuroprotective activities [2–5]. Since reported on the clinical application in attenuating capillary permeability in 1945 [6], there has been a growing interest in the pharmacological studies of hesperetin [7–10].

Methods previously employed for the determination of hesperetin involve high performance liquid chromatography (HPLC) with UV or MS detector [11–13], gas chromatography (GC) with MS detector [14], and micellar electrokinetic capillary chromatography (MEKC) with UV detector [15]. So far, no flow injection chemiluminescence (FI-CL) method has been reported on the determination of hesperetin.

In this work, it was found that hesperetin could inhibit the CL intensity from luminol-dissolved oxygen reaction, and the CL intensity decrements were logarithm over hesperetin concentrations ranging from 0.5 to 1000 ng mL^{-1}, with a detection limit (LOD) of 0.2 ng mL^{-1} (3σ) and the relative standard deviations (RSDs) lower than 2.1%. At a flow rate of 2.0 mL min^{-1}, the whole analysis procedure including sampling and washing could be accomplished in 30 s, offering the sampling efficiency of 120 h^{-1}. This proposed method was employed successfully to directly determine hesperetin in human serum samples with recoveries of 97.0–106.6%, and indirectly measure the hesperidin in 2.5 g pericarpium citri reticulatae (PCR) with content of 8.1 ± 0.2%. The possible CL mechanism of luminol-dissolved oxygen-hesperetin reaction was also given.

2. Experimental

2.1. Reagents. All chemicals were of analytical reagent grade. Deionized water purified in a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout. Luminol (Fluka, Biochemika, Switzerland) was purchased from Xi'an Medicine Purchasing and Supply Station, China. Luminol (2.5×10^{-2} mol L^{-1}) stock solution was prepared in 1.0×10^{-1} mol L^{-1} NaOH solution. Hesperetin was supplied by

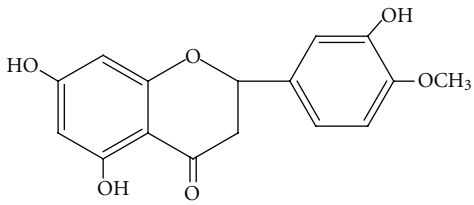

Figure 1: Structure of hesperetin.

Shaanxi Entry-Exit Inspection and Quarantine Bureau (Shaanxi, China). Hesperetin stock solution (10 μg mL^{-1}) was prepared in ethanol and stored at 4°C. The working standard solutions of hesperetin were prepared daily from the above stock solution by appropriate dilution as required.

2.2. Apparatus. The FI-CL system (Xi'an Remax Analysis Instrument Co. Ltd., Xi'an, China) was depicted in Figure 2. The apparatus includes a sampling system (IFFM-E), a CL detector (IFFS-A), and a recorder (a computer with IFFM-E client system). The sampling system provides a peristaltic pump to propel all solutions into the flow lines, and a six-way valve with loops of 100 μL to quantitatively inject luminol. The CL detector, which is placed inside a black box for precluding the interference from ambient light, contains a flow cell and a photomultiplier tube (PMT). The flow cell, which locates closely to the PMT, is a colorless glass tube (15 cm, 1.0 mm i.d.) with a spiral disk shape (2.0 cm i.d.). The CL signal from the flow cell was directly detected by the PMT without wavelength discrimination and the output was recorded on the computer. Polytetrafluoroethylene (PTFE) tubing (1.0 mm i.d.) is used to carry all the reagents.

2.3. General Procedures. As shown in Figure 2, the solutions were propelled on each line at a constant rate of 2.0 mL min^{-1}. The whole flow system was washed by purified water until a stable baseline was recorded. Luminol (100 μL) was then injected into the flow line via the six-way value, and merged with the stream of hesperetin. Thereafter, the mixed solution was delivered into the CL cell, and the CL signal was detected by PMT. The concentrations of hesperetin could be quantified based on the decrements of CL intensity ($\Delta \mathbf{I}_{\mathbf{CL}} = \mathbf{I}_{\mathbf{0}} - \mathbf{I}_{\mathbf{s}}$), where $\mathbf{I}_{\mathbf{0}}$ and $\mathbf{I}_{\mathbf{s}}$ were CL signals in the absence and presence of hesperetin, respectively.

2.4. Sample Preparation

2.4.1. Treatment of PCR Samples. Hesperidin in PCR (the dried ripe pericarp of *Citrus reticulata Blanco*) from local market was transferred to hesperetin according to published method with some minor modifications [16, 17]. 2.5 g PCR were ground into thin powder and transferred into a beaker. 20 mL boiling water and 15 mL saturated lime water were added in. Then the pH of the solution was adjusted to 12-13 using NaOH solution (2.5 mol L^{-1}). After dunking for 18 hours, the solution was filtered through an ordinary filter paper and HCl was introduced into the filtrated solution with final concentration of 2.5 mol L^{-1}. Thereafter, the mixture solution was treated in water bath of 100°C for 60 min. Then suitable aliquots from above solution were taken for the determination.

2.4.2. Preparation of Spiked Human Serum Samples. Human serum samples from healthy volunteers were provided by the Hospital of Northwest University. The spiked samples were prepared by adding known quantities of hesperetin into 0.1 mL of each serum samples. After homogenization, the spiked serum samples with appropriate dilution were taken for hesperetin determination.

3. Results and Discussion

3.1. Relative CL Intensity-Time Profile. The relative CL intensity-time profile of luminol-dissolved oxygen-hesperetin was given in Figure 3 (The concentrations of luminol and NaOH were 2.5×10^{-5} and 2.5×10^{-2} mol L^{-1}, resp.). It can be seen that the CL intensity of luminol-dissolved oxygen reaction reached the maximum of 211 at 5 s and then vanished in the following 20 s; while in the presence of 100 ng mL^{-1} hesperetin, the maximum CL intensity remarkably decreased from 211 to 120 by 43.1%.

3.2. Optimum Experimental Conditions for the Determination of Hesperetin. The effect of luminol concentration on the CL intensity was investigated over a range of 1.0×10^{-7} to 1.0×10^{-4} mol L^{-1}, and 2.5×10^{-5} mol L^{-1} luminol was selected as the optimum concentration giving a stable and strong CL intensity. Considering the nature of luminol CL reaction favoring in alkaline medium, 2.5×10^{-2} mol L^{-1} NaOH was added into the solution to enhance the CL intensity.

The effect of flow rate and mixing tube lengths was also examined. 2.0 mL min^{-1} flow rate and 5.0 cm mixing tube length were chosen in the subsequent experiments as a good compromise of sensitivity, reagent consumption, and reproducibility.

3.3. Performance of Hesperetin Measurement. Under the optimum experimental conditions given above, the standard solutions of hesperetin were determined. It was found that the CL intensity decrements were linear with the logarithm of hesperetin concentrations ranging from 0.5 to 1000 ng mL^{-1}, giving the calibration equation of $\Delta \mathbf{I}_{\mathbf{CL}} = 15.8 \ln \mathbf{C}_{\mathbf{hesperetin}} + 28.9$ ($R^2 = 0.994$) with LOD of 0.2 ng mL^{-1} (3σ) and RSDs <2.1%. At a flow rate of 2.0 mL min^{-1}, one analyzing cycle of hesperetin determination including sampling and washing could be accomplished in 30 s, which offered the sampling efficiency of 120 h^{-1}, accordingly.

3.4. Interference Studies. The interference of potentially interfering species were tested by adding increasing amounts of interfering substance to the standard solution of hesperetin (10 ng mL^{-1}) and the error was controlled at 5% level. The tolerable concentrations of interfering species were 100 μg mL^{-1} for NO_3^-, Ac^-, SO_4^{2-}, ethanol and glucose, 10 μg mL^{-1} for NH_4^+, Mg^{2+} and Ca^{2+}, 500 ng mL^{-1} for uric acid, 300 ng mL^{-1} for rutin, and 10 ng mL^{-1} for quercetin. Compounds abundant in human serum such as

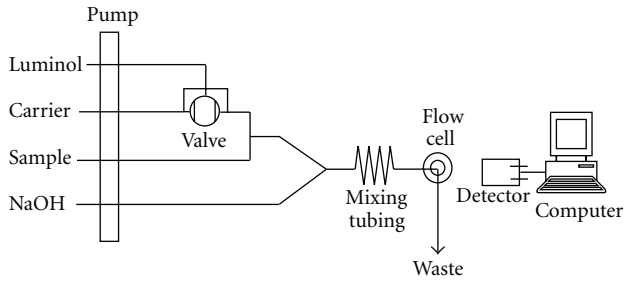

Figure 2: Schematic diagram of the FI-CL system for the determination of hesperetin.

Table 1: Results of hesperidin in PCR ($n = 6$).

Samples number	Added ng mL^{-1}	Found ng mL^{-1}	RSD %	Recovery %	Content in PCR %	
1	0	199	1.9	95.0	8.0	
	100	294	1.4			
2	0	201	1.5	103.9	8.1	
	100	305	1.2			
3	0	205	1.5	107.0	8.3	8.1 ± 0.2
	200	419	0.9			
4	0	197	2.0	99.7	7.9	
	200	396	1.0			
5	0	203	1.9	96.9	8.2	
	300	494	0.7			
6	0	200	1.9	104.0	8.0	
	300	512	0.6			

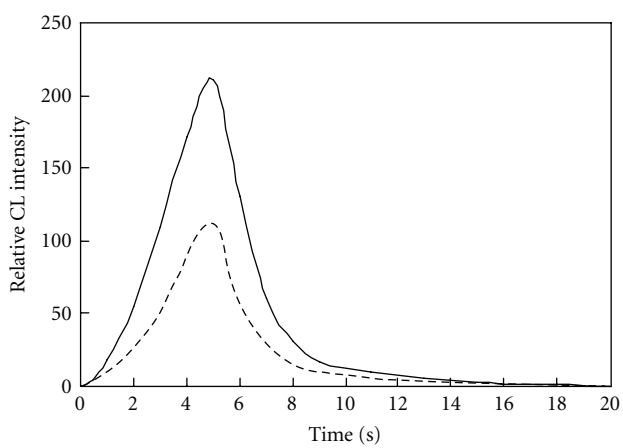

Figure 3: Relative CL intensity: time profile of different reactions. Luminol: 25 μmol L^{-1}; hesperetin: 100 ng mL^{-1}; NaOH: 25 mmol L^{-1}. Solid line: luminol-dissolved oxygen reaction; Dash line: luminol-dissolved oxygen-hesperetin reaction.

salts, lipids, and proteins had no obvious interference for the determination of hesperetin at nanogram levels.

3.5. Possible Mechanism of Luminol-Dissolved Oxygen-Hesperetin CL Reaction. The possible mechanism of luminol-dissolved oxygen-hesperetin reaction was discussed in detail. Firstly, the CL emission spectrum showed that the maximum CL emission wavelength was at 425 nm. This result suggested that luminol could react with dissolved oxygen to yield $^{\bullet}O_2^-$ radicals and be oxidized subsequently to excited 3-aminophthalate by the produced $^{\bullet}O_2^-$ radicals, with CL signals emitted in high pH medium (0.025 mol L^{-1} NaOH) [18]. Additionally, the UV spectra of luminol had no obvious change in the presence of hesperetin, which demonstrated that there was no interaction between luminol and hesperetin. Furthermore, the CL intensities generated by online ultrasonically degassed solutions decreased compared to that generated by the general solutions, indicating that hesperetin could react with $^{\bullet}O_2^-$ radicals. The possible CL mechanism of luminol-dissolved oxygen-hesperetin reaction can be explained as that the anti-oxidant hesperetin can react with the yielded $^{\bullet}O_2^-$ radicals, which reduced the concentrations of $^{\bullet}O_2^-$ radicals leading to obvious inhibition of CL intensity from luminol-dissolved oxygen reaction.

4. Application

4.1. Indirect Determination of Hesperidin in PCR. 2.5 g PCR were pretreated by the modified method which was described in the experimental section and then suitable aliquots from above prepared samples were taken for the indirect determination of hesperidin. In order to evaluate the validity of the proposed method, recovery studies were carried out and the results were listed in Table 1, with recoveries from 95.0 to 107.0% and RSDs < 2.0%. The contents of hesperidin

Table 2: Hesperetin determination in spiked human serum samples ($n = 6$).

Samples number	Added ng mL^{-1}	Found ng mL^{-1}	RSD %	Recovery %	Proposed method/spiked μg mL^{-1}
1	0	19	1.5	97.0	9.7/10.0
	20	39	1.0		
2	0	21	1.4	106.6	10.6/10.0
	20	43	0.9		
3	0	20	1.6	98.2	9.8/10.0
	20	39	1.0		
4	0	21	1.3	102.4	10.5/10.0
	30	52	0.8		
5	0	20	1.5	97.0	9.7/10.0
	30	49	0.9		
6	0	21	1.6	103.7	10.3/10.0
	30	52	0.8		

Table 3: Comparison of different methods for hesperetin determination.

Methods	Linear range ng mL^{-1}	LOD ng mL^{-1}	Samples	References
MEKC-UV	$2.0 \times 10^4 \sim 6.0 \times 10^5$	6000	Propolis	[15]
HPLC-UV	$5.0 \times 10^2 \sim 1.0 \times 10^5$	500	Human urine Rat urine	[13]
	$2.5 \times 10^2 \sim 2.5 \times 10^4$	100	Human urine	[12]
GC-MS	$2.0 \sim 3.0 \times 10^2$	2.0	Human plasma Human urine	[14]
HPLC-MS/MS	$5.0 \sim 1.0 \times 10^3$	0.5	Rat plasma	[11]
FI-CL	$5.0 \times 10^{-1} \sim 1.0 \times 10^3$	0.2	PCR Human serum	This study

in PCR were 7.9–8.3%, which were in the reported range of 3.8% to 12.1% by HPLC [19].

4.2. The Determination of Hesperetin in Spiked Human Serum Samples. Hesperetin in the spiked serum samples prepared in the experimental section were measured, and the results were listed in Table 2. It can be seen that the recoveries of hesperetin were 97.0–106.6% and the RSDs were less than 1.7%, which confirmed the applicability of this method to determine hesperetin in biological samples.

5. Conclusion

A simple and rapid CL method for the determination of hesperetin at nanogram levels was proposed for the first time. A comparison of the presented FI-CL method and the previously reported methods for the determination of hesperetin was given in Table 3, showing this proposed method offered prominent advantages of wide linear range, lower detection limit, and analytical sensitivity. The satisfactory results of indirect hesperidin quantification in PCR and hesperetin determination in human serum samples confirmed the promise of this proposed method for further pharmacological and clinical research.

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