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Ultrasensitive determination for picomolar-level midecamycin in human serum by flow injection chemiluminescence using luminol–BSA system

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Abstract. An ultrasensitive method for determining picomolar midecamycin (MID) by flow injection (FI) chemiluminescence (CL) was first described based on the inhibitory effect of MID on luminol–BSA reaction. It was found that the CL intensity decrements were linear with the logarithm of MID concentrations in the range of 1.0–5000 pmol $\cdot$ l^{-1} with a detection limit as low as 0.3 pmol $\cdot$ l^{-1} (3σ). The relative standard deviation of seven repetitive measurements for 10 pmol $\cdot$ l^{-1} MID was 3.0%. At a flow rate of 2.0 ml $\cdot$ min^{-1}, the whole analysis procedure including sampling and washing could be finished in 30 s, offering the sample efficiency of 120 h^{-1}. This proposed method was successfully applied to determine MID in human serum samples with the recoveries from 96.0 to 110.0%. The CL mechanism of luminol–BSA–MID reaction was also given.

Keywords: Midecamycin, BSA, luminol, chemiluminescence, flow injection

1. Introduction

Since flow injection (FI) analysis first described by Ruzicka and Hansen in 1975, it has revolutionized the performing way of analytical chemistry to a great extent [16]. Combined with chemiluminescence (CL), FI-CL, which possesses advantages of high sensitivity, low limit of detection (LOD), simple handling on-line or real-time monitoring, wide dynamic ranges, reproducibility and automatability as well as less reagent consumption, has become a very useful analytical tool in the science fields of biotechnology, pharmacology, food and environmental chemistry [3,5,12,15]. Luminol, one of the highest quantum yield luminescent compounds, has been extensively employed as the CL light emitting substrate in conjunction with different oxidants, such as metal ion [20,23], metal complex [6,17], nanoparticle [9,26],

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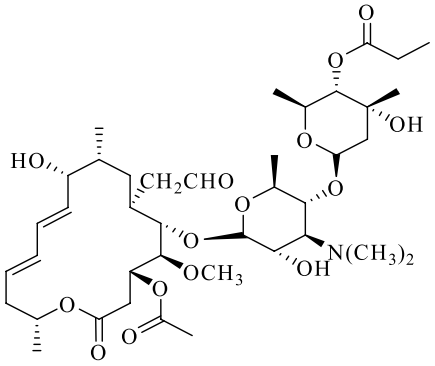

Fig. 1. The molecular structure of MID.

enzyme [4,24] and non-enzyme protein [19]. Recently, it has been reported that the hyperchromic effect of BSA on luminol can accelerate the electrons transferring rate of excited 3-aminophthalate leading to the CL intensity of luminol remarkably enhanced, and this FI-CL system has been developed successfully to assay azithromycin in pharmaceutical preparations [21]. So far there has been no report on the determination of midecamycin (MID) by FI-CL using luminol–BSA system.

MID ($C_{41}H_{67}NO_{15}$, Fig. 1), white crystalline powder with broad-spectrum against pneumococcal, streptococcal and mycoplasmal infections, is a widely used macrolide agent in the therapy of otitis media, urinary tract infection, skin soft tissue infection, and upper and lower respiratory tract infections [1,2,7,13,14]. Electrochemical analysis is the common-used method for the determination of MID, involving capillary zone electrophoresis (CZE) [10], cyclic voltammetry (CV) [8], adsorptive stripping voltammetry (ASV) [22], and polarographic catalytic wave (PCW) [18]. High-performance liquid chromatography with evaporative light-scattering detection (HPLC-ELSD) is also reported on determining MID [11]. In this present work, an ultrasensitive approach for determining MID by FI-CL was established for the first time on the basis of the inhibitory property of MID on luminol–BSA reaction. It was found that the CL intensity decrements were logarithm over MID concentrations, giving the calibration graph range of 1.0–5000 pmol $\cdot$ l^{-1} with the LOD of 0.3 pmol $\cdot$ l^{-1} (3σ). This proposed method was successfully applied to determine MID in human serum samples with the recoveries from 96.0 to 110.0%. The possible CL mechanism of luminol–BSA–MID reaction was also discussed in detail.

2. Experimental section

2.1. Reagents

All chemicals used in the whole experiments were of at least analytical reagent grade. Deionized water was purified in a Milli-Q system (Millipore, Bedford, MA, USA) before use. Luminol (Fluka, Biochemika) was purchased from Xi'an Medicine Purchasing and Supply Station, China. BSA (Sigma-Aldrich) was used directly without any further purification. MID was obtained from National Institute for the Control of Pharmaceutical and Biological Products, China.

MID stock standard solution (2.0×10^{-3} g $\cdot$ ml^{-1}) was prepared in ethanol–water solution (1:4, v/v). The working standard solutions of MID were prepared daily from the stock solution as required. Luminol

stock standard solution (2.5×10^{-2} mol·l^{-1}) was prepared in 1.0×10^{-1} mol·l^{-1} NaOH solution. BSA stock standard solution (1.0×10^{-6} mol · l^{-1}) was prepared with pure water and stored in a refrigerator at 4°C.

2.2. Apparatus

The FI-CL system (Xi'an Remax Analysis Instrument Co. Ltd., Xi'an, China) was schematically illustrated in Fig. 2, including a sampling system (IFFM-E), a CL detector (IFFS-A) and a recorder (a computer with IFFM-E client system). The sample system provided a peristaltic pump to propel all solutions to the flow cell, and a six-way valve with loops of 100 μl to inject quantitative luminol. The CL detector which was inside a black box to prevent ambient light contained a flow cell and a photomultiplier tube (PMT). The flow cell, which was placed close to the PMT, was made by coiling 15 cm length colorless glass tube (1.0 mm i.d.) into 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.) was used to carry all the reagents.

2.3. Procedures

As shown in Fig. 2, all the streams (luminol, BSA, MID and carrier) were inserted into flow lines at a constant rate of 2.0 ml · min^{-1} by the pump. The pure water was used to wash the whole flow system until a stable baseline had been recorded. Then 100 μl of luminol was injected into the carrier stream by six-way valve and thereafter merged with the premixing stream of BSA and MID. The whole mixing solution was delivered to the CL cell in an alkaline medium and the CL intensity was detected by the PMT and luminosity meter. The concentrations of MID were measured by the CL intensity decrements ($\Delta I_{CL} = I_o - I_s$), where I_o and I_s were CL intensities in the absence and presence of MID, respectively.

2.4. Treatment of spiked human serum samples

The human serum samples from healthy volunteers were obtained from the Hospital of Northwest University. To prepare the spiked samples, known quantities of MID were added into 0.10 ml human serum. After homogenization, suitable aliquot from the spiked samples with appropriate dilution were taken for the determination of MID.

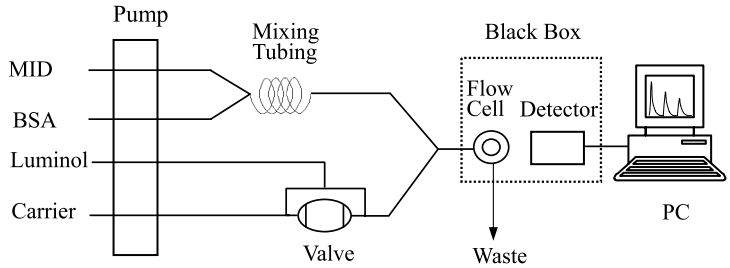

Fig. 2. Schematic diagram of the FI-CL system for the determination of MID. The concentrations of luminol, BSA and NaOH were 5.0×10^{-5}, 5.0×10^{-9} and 2.5×10^{-2} mol·l^{-1}, respectively. The flow rate was 2.0 ml · min^{-1}. The high voltage was −750 V.

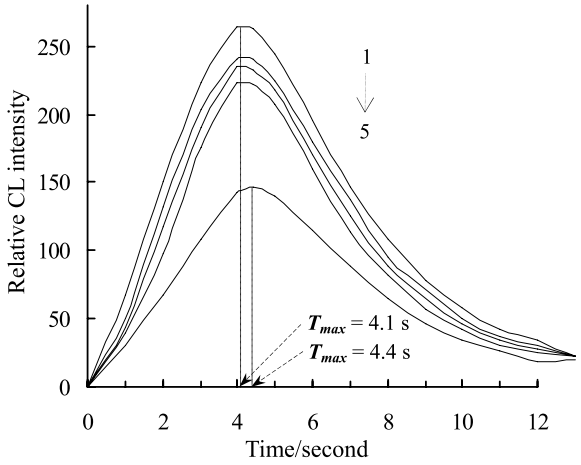

Fig. 3. Relative CL intensity–time profiles of different CL reactions: curve 1: luminol–BSA CL reaction; curves 2–4: luminol–BSA–MID CL reaction; curve 5: luminol–dissolved oxygen CL reaction. The concentrations of luminol and BSA were 5.0×10^{-5} and 5.0×10^{-9} mol $\cdot$ l^{-1}. MID concentrations were 1.0×10^{-12}, 1.0×10^{-11} and 1.0×10^{-9} mol $\cdot$ l^{-1}, respectively.

3. Results and discussion

3.1. The relative CL intensity–time profile

The relative CL intensity–time profile was given in Fig. 3. It can be seen that the time ($T_{\max}$) for reaching maximum CL intensity ($I_{\max}$) of luminol–BSA reaction (curve 1) and luminol–dissolved oxygen reaction (curve 5) were 4.1 and 4.4 s with the $I_{\max}$ of 264 and 146, respectively; the $T_{\max}$ for luminol–BSA reaction in the presence of MID (curves 2–4) was 4.1 s, while the $I_{\max}$ decreased proportionally to MID concentrations. The concentrations of luminol and BSA were 5.0×10^{-5} and 5.0×10^{-9} mol$\cdot$l^{-1}. MID concentrations from curves 2–4 were 1.0×10^{-12}, 1.0×10^{-11} and 1.0×10^{-9} mol $\cdot$ l^{-1}, respectively.

3.2. Optimum conditions of luminol–BSA CL system

It was found that the concentrations of luminol and BSA greatly affected the CL intensity. To obtain a stable and strong CL intensity, 5.0×10^{-5} mol $\cdot$ l^{-1} luminol and 5.0×10^{-9} mol $\cdot$ l^{-1} BSA were selected in this work. Considering the nature of luminol CL reaction favoring under the alkaline medium, 2.5×10^{-2} mol $\cdot$ 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$\cdot$min^{-1} flow rate and 5.0 cm mixing tube length were chosen in the subsequent experiments giving a strong CL intensity with a better precision, a good sensitivity and lower reagent consumption.

3.3. Analytical performance for the determination of MID

Under the optimized experimental conditions given above, the standard solutions of MID were determined. It was found that the CL intensity decrements were proportional to the logarithm of MID concentrations ranging from 1.0 to 5000 pmol$\cdot$l^{-1} with the LOD of 0.3 pmol$\cdot$l^{-1} (3σ). The calibration equation for MID was $\Delta I_{\mathrm{CL}} = 6.1 \lg C_{\mathrm{MID}} + 22.8$, $\mathrm{R}^2 = 0.9921$. The relative standard deviations (RSDs)

of seven replicate determinations of MID were 3.0, 2.4 and 1.7% at 10.0, 100.0 and 500.0 pmol $\cdot$ l^{-1}, respectively. One analyzing cycle of MID determination could be finished in 30 s, which offered a sample efficiency of 120 h^{-1}.

3.4. Interference studies

The interference studies were tested by adding potential interfering substances with increasing amounts into MID standard solution and the error was controlled under 5%. The tolerable ratios of foreign substances with respect to 100 pmol $\cdot$ l^{-1} MID were 100,000 for ethanol, glucose and fructose, 5000 for NH_4^+, Mg^{2+} and Ca^{2+}, 1000 for uric acid, 100 for Fe^{3+} and Fe^{2+}, 10 for azithromycin. Under the optimum experimental conditions, abundant compounds such as protein, salt and lipid in human serum had no obvious interference for MID determination. Thus MID in human serum samples could be determined by FI-CL without separation step, which shortened the analyzing time and eliminated the errors from the separation procedure.

3.5. Possible CL mechanism of luminol–BSA–MID reaction

From Fig. 3, it was clear that the I_{max} of luminol–dissolved oxygen reaction in the presence of BSA (curve 1) increased from 146 to 264 and the T_{max} changed from 4.4 to 4.1 s, showing BSA can accelerate the CL emission from luminol [21]; it was also clear that the I_{max} of luminol–BSA reaction was inhibited in the presence of MID (curves 2–4), however the T_{max} was the same as that of luminol–BSA reaction, indicating there may be interaction between BSA and MID. According to the FI-CL model of protein–small molecule interaction [25], the binding parameters of BSA with MID were obtained with 1.49×10^4 $l \cdot mol^{-1}$ for the binding constant K_{MID} and 0.60 for the binding site number n. Hence, the possible CL mechanism of luminol–BSA–MID reaction can be explained as follows: the interaction between BSA and luminol with a 1:1 complex formed ($K_L = 1.12 \times 10^7$ $l \cdot mol^{-1}$) led to the CL intensity of luminol enhanced and produced the effect of complexing enhancement of CL [25]; while in the presence of MID, the BSA–MID complex ($K_{MID} = 1.49 \times 10^4$ $l \cdot mol^{-1}$, $n = 0.60$) formed online, which may cause the conformation of BSA changed and resulted in the CL intensity inhibition of luminol–BSA reaction.

4. Application

4.1. Determination of MID in spiked human serum samples

MID in the spiked human serum samples prepared in the experimental section were measured and the results were summarized in Table 1. It can be seen that the recoveries for the determination of MID were from 96.0 to 110.0% and the RSDs were less than 2.6%, confirming the applicability of this method for MID determination in biological samples.

5. Conclusion

This paper described a sensitive method for the determination of MID by FI-CL with luminol–BSA system for the first time. Comparing with the reported methods for the measurement of MID shown in

Table 1
The determination of MID in spiked human serum samples[a]

Sample no.	Added/Found ($pg \cdot ml^{-1}$)	RSD (%)	Recovery (%)	By proposed method/spiked ($\mu g \cdot ml^{-1}$)
1	0/31	2.2	96.7	0.62/0.60
	30/60	1.9		
2	0/29	2.5	106.3	0.58/0.60
	30/61	2.0		
3	0/32	2.3	96.0	0.64/0.60
	50/80	1.8		
4	0/29	2.0	104.0	0.58/0.60
	50/81	1.3		
5	0/47	2.6	110.0	0.94/1.00
	30/80	1.4		
6	0/51	1.8	98.0	1.02/1.00
	50/100	1.5		
7	0/98	1.6	103.0	1.96/2.00
	100/203	1.3		
8	0/101	1.5	98.4	2.02/2.00
	100/199	1.4		

[a]The average of five determinations.

Table 2
The comparison of FI-CL and other methods for determining MID

Methods	Linear ranges ($\mu mol \cdot l^{-1}$)	LODs ($nmol \cdot l^{-1}$)	Samples[a]	Refs
ASV	2.5–6.1	1.2×10^3	Tablet, urine	[22]
CZE	1.0–1000	5.0×10^2	Urine	[10]
HPLC-ELSD	0.6–4900	–	Bulk material	[11]
CV	0.5–20	–	Tablet	[8]
PCW	2.0×10^{-2}–1.0×10	6.0	Tablet, urine	[18]
FI-CL	1.0×10^{-6}–5.0×10^{-3}	3.0×10^{-4}	Serum	This study

[a]The biological samples were from human beings.

Table 2, this proposed method exhibited excellent sensitivity with the LOD of 0.3 $pmol \cdot l^{-1}$ which was about four orders in magnitude higher than others. The successful application of determining MID in human serum samples proved the ability of this method for analyzing complex biological matrix with separation-free, indicating its potential value in the pharmaceutical and clinic researches.

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