Determination of Melatonin in Rat Pineal Gland and Drug with Flow-Injection Chemiluminescence

WEI, Shou-Lian^{a,b}(韦寿莲) ZHAO, Li-Xia^c(赵利霞)
CHENG, Xiang-Lei^c(程祥磊) LIN, Jin-Ming^{*b,c}(林金明)

^a Department of Light Industry & Chemistry, Zhaoqing University, Zhaoqing, Guangdong 526061, China

^b Department of Chemistry, Tsinghua University, Beijing 100084, China

^c State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

A flow-injection chemiluminescence (CL) method for the determination of melatonin based on the CL reaction of melatonin with hydrogen peroxide and sodium hypochlorite (NaOCl) in a basic alkaline solution was developed. The possible CL mechanism has been discussed, and a proposal for the reaction pathway was given that singlet oxygen was clarified to be produced in this reaction system and was responsible for the CL emission. Under the optimized conditions, the linear concentration range of application was 1.0×10^{-7} – 2.5×10^{-4} mol·L⁻¹ with a detection limit of 5.0×10^{-8} mol·L⁻¹ ($S/N=3$). The relative standard deviation for eight repeated measurements of 1.0×10^{-6} mol·L⁻¹ melatonin was 2.8%. The interferences of several important biological substances, some indole compound, cations and anions were studied. No interference was found for the anions, glucose, starch, most of cations and low concentration (less than 3.0×10^{-6} mol·L⁻¹) of some biological substances and indole compound. The method was applied to the determination of melatonin in rat pineal gland and drug with satisfactory results. The sample throughput was 90 injections per hour.

Keywords chemiluminescence, melatonin, hypochlorite, rat pineal gland

Introduction

Melatonin (5-methoxy-*N*-acetyltryptamine) is an important hormone, mainly produced and secreted at night by the pineal gland, playing a major role in the synchronization of the sleep/wake cycle. The production of melatonin is regulated by a structure located in the hypothalamus called the suprachiasmatic nucleus (SCN). The activity of the SCN is strongly affected by changes in illumination and, as a consequence, melatonin levels are high during darkness and low in the light and it, therefore, reflects the cycle.¹ Since its structure was first discovered in 1958 by Lerner,² many studies about melatonin in the scientific literature were published, making melatonin one of the hottest subjects of life extension research.^{3,4}

Many analytical methods have been developed for the determination of melatonin and its related indoleamines,^{5–8} in which, chemiluminescence (CL) methods have also been used for certain indole derivatives.^{9–13} For example, Alwarthan¹¹ determined tryptophan using flow injection CL system based on the oxidation of tryptophan with cerium(IV) in sulfuric acid. Lee and Whang¹² have determined 5-hydroxytryptamine with acidic potassium permanganate CL detection. As for the measurement of melatonin, only two CL meth-

ods were reported.^{14,15} Kawatani and Lin¹⁴ reported a CL method of melatonin determination by the reaction of H₂O₂ with MoO₄²⁻, which was immobilized on strongly basic anion-exchange resin. A major limitation of this approach is that the speed of the CL reaction was slow at ambient reaction temperature and relative sophisticated instruments was required. To overcome these problems, Lu and his co-workers¹⁵ described a simple CL method for melatonin determination by the reaction of H₂O₂ and acetonitrile, which caused toxicity and pollution in environment due to a large amount of acetonitrile. Moreover, These two methods also suffered from a low sample throughput since maximum signal was reached at 80–180 s after mixing various reaction reagents. Besides, both of them were not applied for the determination of melatonin in biological samples. Thus a simple, fast, environment-friendly and sensitive analytical method is necessary for the routine assay of melatonin in biological samples and drug.

In the present work, it was found that the addition of melatonin into H₂O₂ and NaOCl mixture caused a strong CL under alkaline conditions and the light intensity showed a good linear relationship with the concentration of melatonin. Based on this observation, a flow-injection analysis CL (FIA-CL) method was de-

* E-mail: jmlin@mail.tsinghua.edu.cn; Tel. & Fax: 0086-010-62792343

Received March 10, 2006; revised October 15, 2006; accepted December 15, 2006.

Project supported by the National Natural Science Foundation of China (No. 20437020) and Zhaoqing Innovative Research Program.

veloped for the determination of melatonin in rat pineal gland and drug.

Experimental

Reagents and apparatus

All reagents were of analytical-reagent grade and used as received. Ultrapure water was obtained from a compact ultrapure water system (18.3 M Ω ·cm, Barnstead, Iowa, USA). Stock solutions of 10⁻² mol·L⁻¹ melatonin (Sigma, USA) and other indole compounds were prepared by dissolving in methanol. Other standard solutions were made by gradually diluting relative stock solutions with water. NaOCl, H₂O₂, HCl, NaOH, HClO₄ and other organic compounds were purchased from Beijing Chemical Reagents Company, China. Naobaijin capsules (Kangqi Inc., Zhuhai, China) were purchased from the drug store. The capsule was dissolved in 100 mL of methanol with stirring for 5 min. After centrifugation, 10 mL of the supernatant were diluted to 100 mL with water and the solution was analyzed by the present method.

Batch CL measurements were carried out by using a BPCL chemiluminescence analyzer (Institute of Biophysics, Chinese Academy of Science, Beijing, China). Light-producing reactions were carried out in 12×75 mm disposable culture tubes, which were placed in front of photomultiplier tube of CL analyzer, containing 10 μ L of different concentrations of melatonin. 100 μ L of 0.1 mol·L⁻¹ Na₂CO₃ and 20 μ L of 0.1 mol·L⁻¹ H₂O₂ were added. A 60 μ L NaOCl solution was injected into the mixed solution to initiate CL. The signal was displayed and integrated for a 3 s interval, in arbitrary units.

Flow-injection system

As shown in Figure 1, the flow system consisted of a peristaltic pump which delivered each solution at equal flow rate (2.0 mL·min⁻¹) using flow tubes and a six-way injection valve with a 50- μ L sample loop, through which sample solution was injected into the NaOCl carrier stream. The background response was obtained by premixing alkaline buffer and hydrogen peroxide at a T-piece, and then the mixed solutions was merged with

Figure 1 Schematic diagram of the flow system for the determination of melatonin. (1) 0.05 mol·L⁻¹ Na₂CO₃-NaHCO₃ solution (pH 10.0); (2) 1×10⁻² mol·L⁻¹ H₂O₂ solution; (3) carrier solution (1% NaOCl solution); (S) melatonin solution; P: pump; V: six-way injection valve; S: sample; W: waste solution; PMT: photomultiplier tube.

the NaOCl carrier at a second T-piece. Sample injections were directly injected into the NaOCl carrier. The final mixture flowed into the flow-cell positioned in front of the PMT of luminometer (Lumiflow LF-800, Microtec NITI-ON, Funabashi, Japan). The flow cell was made by coiling 10.5 cm length of PTFE tubing (1.0 mm i.d.) into a spiral disk shape with a diameter of 2 cm. The output was fed to a computer equipped with the LUM-2000 data processing program (Research Center for Eco-Environmental Science, CAS, China). PTFE tubing of 0.25 mm i.d. and 0.8 mm o.d. was used for all connections. The CL response was continuously monitored.

Extraction of melatonin from rat pineal gland

A great hundred of juvenile male rats (40–50 d) were bought from Guangzhou Epidemic Prevention Institute. The body weights of them were (180±30) g. The rats were sacrificed by decapitation during the dark (between local time 11:00 and 12:00 p.m.). Pineals were removed, quickly frozen on dry ice and stored at -80 °C until assay. Pools of all pineals were sonicated 6 times (each times 8 s, interval 2–3 s) in 24.0 mL of a 0.2 mol·L⁻¹ ice-cold perchloric acid containing 0.2 mol·L⁻¹ EDTA using a Vibra-cell microsonicator (20 kHz, 40 W). Protein in pineal gland was deposited via this process. Half of homogenate was then extracted after 1.0 mL of 1.0 mol·L⁻¹ NaOH alkalisation (adjust the pH) with 16.0 mL of CH₂Cl₂ shaken for 10 min. After centrifugation (10 min, 1500 g, at 4 °C), the aqueous layer was removed by aspiration. This process removed water-soluble interferent, such as 5-hydroxytryptamine, *N*-acetylserotonin, 5-hydroxyindole acetic acid, 5-methoxyindole acetic acid, *etc.* And a 3.5 mL 0.1 mol·L⁻¹ HCl was added to the organic layer, horizontal shaking for 10 min performed the extraction. After centrifugation (10 min, 1500 g, at 4 °C), the aqueous layer was removed by aspiration (remove 5-methoxytryptamine), then, an 20.0 mL of *n*-heptane and 1.2 mL of 5 mol·L⁻¹ HCl solution was added to the organic layer. Horizontal shaking for 10 min performed the extraction. After centrifugation (10 min, 1500 g, at 4 °C), the organic layer was collected and spin-evaporated to 300 μ L for melatonin quantification (use this present method and the referenced method). Extraction recovery was determined by spiking known quantities of melatonin to another half of homogenates from male rats. Spiked samples were treated as previously described.

Results and discussion

It is well known that a weak CL could be observed between H₂O₂ and NaOCl in alkaline solution and singlet oxygen was produced.^{16,17} When melatonin was added into this CL system under optimum experimental conditions, a strong CL was generated and the result was shown in Figure 2a. The maximum CL intensity was obtained less than 3 s after mixing various reagents and then the signal quickly decreased. It took less than

10 s to complete a measurement. Compared with previously reported systems^{14,15} (Figure 2b and 2c), which were in their optimum experimental conditions selected respectively, the analysis efficiency and sensitivity has been remarkably improved using the present method. Therefore, a simple, rapid and sensitive CL method was provided.

Figure 2 Time course of the kinetic profile of the CL reactions. (a) H_2O_2 -NaOCl-melatonin, (b) H_2O_2 -acetonitrile-melatonin, and (c) H_2O_2 - MoO_4^{2-} -melatonin, the concentration of H_2O_2 , Na_2CO_3 and melatonin was 0.01 , 0.02 and $1 \times 10^{-5} \text{ mol}\cdot\text{L}^{-1}$ respectively, the concentration of NaOCl, acetonitrile and MoO_4^{2-} was 1%, 25% and $0.005 \text{ mol}\cdot\text{L}^{-1}$ respectively.

Effect of pH and reaction media

In the CL reaction, the systematic media and pH are important factors. Three different reaction media ($0.05 \text{ mol}\cdot\text{L}^{-1} \text{ Na}_2\text{CO}_3$ - NaHCO_3 , $0.05 \text{ mol}\cdot\text{L}^{-1} \text{ Na}_2\text{CO}_3$, $0.05 \text{ mol}\cdot\text{L}^{-1}$ borate-NaOH) were studied. The results showed that the strongest CL intensity was obtained when using the $0.05 \text{ mol}\cdot\text{L}^{-1} \text{ Na}_2\text{CO}_3$ - NaHCO_3 buffer as reaction medium. While using $0.05 \text{ mol}\cdot\text{L}^{-1} \text{ Na}_2\text{CO}_3$ solution and borate-NaOH solution as reaction media, only about 76% and 65% signal was observed, respectively. Therefore, Na_2CO_3 - NaHCO_3 buffer was chosen as reaction media, and the effect of the concentration of Na_2CO_3 - NaHCO_3 buffer in the range of 0.01 – $0.3 \text{ mol}\cdot\text{L}^{-1}$ was further examined. The CL intensity was the greatest and remained on a plateau in the concentration range of 0.02 – $0.1 \text{ mol}\cdot\text{L}^{-1}$. A concentration of $0.05 \text{ mol}\cdot\text{L}^{-1} \text{ Na}_2\text{CO}_3$ - NaHCO_3 buffer was selected for the subsequent experiments. Figure 3 showed the CL signals depending on pH values. When the pH was in the range of 9.0 – 10.0 , the CL intensity increased with the pH, after that the CL intensity decreased with the increasing of pH. Therefore, the following experiments were done in $0.05 \text{ mol}\cdot\text{L}^{-1} \text{ Na}_2\text{CO}_3$ - NaHCO_3 solutions, pH 10.0.

Effects of the concentrations of H_2O_2 and NaOCl on the CL reaction

The effects of the concentration of H_2O_2 and NaOCl on the CL reaction were studied, as shown in Figure 4a and Figure 4b. The trend of two curves was similar. The CL intensity was largely enhanced when concentration

Figure 3 Effect of pH on the CL intensity. NaHCO_3 - Na_2CO_3 buffer, $0.01 \text{ mol}\cdot\text{L}^{-1} \text{ H}_2\text{O}_2$, 1% (V/V) NaOCl and $10^{-5} \text{ mol}\cdot\text{L}^{-1}$ melatonin.

Figure 4 Effect of the concentration of H_2O_2 and NaOCl on the CL intensity, $0.05 \text{ mol}\cdot\text{L}^{-1} \text{ Na}_2\text{CO}_3$ - NaHCO_3 (pH 10.0) and $10^{-5} \text{ mol}\cdot\text{L}^{-1}$ melatonin. (a) 1% NaOCl; (b) $0.01 \text{ mol}\cdot\text{L}^{-1} \text{ H}_2\text{O}_2$.

of H_2O_2 or NaOCl increased a little at the beginning. The reason was that the productivity of singlet oxygen increased with the increasing of the concentration of H_2O_2 or NaOCl. After that, the signal decreased slowly or reached a plateau. This was possibly due to the consumption of singlet oxygen within the medium when the concentration of H_2O_2 or NaOCl increased.^{16,17} Therefore, $0.01 \text{ mol}\cdot\text{L}^{-1} \text{ H}_2\text{O}_2$ and 1% (V/V) NaOCl were

used as the optimum concentration.

Selection of the flow rate

The flow rate is also an important effective factor in FIA-CL systems. The influence of flow rate on the sensitivity of the proposed CL system was examined by investigating the signal-to-noise ratio over the range 0.5–4.0 mL·min⁻¹. The signal intensity increased with the increase of flow rate from 0.5 to 2.0 mL·min⁻¹. Above 2.0 mL·min⁻¹ flow rate, the CL intensity did not increase significantly, and the baseline noise level increased remarkably. The flow rate of 2.0 mL·min⁻¹ offered the highest signal-to-noise ratio. Therefore 2.0 mL·min⁻¹ flow rate was chosen with superior sensitivity, precision and reducing reagent consumption.

Calibration curve

Under optimum experimental conditions selected as described above, with a batch method, a log-log calibration graph in the concentration range 0.10 $\mu\text{mol}\cdot\text{L}^{-1}$ –0.25 mmol·L⁻¹ showed a linear correlation (Figure 5a), where relative CL intensity is the integrated signal for 3 s. The detection limit of melatonin ($S/N=3$) was $5.0\times 10^{-8}\text{ mol}\cdot\text{L}^{-1}$ with our simple setup. The relative standard deviation (RSD) was 2.8% at $1.0\times 10^{-6}\text{ mol}\cdot\text{L}^{-1}$ melatonin ($n=8$). Ninety samples could be determined per hour. The method proposed here could be advantageous because it did not require the use of a heating bath apparatus and a large amount of organic solvent CH₃CN, the analysis efficiency increased remarkably, and the sensitivity was enough for the routine assay of melatonin in biological samples and drug. Under the same experimental conditions, indole-3-acetic acid could also be sensitively measured with a detection limit of $3.0\times 10^{-6}\text{ mol}\cdot\text{L}^{-1}$ (Figure 5b).

Figure 5 Calibration curve of (a) melatonin and (b) indole-3-acetic acid, 0.01 mol·L⁻¹ H₂O₂, 1% NaOCl and 0.05 mol·L⁻¹ Na₂CO₃–NaHCO₃ (pH 10.0).

Interferences

To demonstrate the usefulness of the present CL method for the determination of melatonin in pharmaceutical preparation and biological samples, the influ-

ence of various interferences concurrently presented in pharmaceutical preparation and biological samples for the determination of $1.0\times 10^{-6}\text{ mol}\cdot\text{L}^{-1}$ melatonin using the proposed method was investigated. The tolerance limit was taken as the amount (concentration ratio) which caused an error of $\pm 5\%$ in the analytical signal of melatonin. Several important biological substances and some indole compounds, such as dopamine, noradrenaline, ascorbic acid, VB₆, glucose, tryptophan and indole were studied. The results are listed in Table 1. No interference was found for the anions, glucose, starch and most of cations. Low concentration (less than $3.0\times 10^{-6}\text{ mol}\cdot\text{L}^{-1}$) of indole-3-acetonitrile, indole-3-acetic acid, dopamine, 5-hydroxytryptamine, 5-methoxytryptamine, *N*-acetyserotonin, 5-methoxyindole acetic acid, 5-hydroxyindole acetic acid, methylindole, noradrenaline, vitamin B₆, ascorbic acid, indole, Fe³⁺, Cu²⁺ and Co²⁺ did not interfere with determination of melatonin. It showed clearly that the present method offered the high selectivity for the determination of melatonin. But due to the very low level of melatonin in rat pineal gland samples, a complete extraction step to concentrate melatonin should be taken when using this simple method.

Sample analysis

To illustrate the usefulness and reliability of the proposed method, it was applied to the determination of melatonin in rat pineal gland and a drug named Naobaijin capsules. Because of the very low level of melatonin in a rat pineal gland (estimated at 200–500 pg⁶) and the potential losses of melatonin during organic extraction, it is necessary to pool at least fifty pineals to achieve such a detectable and quantifiable level. The determination results are shown in Table 2. The determined melatonin amounts were 27.6 ng and 2.95 mg in a sample of pools of 120-rat pineal gland, and a capsule sample, respectively. Melatonin amounts measured in rat pineal gland using the proposed method and the referenced method with fluorescence detection¹⁸ were not significantly different, and the measured melatonin amounts in Naobaijin capsules were in agreement with the labeled values (3 mg/capsule) and the values of the previous methods.¹⁴ Known amounts of the melatonin were added to the samples and then analyzed. From Table 2, it can be seen that the recovery of melatonin was 85.3% and 97.3%–99.0% from rat pineal gland and capsules, respectively. The present method was suitable for determination of melatonin in rat pineal gland samples and drug with good accuracy.

Possible CL mechanism

Many investigations^{16,17} have confirmed that H₂O₂ and NaOCl in a basic solution generated singlet oxygen, an excited state of molecular oxygen. Three singlet oxygen scavengers, NaN₃, dimethylfuran and 1,4-diazobicyclo[2,2,2]octane were used to study the mechanism.

Table 1 Tolerance level of interference in the analysis of $1.0 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1}$ melatonin

Compound	Tolerance ratio (species/melatonin)	Compound	Tolerance ratio (species/melatonin)
Glucose	1000	Starch	1000
Indole-3-acetonitrile	3	Alanine	1000
Indole-3-acetic acid	5	$\text{C}_2\text{O}_4^{2-}$	1000
Methylindole	5	SO_4^{2-}	1000
Indole	10	NO_3^-	1000
2-Methylindole	50	Cl^-	1000
Tryptophan	50	PO_4^{3-}	1000
Vitamin B ₆	5	NH_4^+	1000
Ascorbic acid	4	Mg^{2+}	1000
5-Hydroxytryptamine	5	Ca^{2+}	1000
5-Methoxytryptamine	3	Na^+	1000
Dopamine	4	K^+	1000
Noradrenline	10	Cu^{2+}	10
N-Acetylserotonin	10	Ni^{2+}	20
5-Methoxyindole acetic acid	3	Zn^{2+}	100
5-Hydroxyindole acetic acid	5	Fe^{3+}	10
Co^{2+}	10		

Table 2 Analysis of concentrations of melatonin in rat pineal gland samples and Naobaijin capsule samples

Sample	Labeled amount	Proposed method ^a	Reference method ^a	Added amount	Found amount	Recovery ^{b/} %
Pools of rat pineal gland	—	$27.6 \pm 0.53 \text{ ng}$	28.6 ng^{18}	15.0 ng	12.8 ng	85.3 ± 5.0
Capsule 1#	3 mg	$2.94 \pm 0.07 \text{ mg}$	3.03 mg^{14}	1.00 mg	0.98 mg	98.0 ± 2.3
Capsule 2#	3 mg	$2.95 \pm 0.03 \text{ mg}$	3.06 mg^{14}	1.50 mg	1.46 mg	97.3 ± 3.0
Capsule 3#	3 mg	$2.95 \pm 0.05 \text{ mg}$	3.05 mg^{14}	2.00 mg	1.98 mg	99.0 ± 1.5

^a Average of three measurements $\pm$ S.D.

Indeed, as shown in Figure 6, all of these scavengers strongly affected the reaction mechanism in a different way. About 53% integrated CL intensity for 30 s was decreased by the addition of $0.025 \text{ mol} \cdot \text{L}^{-1}$ dimethylfuran, but the kinetic profile was unchanged in the presence of dimethylfuran. For 1,4-diazobicyclo[2,2,2]-octane, maximum signal was almost the same in its presence and absence, however 75% integrated CL intensity for 30 s was decreased after adding this scavenger. For NaN_3 , 95% CL intensity was inhibited in the presence of $0.025 \text{ mol} \cdot \text{L}^{-1}$ NaN_3 . Besides, hydroxyl radical scavengers, such as DMSO and methanol, did not affect this reaction and thus hydroxyl radical was not involved in this reaction. This phenomenon provided strong evidence that singlet oxygen did play a primary role in chemiexcitation.

In order to further clarify the CL mechanism of the present system, the CL spectra in the absence and presence of melatonin were studied as shown in Figure 7. It can be observed that melatonin could significantly

Figure 6 Time course of the kinetic profile of the CL reaction in (a) H_2O , (b) $0.007 \text{ mol} \cdot \text{L}^{-1}$ 1,4-diazabicyclo[2,2,2]octane, (c) $0.025 \text{ mol} \cdot \text{L}^{-1}$ dimethylfuran and (d) $0.025 \text{ mol} \cdot \text{L}^{-1}$ NaN_3 , $0.01 \text{ mol} \cdot \text{L}^{-1}$ H_2O_2 , 1% NaOCl , $0.05 \text{ mol} \cdot \text{L}^{-1}$ $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$ (pH 10.0) and $10^{-5} \text{ mol} \cdot \text{L}^{-1}$ melatonin.

Figure 7 CL spectra for (a) NaClO and H₂O₂ and (b) NaClO and H₂O₂ with melatonin. $1 \times 10^{-2} \text{ mol} \cdot \text{L}^{-1}$ H₂O₂, $5 \times 10^{-2} \text{ mol} \cdot \text{L}^{-1}$ Na₂CO₃-NaHCO₃, 1% (V/V) NaClO, $1 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$ melatonin.

enhance the CL intensity of the reaction of NaClO-H₂O₂ and maximum emission wavelength was at *ca.* 500 nm which was consistent with the CL spectra reported by Kladna.¹⁹ The fluorescence spectra of melatonin with and without the addition of NaClO-H₂O₂ are shown in Figure 8. The fluorescent maximum emission wavelength of melatonin was located at 347 nm, while the reaction of NaClO-H₂O₂ and melatonin produced a new band with fluorescence emission of maximum wavelength at 420 nm, which was consistent with well-described fluorescent properties of AFMK.^{19,20} The UV absorption of melatonin and the reaction of NaClO-H₂O₂ and melatonin are shown in Figure 9. The absorption of melatonin was at 220 and 275 nm, respec-

Figure 8 Fluorescence spectra. (a) the excitation, (b) emission spectra of $1 \times 10^{-7} \text{ mol} \cdot \text{L}^{-1}$ melatonin, (c) the excitation and (d) emission spectra of the products of NaClO-H₂O₂-buffer-melatonin under conditions: $1 \times 10^{-3} \text{ mol} \cdot \text{L}^{-1}$ H₂O₂, $5 \times 10^{-3} \text{ mol} \cdot \text{L}^{-1}$ Na₂CO₃-NaHCO₃, 0.02% NaClO and $1 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$ melatonin.

Figure 9 UV-spectra. (a) NaClO, (b) H₂O₂ + buffer + melatonin; (c) NaClO + H₂O₂ + buffer + melatonin. Conditions: $1 \times 10^{-3} \text{ mol} \cdot \text{L}^{-1}$ H₂O₂, $5 \times 10^{-3} \text{ mol} \cdot \text{L}^{-1}$ Na₂CO₃-NaHCO₃ and 0.02% NaClO.

tively. However, after rapid mixing of NaClO and H₂O₂-buffer-melatonin, a new band appeared at 245 nm and the characteristic band of melatonin at 220 nm disappeared,²¹ which can be suggested to be due to the pyrrole ring being oxidated to produce a new compound AFMK.^{10,22} The formation of this new product also showed substantial absorbance in the range of 320–400 nm with a broad maximum at about 340 nm, which was very similar with that observed in Refs. 19 and 22. Based on the results above mentioned, the mechanism can be summarized that singlet oxygen was first formed between H₂O₂ and NaOCl, and then it reacted with melatonin to form a peroxide ring. The peroxide ring is further decomposed by H₂O₂ in Na₂CO₃-NaHCO₃ buffer to produce an excited AFMK, which will emit light when it returns to ground state. In addition, singlet oxygen ¹O₂ could be formed as a result of the energy transfer from AFMK* to the molecular oxygen. The possible mechanism is shown in Scheme 1.

Conclusion

The proposed method demonstrated the feasibility of performing the reaction between melatonin and singlet oxygen generated from H₂O₂ and NaOCl for analysis of melatonin in drug and a sample of pools of rat pineal. It was believed that singlet oxygen was formed in this CL reaction and responsible for the CL emission of melatonin. The present method offers several advantages: simpler apparatus, lower reagent consumption, environmental-friendly, higher sample throughput (90 h⁻¹), higher tolerance towards interferences and high enough sensitivity being applicable to rat pineal gland samples and routine quality control analysis in pharmaceuticals.

Scheme 1

Acknowledgements

We would like to express our thanks to Dr. Xiaofeng Xu, Dr. Yinfeng Zhang and Master Zhiyong Zhong from Guangzhou University of Traditional Chinese Medicine for their kindly help with the rat pineal gland operation.

References

- Reiter, R. J.; Acuna-Castroviejo, D.; Tan, D. X.; Burkhardt, S. *Ann. N. Y. Acad. Sci.* **2001**, 939, 200.
- Lerner, A. B.; Case, J. D.; Takahashi, Y.; Lee, T. H.; Mori, W. *J. Am. Chem. Soc.* **1958**, 80, 2587.
- Vijayalaxmi, T. C. R.; Reiter, R. J.; Herman, T. S. *J. Clin. Oncol.* **2002**, 20, 2575.
- Leon, J.; Acuna-Castroviejo, D.; Sainz, R. M.; Mayo, J. C.; Tan, D.-X.; Reiter, R. J. *Life Sci.* **2004**, 75, 765.
- Chanut, E.; Nguyen-Legros, J.; Versaux-Botteri, C.; Trouvin, J.-H.; Launay, J.-M. *J. Chromatogr., B* **1998**, 709, 11.
- Harumi, T.; Akutsu, H.; Matsushima, S. *J. Chromatogr., B* **1996**, 675, 152.
- Harumi, T.; Matsushima, S. *J. Chromatogr., B* **2000**, 747, 95.
- Galian, R. E.; Veglia, A. V.; de Rossi, R. H. *Analyst* **2000**, 125, 1465.
- Sugiyama, N.; Akutagawa, M.; Gasha, T.; Saiga, Y.; Yamamoto, H. *Bull. Chem. Soc. Jpn.* **1967**, 40, 347.
- Chen, G.; Xu, X.; Duan, J.; He, M.; Zhang, F. *Analyst* **1995**, 120, 1699.
- Alwarthan, A. A. *Anal. Chim. Acta* **1995**, 317, 233.
- Lee, Y.; Whang, C. *J. Chromatogr., A* **1997**, 771, 379.
- Ximenes, V. F.; Campa, A.; Catalani, L. H. *Arch. Biochem. Biophys.* **2001**, 387, 173.
- Kawatani, T.; Lin, J.-M.; Yamada, M. *Analyst* **2000**, 125, 2075.
- Lu, J.; Lau, C.; Lee, M. K.; Kai, M. *Anal. Chim. Acta* **2002**, 455, 193.
- Lion, Y.; Delmelle, M.; Van de Vorst, A. *Nature* **1976**, 263, 442.
- Aubry, J. M. *J. Am. Chem. Soc.* **1985**, 107, 5844.
- Anderson, G. M.; Young, J. G.; Cohen, D. J.; Simon, N. Y. *J. Chromatogr., B* **1982**, 228, 155.
- Kladna, A.; Aboul-Enein, H. Y.; Kruk, I. *Free Radical Biol. Med.* **2003**, 34, 1544.
- de Oliveira Silva, S.; Ximenes, V. F.; Catalani, L. H.; Campa, A. *Biochem. Biophys. Res. Commun.* **2000**, 279, 657.
- Beltagi, A. M.; Khashaba, P. Y.; Ghoneim, M. M. *Electroanalysis* **2003**, 15, 1121.
- Rozov, S. V.; Filatova, E. V.; Orlov, A. A. *J. Pineal Res.* **2003**, 35, 245.

(E0603108 ZHAO, X. J.)