Ultrasensitive determination of amoxicillin using chemiluminescence with flow injection analysis

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Abstract. Results presented here reveal that amoxicillin can greatly enhance the chemiluminescence intensity generated from the reaction between luminol and hydrogen peroxide. The increment chemiluminescence signal was linearly dependent on amoxicillin concentration in the range from 10 pg·ml⁻¹ to 2 ng·ml⁻¹ \((r^2 = 0.9978)\) offering a detection limit as low as 3.5 pg·ml⁻¹ \((3\sigma)\). At a flow rate of 2.0 ml·min⁻¹, one analysis cycle, including sampling and washing, can be accomplished in 20 s with a relative standard deviation of less than 5%. The sensitive flow injection method was applied successfully to determine of amoxicillin in pharmaceutical preparations, human urine and serum without any pretreatment procedure, with recovery from 90.0% to 110.0% and relative standard deviations of less than 5.0%.

1. Introduction

Amoxicillin, 4-Thia-1-azabicyclo[3,2,1]heptane-2-carboxylic acid, 6{[amino(4-hydroxyphenyl)acetyl]amino}-3,3-dimethyl-7-oxo-, thihydrate \(2s-\{2\ \alpha; 5\ \alpha, 6\ \beta (s^*)\}\), white, practically odorless, crystalline powder with molecular weight of 419.45 [1]. Owing to the ability of amoxicillin interfering with the bacteria to form cell walls that are vital for the survival of bacteria, it is used to treat infections caused by bacteria, such as pneumonia; bronchitis; gonorrhea and infection of the ears, nose, throat, urinary tract, and skin [2].

Liquid chromatography with MS or UV detection after solid-phase extraction or multi-step liquid-liquid extraction has been reported for the determination of amoxicillin [3–7]. Other analytical techniques including capillary electrophoresis [8], spectrophotometry [9,10], and biosensors [11–13] have also been applied in the determination of amoxicillin. Chemiluminescence (CL) for determination of amoxicillin in pharmaceuticals has also been reported by using the reaction of amoxicillin with KMnO₄ in acidic medium in the presence of quinine sulfate, with a detection limit of 0.02 µg·ml⁻¹ [14]. In this paper, it was found that amoxicillin could evidently enhance the CL generated from the reaction of luminol and hydrogen peroxide. The increment of CL signal was linear with the concentration of amoxicillin...
ranging from 10 pg·ml⁻¹ to 2 ng·ml⁻¹ with a relative standard deviation of less than 3.0%. The proposed method has a rather low limit of detection down to 3.5 pg·ml⁻¹, thus it was applied successfully in an assay of amoxicillin for pharmaceutical preparations, human urine and serum without any pretreatment, with recovery from 90.0% to 110.0% and relative standard deviations (RSDs) of less than 5.0%.

2. Experimental

2.1. Reagents

All the reagents were of analytical grade; Water purified in a Milli-Q system (Millipore, Bedford, MA, USA) was used for the preparation of solutions. Amoxicillin standard solution was obtained from Shaanxi Institute for Drug Control. Luminol (Fluka, Bilchemika) was purchased from Xi’an Medicine Purchasing and Supply Station, China. Hydrogen peroxide was obtained from Xi’an Chemical Reagent Plant.

A standard solution of amoxicillin (0.676 mg·ml⁻¹) was stored at 4°C and protected from light. Luminol was used as supplied to prepare a 2.5 × 10⁻² mol·l⁻¹ stock standard solution by dissolving 4.40 g of luminol with 0.1 mol·l⁻¹ sodium hydroxide to 1.0 l in a brown calibrated flask. Hydrogen peroxide (33.3%) was diluted by pure water to give a final concentration of 0.1 mol·l⁻¹.

2.2. Apparatus

The flow injection system used in this work is shown in Fig. 1. A peristaltic pump (shanghai meter electromotor plant, Model ND-15, 15 rpm) was utilized to pump each of all flow streams at a flow rate of 2.0 ml·min⁻¹. PTFE tubing (1.0 mm i.d.) was used throughout the manifold for carrying the CL reagents. A six-way valve with loop of 100 µl was employed for sampling. The CL emission cell is a spiral glass tube (1.0 mm i.d., 15 cm length) producing a large surface area exposed to the adjacent photomultiplier tube (PMT) (Hamamatsu, Model IP28). Extreme precautions were taken to ensure that the cell compartment and the photomultiplier tube were light-tight. The CL signal produced in the CL emission cell 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 Plant, Model XWT-206).

![Fig. 1. Schematic diagram of the flow-injection system for amoxicillin determination.](image-url)
2.3. Procedures

The carrier water and the solutions (NaOH, sample, hydrogen peroxide and luminol) were propelled at a constant flow rate of 2.0 ml·min\(^{-1}\) on each flow line. The pump was started to wash the whole flow system until a stable baseline was recorded. The luminol solution firstly mixed with hydrogen peroxide, and then, the mixture was injected into the carrier stream by a six-way valve, which was then merged with the amoxicillin stream. The mixed solution was delivered to the CL cell in an alkaline medium, and the peak height of the CL signal was detected with the PMT and luminometer. The concentration of the sample was quantified by the increment of CL intensity (\(\Delta I = I_s - I_o\)), where \(I_o\) and \(I_s\) were CL signals in the absence and in the presence of amoxicillin, respectively.

2.4. Determination of amoxicillin in pharmaceutical preparations

The preparations were purchased from the local market and weighed then grounded to a fine powder and mixed. A sample equivalent to approximately 40 mg preparations was weighed accurately and made up to 50 ml with water. As the proposed method, the sample was then diluted to the concentration with the calibration range without pretreatment.

2.5. Determination of amoxicillin in spiked human urine and serum samples

The urine samples collected from two apparently healthy male volunteers and the serum samples supplied by the Hospital of Northwest University were spiked before determination. Known quantities of amoxicillin were spiked into 1.0 ml of urine or serum to prepare the spiked samples. After homogenization, the solutions were diluted with a factor of 2\(\times 10^5\) for urine sample and 5\(\times 10^5\) for serum sample, and then the samples were determined by the proposed method directly.

3. Results and discussion

3.1. CL intensity–time profile

Before carrying out the flow injection method, the kinetic curve was examined by static method, using 5.0 \(\times 10^{-7}\) mol·l\(^{-1}\) luminol and 5.0 \(\times 10^{-5}\) mol·l\(^{-1}\) H\(_2\)O\(_2\). As shown in Fig. 2, the mixed solution of luminol and hydrogen peroxide gave an evident CL signal in alkaline medium, which reached a maximum intensity at 2 s after the mixing of reactants, and then became extinguished within 15 s thereafter. In the presence of amoxicillin (100 pg·ml\(^{-1}\)), the CL intensity approached the maximum at 3 s and then vanished in the following 18 s, giving a maximum intensity 2.5-fold as that in the absence of amoxicillin.

3.2. Effects of NaOH concentration

Owing to the nature of the luminol reaction, which is more favored under alkaline conditions, NaOH was introduced into the CL cell through a flow line to improve the sensitivity of the system. The effect of NaOH concentration on CL was tested by measuring the CL intensity with a series of NaOH solutions from 0.01 to 0.25 mol·l\(^{-1}\) in the presence of 100 pg·ml\(^{-1}\) amoxicillin. The CL intensity approached a maximum at about 0.025 mol·l\(^{-1}\) NaOH, thus this concentration was employed in subsequent experiments.
Fig. 2. Kinetic CL intensity-time profile in static system. ○: CL intensity in the absence of amoxicillin. ●: CL intensity in the presence of amoxicillin (100 pg·ml⁻¹).

3.3. Effects of luminol and hydrogen peroxide concentration

The CL intensity was tested for different luminol solutions from 1.0 × 10⁻¹⁰ to 1.0 × 10⁻⁶ mol·l⁻¹ in the presence of 100 pg·ml⁻¹ amoxicillin. With increasing luminol concentration the CL signal increased steadily until luminol was 5.0 × 10⁻⁷ mol·l⁻¹, after which the CL intensity tended to be stable. Therefore 5.0 × 10⁻⁷ mol·l⁻¹ luminol solution was selected as optimum for the present system.

The effect of hydrogen peroxide concentration on the CL intensity was also investigated in the presence of 100 pg·ml⁻¹ over the ranges of 1.0 × 10⁻⁷–1.0 × 10⁻⁴ mol·l⁻¹. With the increasing concentration of hydrogen peroxide, the CL intensity increased steeply and reached a maximum intensity at 5.0 × 10⁻⁵ mol·l⁻¹ of hydrogen peroxide. Then the intensity decreased considerably with hydrogen peroxide concentrations higher than 5.0 × 10⁻⁵ mol·l⁻¹. Therefore, 5.0 × 10⁻⁵ mol·l⁻¹ hydrogen peroxide was chosen according to the experiment in the subsequent experiments.

3.4. 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 good precision and lower reagent consumption in the subsequent work. The length of the mixing tube was also adjusted to yield maximum light emission in the CL cell. It was observed that a 5.0 cm of mixing tube afforded the best results with regards to sensitivity and reproducibility. Accordingly, 5.0 cm was then selected as the optimum length of mixing tube.

3.5. Interference studies

The effect of foreign species was tested by analyzing a standard solution of amoxicillin (100 pg·ml⁻¹) to which increasing amounts of interfering species were added. The tolerable limits of a foreign species caused a relative error of less than 5.0%, were less than 30 µg·ml⁻¹ for glutin, barbiturate, oxalic acid, urea and acetone, 10 µg·ml⁻¹ for starch, lactose, cellulose, stearic acid, agar, talc, citric acid, fructose and...
4. Applications

4.1. Determination of amoxicillin in pharmaceutical preparations

Following the procedure described above, the proposed method was applied to the determination of amoxicillin in the pharmaceutical preparations from the local market, and the results were listed in Table 1. By adding a known amount of amoxicillin to the sample before the recommended treatment, the recovery studies were performed on each of the analyzed samples that range from 90.0% to 106.5%.

4.2. Determination of amoxicillin in spiked human urine and serum samples

The proposed CL method using luminol–hydrogen peroxide system was also used to determine amoxicillin in spiked samples of urine and serum. Seven spiked urine samples and six spiked serum samples were determined for amoxicillin by standard addition method. The results from determination of amoxicillin were listed in Tables 2 and 3. The method was verified by determination of recoveries, which varied from 94.0% to 110.0% and from 93.7% to 104.0%. It was obvious that the proposed method was very sensitive for amoxicillin determination in biological fluids, especially in serum sample and urine sample.

5. Conclusion

The results obtained clearly demonstrate that the proposed method offers advantages of simplicity, rapidity, widely linear range as well as high sensitivity for the determination of amoxicillin. The satisfactory performance in an assay of amoxicillin in pharmaceutical preparations and urine and serum demonstrated that the method was practical and suitable in quality control analysis and clinical research.
Table 2
Results of amoxicillin in spiked human urine samplesa

| Sample No. | Added pg·ml⁻¹ | Found pg·ml⁻¹ | RSD % | Recovery % | By proposed method/spiked µg·ml⁻¹ |
|------------|---------------|---------------|-------|------------|----------------------------------|
| 1          | 0             | 49.2          | 3.99  | 108.0      | 9.84/10.0                       |
| 2          | 30            | 51.0          | 2.32  | 94.0       | 10.20/10.0                      |
| 3          | 50            | 98.0          | 2.95  | 98.8/10.0  |                                  |
| 4          | 70            | 125.6         | 4.32  | 108.8      | 10.11/10.0                      |
| 5          | 50            | 125.8         | 1.53  | 106.0/10.0 |                                  |
| 6          | 100           | 166.7         | 3.61  | 96.3       | 10.06/10.0                      |
| 7          | 70            | 166.0         | 3.15  | 95.3       | 9.93/10.0                       |
| 8          | 100           | 393.1         | 1.77  | 103.9      | 9.84/10.0                       |

aThe average of five determinations.

Table 3
Results of amoxicillin in human serum samplesa

| Sample No. | Added pg·ml⁻¹ | Found pg·ml⁻¹ | RSD % | Recovery % | By proposed method/spiked µg·ml⁻¹ |
|------------|---------------|---------------|-------|------------|----------------------------------|
| 1          | 0             | 50.6          | 1.29  | 93.7       | 10.12/10.0                      |
| 2          | 30            | 78.7          | 2.21  |            |                                  |
| 3          | 50            | 101.5         | 4.03  |            |                                  |
| 4          | 70            | 140.0         | 3.82  |            |                                  |
| 5          | 100           | 165.9         | 2.14  |            |                                  |
| 6          | 70            | 168.0         | 1.73  |            |                                  |
| 7          | 100           | 200.0         | 3.94  |            |                                  |

aThe average of five determinations.

Acknowledgements

The authors gratefully acknowledge the financial support from Shaanxi Province Nature Science Foundation and Ministry of Education, China, Grant No. 2003B15 and No. 04Jk145.
References

[1] USP Dictionary of USAN and International Drug Names, The United States Pharmacoperial Convention, Inc., Rockville, MD, 2002.
[2] http://www.nlm.nih.gov/medlineplus/druginfo/medmaster/a685001.html.
[3] C.K. Fagerquist, A.R. Lightfield and S.J. Lehotay, Confirmatory and quantitative analysis of -lactam antibiotics in bovine kidney tissue by dispersive solid-phase extraction and liquid chromatography–tandem mass spectrometry, Anal. Chem. 77(5) (2005), 1473–1482.
[4] K.H. Yoon, S.Y. Lee, W. Kim, J.S. Park and H.J. Kim, Simultaneous determination of amoxicillin and clavulanic acid in human plasma by HPLC-ESI mass spectrometry, J. Chromatogr. B 813(1–2) (2004), 121–127.
[5] R. Lindberg, P.-Å. Jarnheimer, B. Olsen, M. Johansson and M. Tysklind, Determination of antibiotic substances in hospital sewage water using solid phase extraction and liquid chromatography/mass spectrometry and group analogue internal standards, Chemosphere 57(10) (2004), 1479–1488.
[6] M. Becker, E. Zittlau and M. Petz, Residue analysis of 15 penicillins and cephalosporins in bovine muscle, kidney and milk by liquid chromatography-tandem mass spectrometry, Anal. Chim. Acta 520(1–2) (2004), 19–32.
[7] J.D. Cahill, E.T. Furlong, M.R. Burkhardt, D. Kolpin and L.G. Anderson, Determination of pharmaceutical compounds in surface- and ground-water samples by solid-phase extraction and high performance liquid chromatography-electrospray ionization mass spectrometry, J. Chromatogr. A 1041(1–2) (2004), 171–180.
[8] G. Pajchel, Pawkowskik and S. Tyski, CE versus LC for simultaneous determination of amoxicillin/clavulanic acid and ampicillin/sublactam in pharmaceutical formulations for injections, J. Pharm. Biomed. Anal. 29(1–2) (2002), 75.
[9] H. Salem, Selective spectrophotometric determination of phenolic-lactam antibiotics in pure forms and in their pharmaceutical formulations, Anal. Chim. Acta 515(2) (2004), 333–341.
[10] I.F. Al-Momani, Flow-injection spectrophotometric determination of amoxicillin, cephalexin, ampicillin and cephadrine in pharmaceutical formulations, Anal. Lett. 37(10) (2004), 2099–2110.
[11] G. Cacciatore, M. Petz, S. Rachid, R. Hakenbeck and A.A. Bergwerff, Development of an optical biosensor assay for detection of -lactam antibiotics in milk using the penicillin-binding protein 2x*, Anal. Chim. Acta 520(1–2) (2004), 105–115.
[12] E. Gustavsson, J. Degalaen, P. Bjurling and A. Sternesjo, Determination of -lactams in milk using a surface plasmon resonance-based biosensor, J. Agr. Food Chem. 52(10) (2004), 2791–2796.
[13] E. Gustavsson and A. Sternesjo, Biosensor analysis of -lactams in milk: comparison with microbiological, immunological and receptor-based screening methods, J. AOAC Int. 87(3) (2004), 614–620.
[14] J.X. Du, Y.H. Li and J.R. Lu, Chemiluminescence flow-injection analysis of amoxicillin by a permanganate-based reaction, Anal. Lett. 35(14) (2002), 2295–2304.
