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DETERMINATION OF AMOXICILLIN, AMPICILLIN AND PENICILLIN G USING A FLOW INJECTION ANALYSIS METHOD WITH CHEMILUMINESCENCE DETECTION

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The *chemiluminescence* (CL) reactions coupled with flow injection techniques can provide good solutions for development of cheap and fast methods for antibiotic determination. In this work, three β-lactam antibiotics, namely: amoxicillin, ampicillin and penicillin G, were determined by using a *flow injection analysis* (FIA) method based on CL reaction of β-antibiotics with luminol in basic environment, in presence of potassium ferricyanide and ferrocyanide. The novelty of the method is represented by 10 to 350-fold enhancement of the analytical signal using an on-line UV irradiation step of the injected sample. The flow injection system parameters were studied and optimized (concentrations of luminol, Na_2CO_3, $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$, injected sample volume and total flow rate). Ampicillin and penicillin G were determined between 0.05 and 100 ppm and amoxicillin in the 0.25 - 100 ppm range. The developed method proved to be simple, cheap, reproducible and fast due to the UV irradiation of the analyzed samples that determine a lower detection limit in comparison with similar chemiluminometric methods reported in literature. Samples of pharmaceuticals were analyzed and applying the recovery test good results were obtained.

INTRODUCTION

β-lactam antibiotics were determined in the last period by techniques such as: spectrophotometry, [1-7] capillary electrophoresis,[8] calorimetry, [9] polarography,[10] titrations (conductometric with perchloric acid, [11] iodometric, [12] with EDTA [13]), pH-metry,[14] potentiometry,[15] amperometry,[16] fluorimetry,[17] chromatography (micellar electrokinetic chromatography – MEKC,[18] HPLC with solid phase extraction coupled with a diode array detector SPE-HPLC-DAD,[19] isocratic HPLC, [20] HPLC-ESI-MS, [21] ion-paired chromatography with MS,[22] GC-MS [23]), etc. These techniques usually are time-consuming, expensive and additional steps of sample pretreatment are often needed. For these reasons, in the last years several faster and cheaper methods, like chemiluminescence-based techniques, have emerged.

Chemiluminescent (CL) methods for antibiotics determination can be grouped in three categories:

a. Methods based on chemiluminescence reagents use (*e.g.*, luminol)

A method based on the luminol - periodate reaction[24] allowed ampicillin and amoxicillin determination in the 200 – 1000, respectively 100 - 10000 ppm concentration ranges. Another reaction is the one between luminol and the antibiotic, in basic environment, in presence of potassium ferricyanide and ferrocyanide. [25-26] The ferricyanide acts as both catalyst and co-oxidant, while ferrocyanide forms a redox buffer with ferricyanide, reducing the strong CL emission of the luminol – ferricyanide reaction. The method allows the determination of ampicilin in the 5 - 1000 ppm range.

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b. Methods based on direct oxidation of the antibiotic with an appropriate reagent

β-lactam antibiotics can be determined using the oxidation reaction with potassium superoxide. [27] Penicillin G, ampicillin and amoxicillin were determined in the 10 - 100 ppm concentration range using this method. By using potassium permanganate as an oxidizing reagent and in presence of different sensitizers, several antibiotics were determined chemiluminometrically. Thus, cephadroxil was determined in the 0.1 - 30 ppm concentration range, with a 0.05 ppm detection limit (LOD), in presence of quinine. [28] Amoxicillin was determined in presence of quinine sulfate, [29] in the 0.05 - 10 ppm concentration range, with a LOD of 0.02 ppm and of formaldehyde, [30] in the 5.48×10^{-8} - 2.74×10^{-6} mol/L concentration range, with a LOD of 4.1×10^{-8} mol/L.

Cephoxitin, cephoperazone, cephazolin, cephalexin, cephadroxil and cefachlor were determined in concentrations of 0.1 to 10-15 ppm, with LODs of 0.03 - 0.08 ppm, using the tris(2,2'-bipyridil) ruthenium(II) / potassium permanganate system, [31] catalyzed by Mn (II) ions. Ciprofloxacin may be determined after oxidation with Ce (IV), [32] in the 9×10^{-8} - 10^{-6} M concentration range, with a LOD of 3.1×10^{-10} M.

c. Methods based on electrochemiluminescence (ECL)

Cephadroxil was determined by ECL in the $5 \times 10^{-8} - 1 \times 10^{-4}$ mol/L concentration range, using tris(2,2'-bipyridil)ruthenium(II). [33] For concentrations lower than 1×10^{-6} mol/L, the method requires the removal of oxygen from solutions, a step that complicates the method and extends the time of analysis. The chinolones and fluorochinolones were determined by combining ECL with cyclic voltammetry, also using tris(2,2'-bipyridil)ruthenium(II). [34]

The aim of this paper was to develop a sensitive determination method for β-lactam antibiotics that is simple and fast. In order to reach this goal, a flow injection analysis system with chemiluminescence detection (FIA-CL), based on the luminol – ferricyanide / ferrocyanide reaction with analyte was assembled and optimised. In order to lower the detection limit (LOD) of the antibiotics, the UV irradiation influence of the injected sample on the analytical signal was investigated. Literature data confirms that after analytes UV irradiation, sometimes a considerable increase of the CL signal may be obtained. [35-37]

EXPERIMENTAL

Reagents and chemicals

Amoxicillin trihydrate, ampicillin trihydrate and penicillin G trihydrate (Riedel-de Haën) were used.. Structures of the antibiotics are presented in figure 1. 100 ppm stock solutions of antibiotics in bi-distilled water were prepared daily. As chemiluminescent reagent a 1 mM luminol solution prepared by dissolving the corresponding amount of luminol (Fluka), in a potassium hydroxide (Fluka) solution was used. The solution was prepared daily. As CL catalysts 0.2 M potassium hexacyanoferrate(II) (Merck) and 0.5 mM potassium hexacyanoferrate(III) (Riedel-de Haën) in bi-distilled water were used.

Apparatus

Figure 2 presents the used FIA assembly, which consists of a peristaltic pump with 4 channels (Gilson) with tygon pump tubes (1.42 mm i.d.), a 6-ways injection valve Rheodyne type, model 5051, a laboratory-made chemiluminometer based on a Hamamatsu Photonics H9319-01 photomultiplier tube (PMT) that contains a high voltage source and an electronic amplification system. The PMT is operated by direct connection to a PC. The flow cell formed by a coiled PTFE tube (0.5 mm i.d. and 50 cm length) was placed just in front of the PMT's window. The chemiluminescence signals are recorded in an EXCEL format. A PMT voltage of 800 volts was applied in all determinations. An UV lamp (Merck) (254 nm for thin layer chromatography, 6W, 11 cm length and 1.5 cm diameter) was used. Around the tube of the UV lamp was coiled a 150 cm length, 0.5 mm i.d. PTFE tube. All the tubes used in the FIA assembly were PTFE tubes, 0.5 mm i.d.

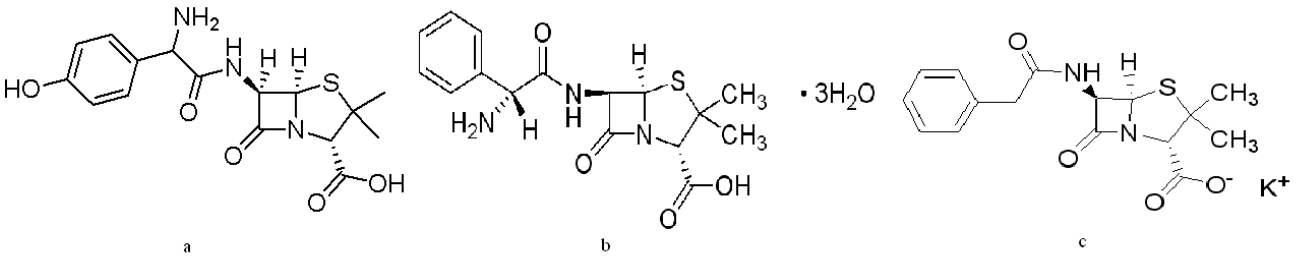

Fig. 1 – Amoxicillin (a), ampicillin (b) and penicillin G structure (c).

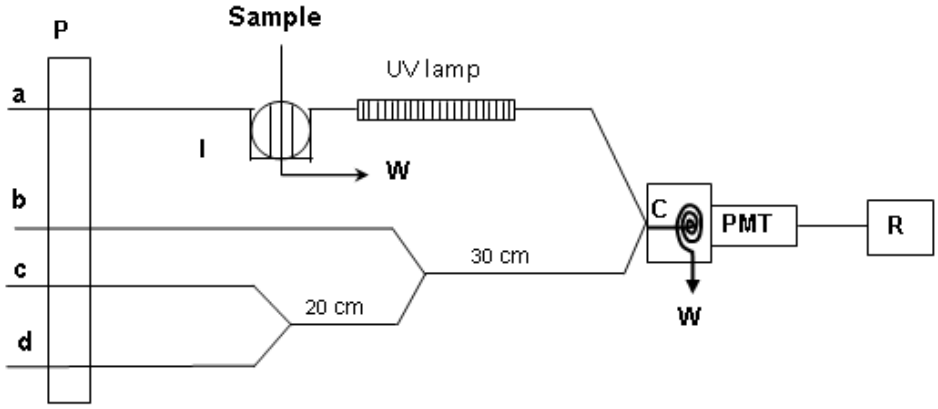

Fig. 2 – Flow injection analysis assembly with CL detection for the determination of amoxicillin, ampicillin and penicillin G. P – peristaltic pump; I – injection valve; W – waste; C – chemiluminometric flow cell; PMT – photomultiplier tube; UV lamp (6W) with a 150 cm teflon tube coiled around it; R – recorder; a – bidistilled water carrier flow; b – potassium hexacyanoferrate(II) solution flow; c – luminol/potassium hydroxide solution flow; d – potassium hexacyanoferrate(III) solution flow.

Procedure

It was used the FIA assembly system presented in figure 2. Carrier flow (a) consisted of bi-distilled water, flow (*b)* was a 0.2 M potassium hexacyanoferrate(II) solution, flow (*c)* was a 1 mM luminol solution dissolved in 0.2 M potassium hydroxide, and flow (*d)* was a 0.5 mM potassium hexacyanoferrate(III) solution. The flow rates were the same for each channel, 0.65 mL/min. The sample with a 250 μL volume was injected in the carrier (*a*), passed the tube coiled around the UV lamp and then mixed the other reagents just before the entrance in the flow cell. The data was collected and then imported in Microsoft EXCEL, FIA characteristic peaks being obtained. Five determinations were carried out for each analyzed sample.

RESULTS AND DISCUSSION

Method optimization

In figure 3 the influence of the total flow rate on the chemiluminescence signal is presented. An increase of the CL signal with the flow rate up to 2.5 mL/min was observed for ampicillin and penicillin G. For higher flow rates, a plateau was reached for penicillin G and a CL signal decrease was observed for ampicillin. In case of amoxicillin, the CL signal slowly increased with the flow rate up to 4 mL/min and then it reached a plateau. As a compromise for all three antibiotics, a total flow rate of 2.6 mL/min was selected.

In figure 4 is presented the influence of the carrier flow rate on the height of the registered FIA signals. The flow rate on the other channels was kept constant.

Low carrier flow rates provide a longer irradiation time of the sample, which can decompose the reactive species of the antibiotics generated by irradiation, and the analytical signal is low. By increasing the flow rate, the signal increased until an optimum value of about 0.65 mL/min and then it decreased again owing to an insufficient irradiation of the sample.

The influence of potassium hexacyanoferrate (II) solution concentration was studied in the 0.01 - 0.5 M range, and was determined to be optimum for 0.2 M. The influence of potassium hexacyanoferrate (III) solution concentration was studied for 0.1 - 1 mM concentration range. The highest CL signals and a good stability of the background noise were obtained for 0.5 mM potassium hexacyanoferrate (III).

The CL signal increases with the luminol concentration, but the background value and noise also increase. In order to study the influence of luminol concentration on the analytical signal it was considered more appropriate to represent the signal/3SD background noise ratio versus luminol concentration as a better indicator for the luminol concentration influence. Therefore, as it can be observed from figure 5, the highest ratio is obtained for the 1mM concentration of luminol.

The influence of the potassium hydroxide concentration on the analytical signal is presented in figure 6, by representing signal/3SD background noise versus potassium hydroxide concentration. It can be observed that the 0.2 M KOH concentration represents the optimum one in order to obtain a higher analytical signal.

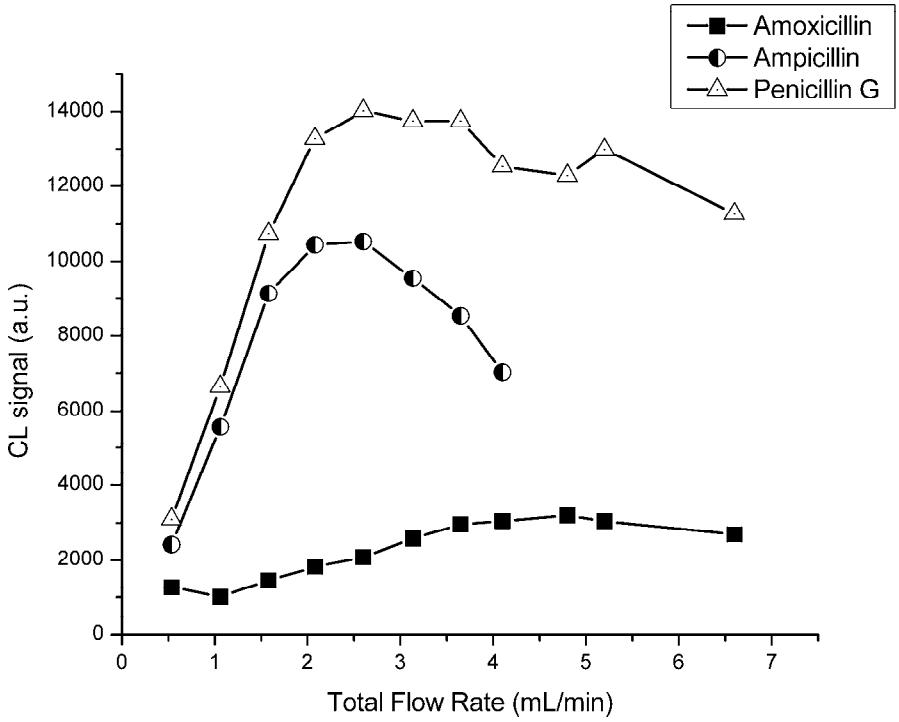

Fig. 3 – Influence of the total flow rate on the chemiluminescence signal.
Conditions as in working procedure, except the 550 µL injected volume; $c_{antibiotic}$ = 2 ppm

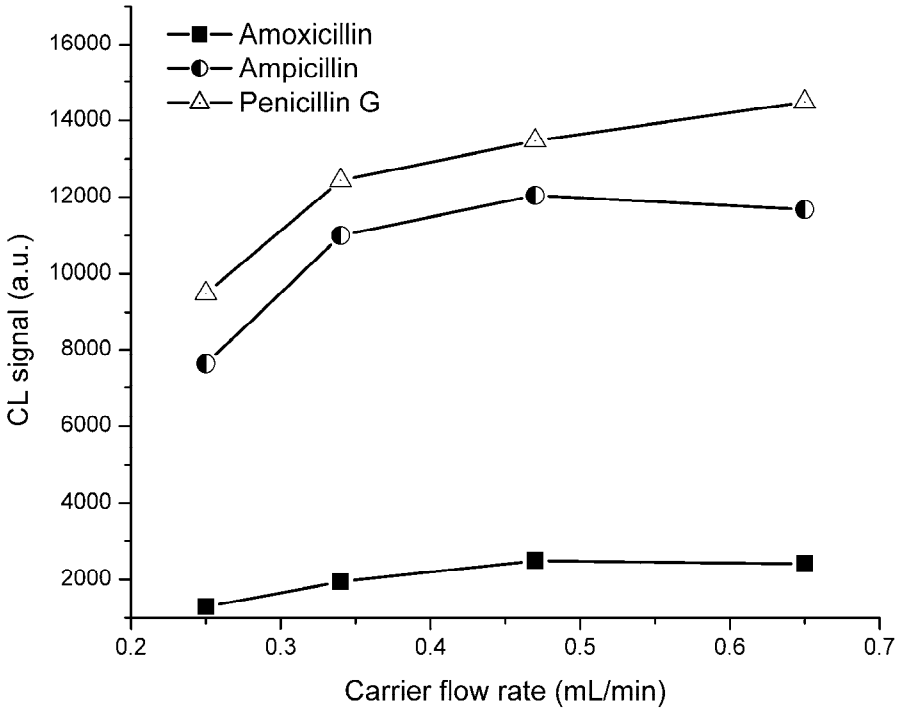

Fig. 4 – Influence of the carrier flow rate on the chemiluminescence signal.
Conditions as in working procedure, except the 550 µL injected volume; $c_{antibiotic}$ = 2 ppm

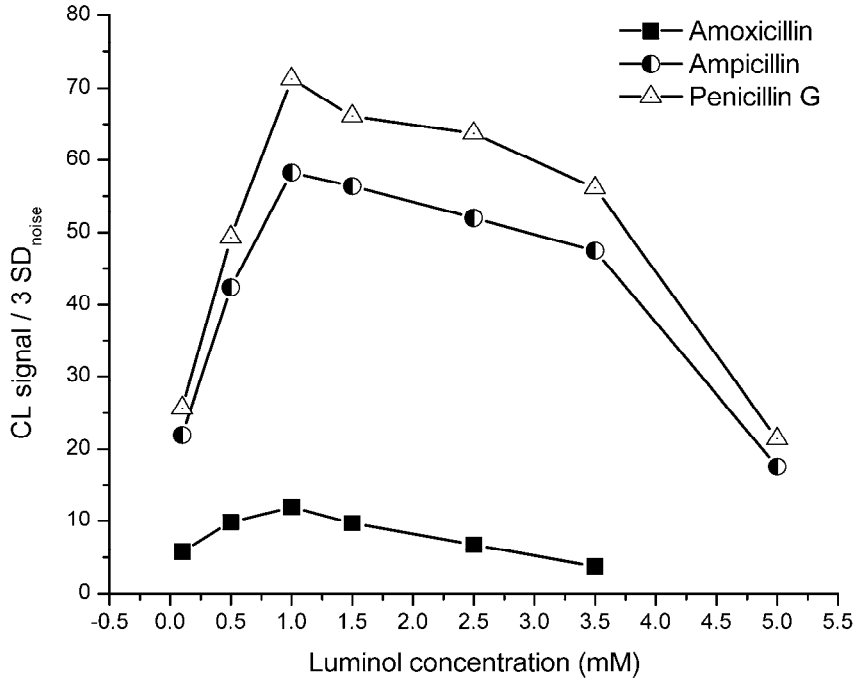

Fig. 5 – Influence of the luminol concentration on the chemiluminescence signal. Conditions as in working procedure, except the 550 µL injected volume; $c_{antibiotic}$ = 2 ppm

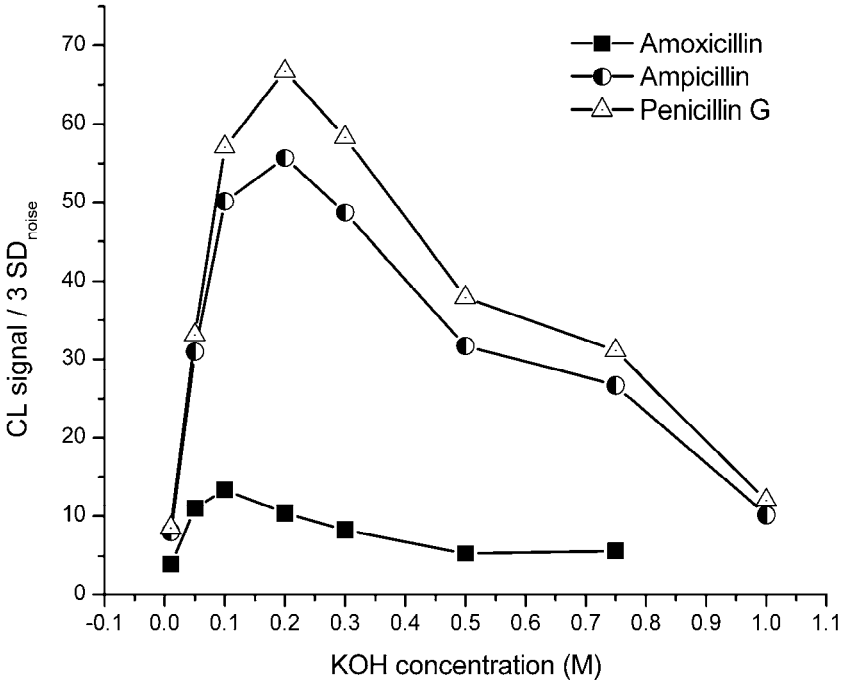

Fig. 6 – Influence of the potassium hydroxide concentration on the chemiluminescence signal. Conditions as in working procedure, except the 550 µL injected volume; $c_{antibiotic}$ = 2 ppm

The influence of the injected sample volume on the analytical signal was studied (data not showed). For small volumes of injected samples, the analytical signal significantly increases with the sample volume. A 250 µL-injected volume was selected as optimum because higher volumes do not generate a significant increase of the CL signal. For 100 ppm concentration of antibiotic, penicillin G signal was amplified 367 fold upon UV irradiation, while ampicillin irradiation generated a

171-fold increase of the signal, compared to the signals obtained without irradiation. Amoxicillin UV irradiation provided only a 10-fold increase of the signal.

Method performances

Penicillin G and ampicillin signals are linear for two concentration intervals, 0.05-3 ppm (y = 4613.9x + 411.86) and 5-100 ppm (y = 10518x + 6443.8) for penicillin G, respectively 0.05-2 ppm (y = 5110.7x + 89.986) and 5-50 ppm (y = 10091x - 15517) for ampicillin. For amoxicillin, second-degree equation dependence was obtained ($y = -13.598x^2 + 515.87x + 238.67$).

The precision of the method was as it follows: $RSD_{amoxicillin}$ = 4.19% for $c_{amoxicillin}$= 5 ppm; $RSD_{ampicillin}$ = 1.33% for $c_{ampicillin}$= 5 ppm and $RSD_{penicillin\ G}$ = 1.17% for $c_{penicillin\ G}$ = 2ppm n = 10 in all cases).

Analysis of pharmaceuticals

Three capsules of each antibiotic were mixed and weighted (for ampicillin, Antibiotice S.A. Iaşi and amoxicillin, Antibiotice S.A. Iaşi, GlaxoSmithKline and PharmaTech). For penicillin G (injectable, Antibiotice S.A. Iaşi), the content of a vial was homogenized and weighted. A portion (0.025g) of each antibiotic mix was rendered soluble with 100 mL of distilled water then filtered using a Filtrak 388 filter to a 250 mL volume.

Recovery method was applied for a 1:200 diluted sample stock solution spiked with standard solutions with concentrations of 0.25, 0.5, 0.75 and 1 ppm, and for a 1:10 diluted sample stock solution spiked with penicillin G to obtain added antibiotic concentrations of 5, 10 and 25 ppm (table 1).

By applying the standard addition method, it was calculated that 82.56% of the analyzed product is represented by penicillin G, while by interpolating the value obtained for the sample solution into the calibration graph, an 83% percent of active product was found in the sample. The Penicillin G recovery was between 91.7 % and 107.0 %, which indicates reduced matrix effects using the proposed method of penicillin G analysis. The percentage recovery of a known quantity of antibiotic introduced in the analyzed sample was calculated with the formula:

$$\%\ \text{recovery} = C_{measured}/C_{expected},\ \text{where}\ C_{expected} = C_{standard}+C_{unspiked\ antibiotic}$$

Table 1

Penicillin G determination in pharmaceutical samples

Sample dilution	Antibiotic added (ppm)	Antibiotic found in sample before spiking (ppm)	Expected value (ppm)	Measured value (ppm)	Recovery (%)
1/200	0.25	0.38	0.63	0.580 ± 0.017	92.1
1/200	0.50	0.38	0.88	0.807 ± 0.019	91.7
1/200	0.75	0.38	1.13	1.076 ± 0.039	95.2
1/200	1	0.38	1.38	1.456 ± 0.109	105.5
1/10	5	8.30	13.30	13.90 ± 0.006	104.5
1/10	10	8.30	18.30	19.44 ± 0.095	106.2
1/10	25	8.30	33.30	35.64 ± 0.321	107.0

The same protocol was applied for the samples of ampicillin (table 2). Good recoveries between 96 and 105% were obtained in both ranges of concentration. The percent of active product in the sample calculated using the calibration graph was 78.78% and by using the standard addition method, a value of 78.08% was obtained. A content of 390 mg of ampicillin in the analysed capsules (582.4 mg) was determined, yielding a 67% percent of active product per capsule.

For amoxicillin samples, recovery data also showed an insignificant influence of the matrix, the

recoveries being in the range of 90 - 105% (data not presented). Using the calibration graph, the concentration of the sample was calculated to be 77.5%, comparing with 81.5% declared by the producer.

Table 2

Ampicillin determination in pharmaceutical samples

Sample dilution	Antibiotic added (ppm)	Antibiotic found in sample before spiking (ppm)	Expected value (ppm)	Measured value (ppm)	Recovery (%)
1/200	0.20	0.394	0.594	0.604 ± 0.008	101.7
1/200	0.40	0.394	0.794	0.830 ± 0.012	104.5
1/200	0.60	0.394	0.994	1.044 ± 0.029	105.0
1/200	0.80	0.394	1.194	1.237 ± 0.016	103.6
1/10	5.00	8.757	13.757	13.860 ± 0.247	100.8
1/10	10.0	8.757	18.757	18.470 ± 0.305	98.5
1/10	25.0	8.757	33.757	32.460 ± 0.243	96.2

CONCLUSIONS

An originally FIA system was designed and optimized for the determination of β-lactam antibiotics: ampicillin, amoxicillin and penicillin G. Calibration graphs were obtained in the 0.05 - 100 ppm concentration range for ampicillin and penicillin G, and 0.25 - 100 ppm for amoxicillin. The precision of the method was very good for ampicillin 5ppm and penicillin G 2ppm (RSD < 1.33%) and more than acceptable for amoxicillin 5ppm (RSD = 4.19%). Low determination limits (0.05 ppm) were obtained for ampicillin and penicillin G, due to the CL signal amplification by the UV irradiation (150 - 350 fold amplification). For amoxicillin a 10-fold amplification was observed, probably due to the presence of the hydroxyl group bounded to the benzene ring. The method throughput was of about 20 determinations / hour with a low consumption of reagents and sample.

Penicillin G, ampicillin and amoxicillin in commercial pharmaceutical products were analyzed by using the proposed methods. By introducing standard quantities of antibiotics in the analyzed samples, recoveries between 89 and 107% were obtained. By analyzing commercially available products, active concentrations close to the ones declared by the producers were determined. The method may be applied with good sensitivity and reproducibility for detection of studied antibiotics after a chromatographic separation of these and for analysis of relative pure solutions of the studied antibiotics.

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REFERENCES

1. L. Xu, H. Wang and Y. Xiao, *Spectrochim. Acta*, **2004**, *60*, 3007-3012.
2. A. Fernández-González, R. Badía and M. E. Díaz-García, *J. Pharm. Biomed. Anal*., **2002**, *29*, 669-679.
3. F. Belal, M. M. El-Kerdawy, S. M. El-Ashry and D. R. El-Wasseef, *Farmaco*, **2000**, *55*, 680-686.
4. M. I. H. Helaleh, *Mikrochim. Acta*, **1998**, *129*, 29-32.
5. M. Q. Al-Abachi, H. Haddi and A. M. Al-Abachi, *Anal. Chim. Acta*, **2005**, *554*, 184-189.
6. H. Salem, *Anal. Chim. Acta*, **2004**, *515*, 333-341.
7. G. Hoizey, D. Lamiable, C. Frances, T. Trenque, M. Kaltenbach, J. Denis and H. Millart, *J. Pharm. Biomed. Anal.*, **2002**, *30*, 661-666.
8. G. Pajchel, K. Pawlowski and S. Tyski, *J. Pharm. Biomed. Anal.*, **2002**, *29*, 75-81.
9. J. Han, S. Gupte and R. Suryanarayanan, *Int. J. Pharm.*, **1998**, *170*, 63-72.
10. F. Belal, M. S. Rizk and M. Eid, *J. Pharm. Biomed. Anal.*, **1998**, *17*, 275-282.
11. E. Kilic, F. Koseoglu, A. Kenar and M. A. Akay, *J. Pharm. Biomed. Anal.*, **1995**, *13*, 1453-1458.
12. M. A. J. van Opstal, R. Wolters, J. S. Blauw, P. C. van Krimpen, W. P. van Bennekom and A. Bult, *J. Pharm. Biomed. Anal.*, **1990**, *8*, 49-60.
13. S. S. M. Hassan, M. T. M. Zaki and M. H. Eldesouki, *Talanta*, **1979**, *26*, 91-95.
14. A. V. Lapierre, R. A. Olsina and J. Raba, *Anal. Chim. Acta*, **1999**, *396*, 143-149.

15. B. Olsson, *Anal. Chim. Acta*, **1988**, *209*, 123-133.
16. M. Stredansky, A. Pizzariello, S. Stredanska and S. Miertus, *Anal. Chim. Acta*, **2000**, *415*, 151-157.
17. A. Fernandez-Gonzalez, R. Badia and M. E. Diaz-Garcia, *Anal. Chim. Acta*, **2003**, *484*, 223-231.
18. M. I. Bailon Perez, L. Cuadros Rodriguez and C. Cruces-Blanco, *J. Pharm. Biomed. Anal.*, **2007**, *43*, 746-752.
19. S. Babic, D. Asperger, D. Mutavdzic, A. J. M. Horvat and M. Kastelan-Macan, *Talanta*, **2006**, *70*, 732-738.
20. H. Liu, H. Wang and V. B. Sunderland, *J. Pharm. Biomed. Anal.*, **2005**, *37*, 395-398.
21. K. H. Yoon, S. Y. Lee, W. Kim, J. S. Park and H. J. Kim, *J. Chromatogr. B: Biomed. Appl.*, **2004**, *813*, 121-127.
22. R. D. Voyksner, K. L. Tyczkowska and A. L. Aronson, *J. Chromat. B: Biomed. Appl.*, **1991**, *567*, 389-404.
23. H. L. Wu, M. Masada and T. Uno, *J. Chromatogr., A*, **1977**, *137*, 127-133.
24. H. Kubo and M. Saitoh, *Anal. Sci.*, **1999**, *15*, 919-921.
25. A. I. Chivulescu, A. F. Danet and S. Kalinowski, *Rev. Chim.*, **2005**, *56*, 1-4.
26. H. Kubo and M. Saitoh, *Anal. Chim. Acta*, **1999**, *389*, 89-94.
27. J. Sun, S. G. Schulman and J. H. Perrin, *Anal. Chim. Acta*, **1997**, *338*, 1-2.
28. F. A. Aly, N. A. Alarfaffj and A. A. Alwarthan, *Talanta*, **1998**, *47*, 471-478.
29. J. Du, Y. Li and J. Lu, *Anal. Lett.*, **2002**, *35*, 2295-2304.
30. W. Cao, J. H. Yang, C. X. Sun, Y. J. Chen and Q. F. Gao, *Luminescence*, **2005**, *20*, 20-24.
31. C. Thongpoon, B. Liawruangrath, S. Liawruangrath, R. A. Wheatley and A. Townshend, *Anal. Chim. Acta*, **2005**, *553*, 123-133.
32. N. Lian, H. Zhao, C. Sun, S. Chen, Y. Lu and L. Jin, *Microchem. J.*, **2003**, *74*, 223-230.
33. I. N. Tomita and L. O. S. Bulhoes, *Anal. Chim. Acta*, **2001**, *442*, 201-206.
34. M. S. Burkhead, H. Wang, M. Fallet and E. M. Gross, *Anal. Chim. Acta*, **2008**, *613*, 152-162.
35. I. S. Ricart, G. M. Anton-Fos, M. J. Duart, J. V. Garcia Mateo, L. Zamora and J. Martinez Catalayud, *Talanta*, **2007**, *72*, 378-386.
36. J. R. Albert-Garcia, M. Catala Icardo and J. Martinez Catalayud, *Talanta*, **2006**, *69*, 608-614.
37. B. Gomez-Taylor, M. Palomeque, J. V. Garcia Mateo and J. Martinez Catalayud, *J. Pharm. Biomed. Anal.*, **2006**, *41*, 347-357.