Monitoring sub-nanogram amount of acetylspiramycin in human urine using flow injection analysis with chemiluminescence detection

LUAN Xin-jun[1] , SONG Zheng-hua[1][*] , XIAO Zhen[2]

(1 . *Department of Chemistry , Northwest University , Xi′an 710069 , China ; 2 . Shaanxi Institute for Drug Control , Xi′an 710061 , China*)

Abstract: **Aim**   To establish a new and simple flow injection method for the rapid determination of acetylspiramycin (ASPM) . **Methods**   ASPM was determined by chemiluminescence (CL) method combined with flow injection (FI) technology , which was based on the inhibitive effect of ASPM on the chemiluminescence reaction of the luminol-K_3Fe (CN)$_6$ system . **Results**   The decrease of chemiluminescence intensity was proportional to the logarithm of ASPM concentration (0.1 - 100) $\mu g \cdot L^{-1}$, the detection limit was 40 ng $\cdot L^{-1}$ (3σ) . The whole process , including sampling and washing , could be completed in 0.5 min with a RSD less than 3.0 % ($n = 5$) . **Conclusion**   The FI-CL method is of both high sensitivity and good selectivity giving a throughput of 120 h^{-1} . The proposed method was applied successfully to the determination of ASPM in pharmaceutical preparations and human urine without any pre-treatment . It was found that the ASPM concentration reached its maximum after being orally administrated for two hours .

Key words : acetylspiramycin ; flow injection ; chemiluminescence ; serum ; urine

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Acetylspiramycin (ASPM) is a macrolide antimicrobial agent with activity against Gram-positive organisms , including *Streptococcus pyogenes* (group A beta-hemolytic streptococci) , *S . viridans* , *Corynebacterium diphtheriae* , and methicillin-sensitive *Staphylococcus aureus* and also some Gram-negative bacteria , such as *Neisseria meningitidis* , *Bordetella pertussis* and *Campylobacter* . Especially , acetylspiramycin has been found to be safe for pregnant woman , fetus and the newborn for treatment of toxoplasmosis[1,2] . Many methods have been reported for the quantitative determination of ASPM , including microbiological assay[3] , fluorimetry[4] , spectrophotometry[5] , HPLC[6] and electroanalysis[7-9] .

Chemiluminescence (CL) combined with flow injection (FI) system is simple , rapid , sensitive , and suitable for pharmaceutical monitoring[10-12] . However , no report has been available on the CL determination of ASPM so far . It was found that the CL intensity generated by luminol reacting with K_3Fe(CN)$_6$ could be inhibited in the presence of ASPM . The decrement of CL was linear over the logarithm of the ASPM concentration from 0.1 to

$100 \ \mu g \cdot L^{-1}$ with a relative standard deviation of less than 3. 0 % . At a flow rate of 2. 0 mL· min^{-1} , the procedure could be performed within 0. 5 min , including sampling and washing , giving a throughput of about 120 times per hour . The proposed method was applied successfully to determine ASPM in pharmaceutical preparations and human urine .

Materiails and methods

Materials　All chemicals were of analytical reagent grade . Water was purified by the Milli-Q (Millipore , Bedford , MA , USA) . ASPM was supplied by Shaanxi Institute for Drug Control . A standard solution of ASPM $(1. 0 \ g \cdot L^{-1})$ was stored at 4 ℃ , from which working strength solutions were prepared freshly . Luminol (Fluka) was dissolved to an appropriate concentration with 0. 2 mol· L^{-1} NaOH solution . 1. 0 mmol· L^{-1} stock solution of $K_3 Fe(CN)_6$ was prepared .

Apparatus　The FI system used is shown in Figure 1 . A peristaltic pump (Shanghai Meter Electromotor Plant , Model ND-15 , 15 r· min^{-1}) was used to generate the flows . PTFE tubing (1 mm ID) was used throughout the system . One hundred μL mixed solution of ASPM with $K_3 Fe (CN)_6$ was injected by a six-way valve . The CL emission cell is a spiral glass tube (15 cm × 1 mm ID) to produce a large surface area exposed to the adjacent photomultiplier tube (PMT) (Hamamatsu , Model IP28) . The CL signal was detected without wavelength discrimination , and the PMT output was amplified and quantified by a luminosity meter (Xi′ an Keri Electron Device Ltd . , Model GD-1) connected to a recorder (Shanghai Dahua Instrument and Meter Plant , Model XWT-206) .
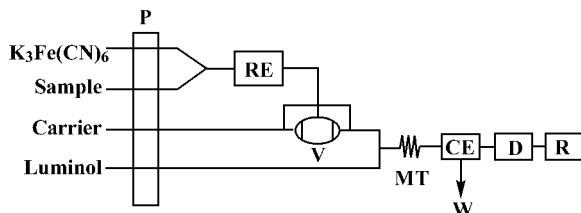
P : Peristaltic pump ; RE : Reaction cell ; V : Injection valve ; CE : CL cell ; D : Detector ; R : Recorder ; W : Waste ; MT : Mixing tubing

Figure 1　Schematic diagram of the flow-injection system for acetylspiramycin (ASPM) determination

Procedures　The carrier (water) and the reagents (luminol , sample and $K_3 Fe(CN)_6$) were propelled at a flow rate of 2 mL· min^{-1} . One hundred μL of the reacted solutions of ASPM and $K_3 Fe(CN)_6$ were injected into the carrier stream , which was then mixed with the luminol

stream . The mixed solution was delivered to the CL cell , and the decrease of the CL signal was detected with luminometer . The concentration of the sample was quantified by decreased CL intensity ($\triangle I = I_0 - I_S$) , where I_0 and I_S were CL signals in the absence and in the presence of ASPM , respectively .

Results and discussion

1　Kinetic curves of CL reaction

To determine the characteristic of the CL reaction , the dynamical profile of the CL was tested in a static system , using $1. 0 × 10^{-7}$ mol· L^{-1} luminol and $1. 0 × 10^{-7}$ mol· L^{-1} $K_3 Fe(CN)_6$. The CL reached its maximum intensity at 26 s and then became extinguished within 200 s after mixing luminol with $K_3 Fe(CN)_6$.

2　Effect of luminol and $K_3 Fe(CN)_6$ concentration

The maximum CL intensity could be obtained at luminol concentrations higher than $8. 0 × 10^{-6}$ mol· L^{-1} . Luminol ($1. 0 × 10^{-5}$ mol· L^{-1}) was then used in this experiment . It was shown that the CL intensity reached its minimum while the concentration of $K_3 Fe(CN)_6$ was 2. 0 $× 10^{-6}$ mol· L^{-1} , which was then chosen as the following procedures .

3　Effect of NaOH concentration

The CL reaction of luminol and $K_3 Fe (CN)_6$ was performed necessarilly in alkaline medium . The maximum CL intensity was found with 0. 5 mol· L^{-1} NaOH , thus the concentration was chosen as optimum condition .

4　Effect of flow rate and the length of mixing tubing

The CL intensity increased with the increase of total flow rate , and the rate of 2. 0 mL· min^{-1} was chosen as a compromise between better precision and lower reagent consumption . It was found that a 10 cm of mixing tubing afforded best results regarding sensitivity and reproducibility .

5　Performance of the system for ASPM measurements

Under the optimized conditions , the calibration graph of decrement of CL intensity ($\triangle I$) versus logarithm of ASPM concentration (μg· L^{-1}) was linear in the range from 0. 1 to 100 μg· L^{-1} ($\triangle I = - 85. 753 \ lg \ C_{ASPM} + 384. 31$; $r^2 = 0. 998 \ 6$) . The relative standard deviation (RSD) of five determinations was 1. 69 % (1. 0 μg· L^{-1}) ASPM and the limit of detection was 40 ng· L^{-1} ASPM.

6　Selectivity Study

The influence of foreign species was investigated by analyzing a standard solution of 5. 0 μg· L^{-1} ASPM to which increasing amounts of interfering species were

added . The tolerable limit of a foreign species was taken as a relative error less than 5 % , and the results are listed in Table 1 .

Table 1   Tolerable concentration ratios of interfering species with respect to ASPM (5. 0 μg· L⁻¹)

Substance	Tolerable concentration ratio
K^+ ,Na^+ ,Cl^- ,NO_3^- ,Ac^- ,CO_3^{2-} ,HCO_3^- ,Br^- ,SO_4^{2-} ,PO_4^{3-}	>1 000
Methanol , urea , phenylbarbital , ethanol	>1 000
Oxalate , sulfosalicylic acid , sodium benzenesulfonate	>1 000
Mg^{2+} ,Co^{2+} , Ni^{2+} ,sucrose ,malic acid	500
Zn^{2+} ,Pb^{2+} ,Ca^{2+} ,glucose ,starch ,dextrin ,sodium benzoic acid	100
Cu^{2+} , uric acid	35
Fe^{3+} , Fe^{2+}	5

7   Determination of ASPM in pharmaceutical preparations

Two different preparations were purchased from the local market and the labeled content were both 100 mg per tablet . The ASPM contents were listed in Table 2 , and the results obtained by the proposed method were in well agreement with that obtained by the UV .

Table 2   Results of determination of ASPM in pharmaceutical tablets ($n = 5$)

Sample No.	Added /μg· L⁻¹	Found /μg· L⁻¹	RSD / %	Recovery / %	Average content in tablets / (mg/ pill) Proposed method	UV
1	0	0.97	2.98	96.0	97	98
	1.00	1.93	2.24			
	0	1.05	2.87	101.5	105	103
	2.00	3.08	2.21			
	0	1.04	2.93	103.6	104	106
	3.00	4.15	1.83			
2	0	0.95	2.95	97.0	95	98
	1.00	1.92	2.09			
	0	0.99	3.00	103.0	99	102
	2.00	3.05	2.18			
	0	0.98	2.97	99.3	98	97
	3.00	3.96	2.15			

8   Determination of ASPM in spiked serum

The proposed method was applied successfully to the analysis of ASPM in spiked human serum . The concentration of the analyte in the samples and the percent recoveries in each case are listed in Table 3 . And t-test was carried out on the results to evaluate the validity of the proposed method for the determination of ASPM in human serum . As indicated , the proposed CL method showed considerable precision and accuracy .

Table 3   Results of assay for ASPM in spiked human serum ($n = 5$)

Sample No.	Added /μg· L⁻¹	Found /μg· L⁻¹	RSD / %	Recovery / %	t-test ($t_{0.05,4}$ = 2.78)	Average content in tablets/ mg· L⁻¹ Proposed method	Spiked
1	0	0.98	2.94	96	2.48	98	100
	1.00	1.94	2.58				
2	0	2.05	2.72	94	2.51	205	200
	1.00	2.99	2.12				
3	0	3.06	2.24	93	2.14	306	300
	1.00	3.99	1.98				
4	0	1.04	2.71	104	2.08	104	100
	1.00	2.08	2.12				
5	0	1.98	2.30	97	2.24	198	200
	1.00	2.95	1.86				
6	0	2.96	2.65	94	1.13	296	300
	1.00	3.90	2.29				
7	0	1.04	2.89	102	1.75	104	100
	1.00	2.06	2.38				
8	0	1.96	2.67	98	1.12	196	200
	1.00	2.94	2.18				
9	0	2.97	2.32	99	1.27	297	300
	1.00	3.96	1.95				
10	0	1.03	2.63	97	2.22	103	100
	1.00	2.00	2.07				
11	0	2.03	1.99	99	0.59	203	200
	1.00	3.02	2.27				
12	0	2.99	2.01	96	1.59	299	300
	1.00	3.95	1.79				
13	0	1.04	2.87	103	2.12	104	100
	1.00	2.07	2.15				
14	0	1.97	2.36	103	1.43	197	200
	1.00	3.00	1.99				
15	0	3.00	1.78	94	2.21	300	300
	1.00	3.94	2.38				
16	0	1.05	2.92	95	2.50	105	100
	1.00	2.00	2.66				
17	0	1.99	2.88	96	2.15	199	200
	1.00	2.95	2.35				
18	0	2.95	2.62	95	1.65	295	300
	1.00	3.90	2.49				

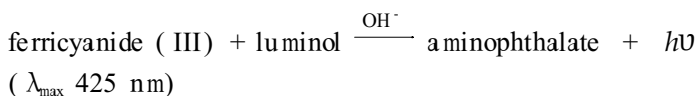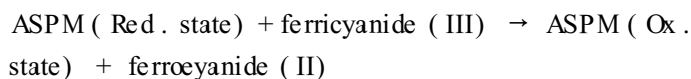## 9   Monitoring excretive ASPM in human urine within 8 h

Two apparently healthy male volunteers were administered ASPM tablets in the morning with empty stomach . According to the marked content , the net dosage of ASPM they took is 200 mg per capita . From then on , the first-voided urine samples were collected in dark glass bottles at different times . Without any pre-treatment procedures urinary ASPM could be determined simply by the proposed method after dilution with a factor of 5×10^3 . The excretive profile of ASPM in urine was studied as shown in Table 4 . It can be observed that the total ASPM excreted through urine was about 1. 2 mg in a total volume of 0. 7 liter in 8 h . The ASPM concentration reached its maximum after two hours and dropped sharply within a few hours .

Table 4　Determination of ASPM in human urine samples ($n = 5$)

Time /h	ASPM Found and Supplement /$\mu g \cdot L^{-1}$	Found /$\mu g \cdot L^{-1}$	RSD / %	Recovery / %	ASPM in urine $C(mg \cdot L^{-1})$ / $V(mL)$	Excretive ASPM (mg) in urine
0.5	0	0	-	108	0/60	0
	1.00	1.08	2.83			
1	0	0.31	8.93	113	1.55/75	0.116
	1.00	1.44	2.78			
1.5	0	0.59	5.92	95	2.95/85	0.251
	1.00	1.54	2.35			
2	0	0.64	6.57	108	3.22/100	0.322
	1.00	1.72	2.58			
2.5	0	0.51	6.82	94	2.55/85	0.212
	1.00	1.45	2.95			
3	0	0.44	7.34	116	2.20/70	0.154
	1.00	1.60	2.95			
4	0	0.19	11.3	88	0.95/80	0.076
	1.00	1.07	3.89			
5	0	0.08	15.85	114	0.40/80	0.032
	1.00	1.22	2.86			
6	0	0	-	96	0/65	0
	1.00	0.96	2.96			
					Total :1.66/700	Total :1.163

Possible mechanism

It was found that the rate of the reaction of $K_3Fe(CN)_6$ with ASPM in solution was very fast reaction process of $K_3Fe(CN)_6$ with ASPM was followed by UV at 232 nm and the results are listed in Table 5. It was obvious that the absorption intensity of ASPM increased greatly in the presence of $K_3Fe(CN)_6$. It was also testified that the products of reaction between $K_3Fe(CN)_6$ and ASPM could not oxidize luminol hemiluminescently. Hence, the mechanism of the inhibit effect of ASPM on luminol-ferricyanide CL system could be presented as below:

ASPM (Red. state) + ferricyanide (III)　→　ASPM (Ox. state) + ferroeyanide (II)

ferricyanide (III) + luminol $\xrightarrow{OH^-}$ aminophthalate + $h\upsilon$ (λ_{max} 425 nm)

Table 5　Results of determination $K_3Fe(CN)_6$ + ASPM by UV at 232 nm

Species*	A ($n = 5$)
$K_3Fe(CN)_6$	0.001
ASPM	0.121
$K_3Fe(CN)_6$ + ASPM	0.224

* The same concentration and injection volume

Conclusion

Compared to the other methods, the reported FI-CL method for the determination of ASPM obtained some results clearly demonstrating that the method offers advantages of simplicity, rapidity, wide linear range as well as high sensitivity for the determination of ASPM.

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