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Chemiluminescence detection coupled to high-performance frontal analysis for the determination of unbound concentrations of drugs in protein binding equilibrium

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Abstract

High-performance frontal analysis coupled with chemiluminescence detection (HPFA–CL) was developed for the determination of unbound oxacillin concentration in human serum albumin solution. The HPFA system consisted of an ISRP column and a mobile phase of 67 m*M* potassium phosphate buffer of pH 7.4 and ionic strength of 0.17. The luminol–H_2O_2–Co^{2+} system was used in the chemiluminescence detection. An enhancement of luminol chemiluminescence by oxacillin was investigated and employed for determining the concentration of oxacillin in the HPFA eluate. Sample solutions were directly injected onto the column; the drug was eluted as a zonal peak with a plateau region. The unbound drug concentrations were determined by using the height of the plateau. The results agreed with those obtained with conventional ultrafiltration–HPLC method. Good reproducibility was confirmed by the within run and between run RSD≤7.4%. HPFA–CL provided a selective method for determination of unbound drug concentration in protein binding equilibrium. © 2002 Published by Elsevier Science B.V.

Keywords: Chemiluminescence detection; Detection, LC; Frontal analysis; Drug–protein binding; Oxacillin; Albumin; Proteins; Antibiotics

1. Introduction

It is well known that drugs are bound more or less to plasma proteins such as albumin and α_1-acid glycoprotein in the blood. Protein binding is a reversible and kinetically rapid process, and the concentrations of the bound and unbound drug are in an equilibrium state. Only the unbound drugs can diffuse from the blood, reach the action site and exhibit pharmacological activity and/or side effects. Therefore determination of the concentration of unbound drug is important for pharmacokinetic and pharmacodynamic studies [1–3].

Several methods have been developed for determining the concentration of unbound drug, such as equilibrium dialysis, ultrafiltration, ultracentrifugation, gel filtration and microdialysis [4]. Equilibrium dialysis and ultrafiltration method have been commonly used because of their relatively simple procedure and device needed. However, these methods have potential problems such as the adsorption of drug onto membranes and the leakage of bound drug through membranes. In addition, the equilibrium

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between bound and unbound drug might shift during the experiment due to a volume shift.

There are chromatographic and electrophoretic methods for studying the binding of drugs to proteins, which have been intensively reviewed [5]. Among these methods, high-performance frontal analysis (HPFA) was reported for determining unbound drug concentration in plasma or in protein-containing solution by direct sample injection [6–12]. HPFA based on gel filtration does not suffer from protein leakage and drug adsorption on the membrane, which causes erroneous result in the conventional ultrafiltration and dialysis method. HPFA is advantageous for the analysis of strongly bound drugs, because the bound drug is transformed into unbound form in the column, which improves the measurement of low levels of unbound drug [13]. In addition, HPFA can be easily incorporated into an on-line column-switching system which is particularly useful in the stereoselectivity study of protein binding of chiral drugs.

In HPFA, drug–protein mixed solution is directly injected onto a restricted-access type HPLC column. This special stationary phase excludes proteins but retains small drug molecules on the hydrophobic legends in the pores [14]. When the sample solution in a state of drug protein binding equilibrium is injected onto the column, bound drug is released quickly from protein by dilution in the mobile phase. After continuous injection of enough volume of sample solution, an equilibrium zone is generated at the top of the column, where the drug concentration in the mobile phase reaches steady state and the drug–protein binding equilibrium in the interstices of the packing materials is the same as that in the original sample solution [15]. As a result, the size-excluded protein is eluted first and the drug is eluted as a trapezoidal peak with a plateau region. The drug concentration in the plateau region is the same as that of unbound drug in the sample solution. Therefore the unbound drug concentration can be determined from the plateau height. However, when more than one drug is administered or in the protein-containing solution the unbound drugs are eluted almost in one plateau region. The determination of unbound concentration of one drug may be interfered with by the existence of another drug. One method to overcome this problem is a heart-cut of the plateau region followed by on-line HPLC measurement [16]. An alternative solution may be employing highly selective detection.

Chemiluminescence (CL) is a powerful and interesting detection technique that is both highly selective and sensitive. The chemiluminescence system of lunimol–hydrogen peroxide (H_2O_2)–Co^{2+} has been widely used in the analysis of chemical and biological samples and applied to pharmaceutical analysis [17,18]. The origin of luminescence in this system can be briefly described as the interaction of luminol, under alkaline conditions, with oxygen to form an adduct which decomposes to form excited aminophthalate emitting luminescence. The Co^{2+} ion is a catalyst in this reaction, which accelerates the decomposition of H_2O_2 to supply the reactive form of oxygen needed for luminol chemiluminescence. This luminescence system has been coupled to HPLC as a detection system [19–21]. It was reported that some β-lactam antibiotics such as penicillins and cephalothin enhanced the luminescence intensity and duration of this system [22,23]. In this study, oxacillin was also found to possess the ability to prolong and enhance the luminescence from luminol. This chemiluminescence system was developed into a CL detection system, which was for the first time successfully connected with HPFA. HPFA–CL was applied to the determination of the unbound fraction of oxacillin in its human serum albumin equilibrated solution.

2. Experimental

2.1. Reagent and materials

Oxacillin was purchased from The Institute of Drug and Biological Product Control of China (Beijing, China). Human serum albumin (HSA, fatty acid free) and luminol (3-aminophthalhydrazide) were supplied by Sigma (St. Louis, MO, USA). ISRP column (GFFII-S5-80, 15 cm×4.6 mm I.D., 5 μm) was from Regis (Morton Grove, IL, USA). Potassium phosphate and H_2O_2 (30%) were purchased from Shenyang Chemicals (Shenyang, China).

2.2. Apparatus

The mobile phase was delivered by a PU980 HPLC pump (Jasco, Tokyo, Japan) and the chemi-

luminescence reagents were pumped by peristaltic pump (LZ1010, Zhaofa Institute, Shenyang, China). Chemiluminescence was measured with a chemiluminescence detector (GD-1, Xi'an Rui Mai Company, Xi'an, China). The chromatographic data were acquired with a Ckchrom workstation (Scientific System). The sample solution was injected using a Rheodyne 7125 injector (Cotati, CA, USA) with a 1-ml loop.

2.3. Preparation of sample solutions

Oxacillin stock solution was prepared by dissolving the compound in methanol. According to the final concentration required, various volumes of the stock solution were placed in 10-ml screw-capped glass vials and the methanol was evaporated under a nitrogen stream. HSA solution of 1 ml (67 m*M* phosphate buffer, pH 7.4; ionic strength, 0.17) was usually added into the vial. The sample solutions were gently mixed and then kept at 20°C for 1 h before measurement.

2.4. Preparation of luminol and hydrogen peroxide solutions

The luminol solution (1 m*M*) was prepared by dissolving 18 mg 3-aminophthalhydrazide in a mixture of about 5 ml deionized water and 6 ml of 2 *M* sodium hydroxide with stirring, and then adjusting to volume with water in a 100-ml volumetric flask. The luminol solution of 100 ml was mixed with 10 ml of 19 μ*M* $CoCl_2$ immediately before use.

The hydrogen peroxide solution (90 m*M*) was prepared by pipetting 1.0 ml of 30% hydrogen peroxide solution into a 100-ml volumetric flask and then adjusting to volume with deionized water.

2.5. Determination of unbound oxacillin concentration by HPFA–CL

Fig. 1 shows the schematic diagram of the HPFA–CL system. The operation conditions are given in Table 1. The oxacillin–HSA solution (900 μl) was injected onto the column without any pretreatment through the injector loop. Oxacillin was eluted as a zonal peak with a plateau region after HSA elution. The height of the plateau region represents the unbound drug concentration in the sample solution.

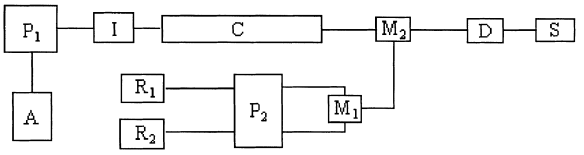

Fig. 1. Schematic diagram of the HPFA–CL detection system. (A) Mobile phase for HPFA; P_1, HPLC pump; P_2, peristaltic pump; I, injection valve; D, CL detector; R_1, luminol and Co^{2+} mixed solution; R_2, H_2O_2 solution; C, ISRP GFFII-S5-80 column; S, chromatography data system; M_1,M_2, T junction mixer.

By comparing the plateau height with peak height of oxacillin sample solution without HSA, the unbound drug concentration was calculated.

2.6. Determination of unbound oxacillin concentration by ultrafiltration

Ultrafiltration using NANOSEP Microconcentrators centrifugal (Pall Filtron, Northborough, MA, USA) was employed as reference standard method to determine the unbound drug concentration. The sample solutions of 500 μl were transferred into the sample reservoir and centrifuged at 2000 *g* for 3 min. To eliminate the effect of undesirable drug adsorption onto the membrane, the second ultrafiltrate was used as sample of HPLC measuring the unbound drug concentration. The HPLC system consisted of an ODS column (Varian, 15 cm×4.6 mm I.D., 5 μm), a mobile phase of 1 m*M* potassium dihydrogenphosphate (pH 6.5)–CH_3OH (65:35, v/v) and UV detection at 254 nm. The flow-rate was 0.4 ml/min.

3. Results and discussion

3.1. Chemiluminescence promoted by oxacillin

The homemade static CL system is shown in Fig. 2. The luminol solution (100 μl) admixed with Co^{2+} was pipetted into the cuvette in a light-proof enclosure, followed by addition of 10 μl oxacillin (2.9 m*M*) and 100 μl H_2O_2. The luminescence intensity was monitored with a photomultiplier tube also in the enclosure. The signal was recorded by a chromatography data system.

As shown in Fig. 3, for the luminol–H_2O_2–Co^{2+}, only a brief pulse of light was observed, but with the

Table 1
HPFA–CL conditions

Subsystem	Condition
HPFA	Column: ISRP column (GFFII-S5-80,15 cm×4.6 mm I.D., 5 μm) Mobile phase: 67 m*M* potassium phosphate buffer (pH 7.4, ionic strength 0.17) Flow rate: 0.3 ml/min Temperature: 20°C
Post-column	Luminol: concentration 1 m*M*, flow-rate 0.2 ml/min
CL system	H_2O_2: concentration 90 m*M*, flow-rate 0.2 ml/min

addition of oxacillin the intensity of luminescence was significantly enhanced (about 40 times) and the duration of luminescence lengthened. The time to attain the maximum luminescence was 4–5 s.

The parameters affecting chemiluminescence enhanced by oxacillin were optimized. The CL intensity was increased with increasing concentration of Co^{2+} in luminol solution up to 1.2 μ*M* and then unchanged in the range from 1.2 to 1.7 μ*M* (Fig. 4). The effect of H_2O_2concentration was investigated from 50 to 260 m*M*. At H_2O_2 concentrations of 50, 70, 90, 110, 160, 230, 260 m*M* investigated, the intensity was 3467, 3985, 4400, 4913, 5656, 5650, 5652 mV, respectively. Increasing the H_2O_2 concentration resulted in an initial increase in the CL intensities of both the sample solutions and reagent blank, and the detected intensity reached maximum with concentrations over 160 m*M*. However, the

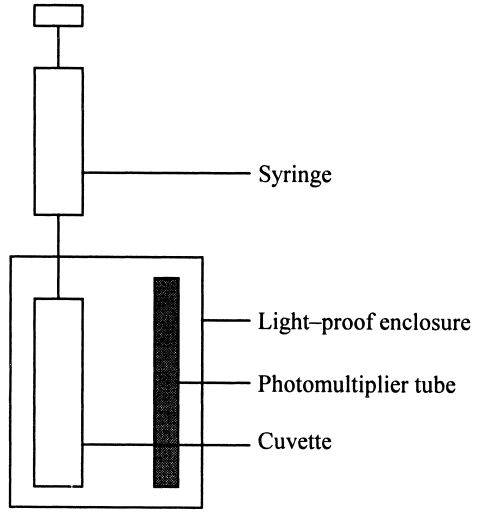

Fig. 2. Schematic diagram of setup for static chemiluminescent measurement.

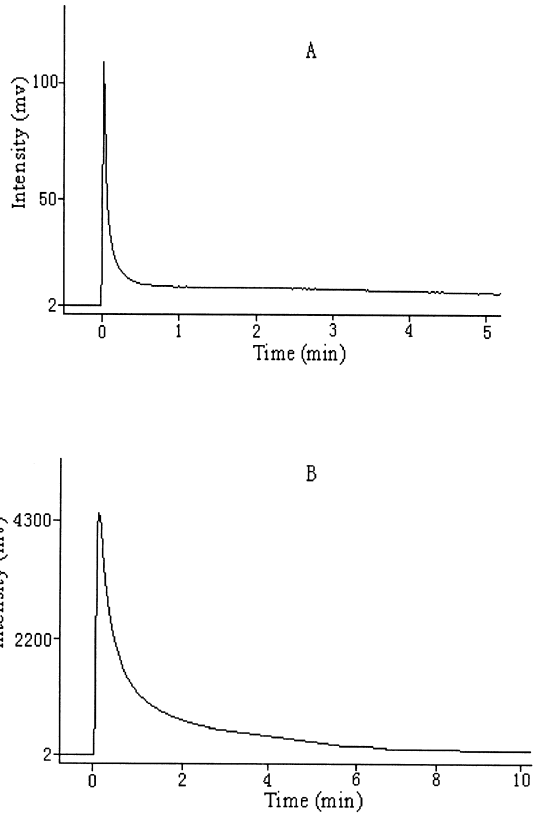

Fig. 3. Chemiluminescence profiles of (A) luminol–H_2O_2–Co^{2+} and (B) luminol–H_2O_2–Co^{2+}–oxacillin systems. The concentrations were as follows: oxacillin, 0.138 m*M*; Co^{2+}, 0.810 μ*M*; H_2O_2, 42.9 m*M*; and luminol, 0.476 m*M*. These concentrations were final concentrations in the cuvette, which were calculated from the initial reagent concentrations, volumes and the total volume of reaction mixture.

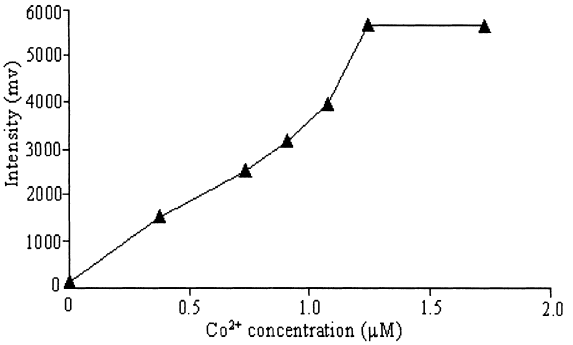

Fig. 4. Relationship between chemiluminescence intensity and Co^{2+} concentration in luminol. Co^{2+} concentration: 0–1.7 μ*M*. Other conditions were the same as in Fig. 3.

baseline noise level increased with the increasing of H_2O_2 concentration, a concentration of 90 m*M* was selected for the highest signal-to-noise ratio. Luminol concentration of 1 m*M* was found optimal after investigating its effect. The effect of solvent was also investigated. The maximum CL intensity was observed in water, and phosphate buffer (67 m*M*, pH 7.4, ionic strength 0.17) reduced the chemiluminescence of luminol to about 50%, while methanol quenched the luminescence severely.

3.2. Determination of unbound oxacillin in the HSA solution by HPFA–CL

In the HPFA, phosphate buffer of physiological pH and ionic strength was used as mobile phase without any organic modifier to provide a similar medium for the equilibrium zone in the column to that in the samples. Although phosphate buffer inhibited the chemiluminescence of luminol to some extent, as mentioned above, the luminescence enhanced by oxacillin was still easily detected. A low flow-rate of mobile phase (0.3 ml/min) was employed, which avoided a high back pressure of the column. Accordingly, a flow-rate of 0.2 ml/min was chosen for both 90 m*M* H_2O_2 and 1 m*M* luminol solution containing 1.7 μ*M* Co^{2+}. At these optimal flow-rates, a 30-cm spiral reaction coil (0.5 mm I.D.) between the mixer (M_2 in Fig. 1) and detector (D in Fig. 1) was required to allow the reacting mixture to reach the detector in approximate 5 s, when the maximum chemiluminescence was achieved.

At such a low flow-rate samples diffuse into the mobile phase in the injector loop, which causes a tailing peak. The ''injector-reswitching technique'' [13] was used to overcome this problem. In this study the 1-ml volume injector loop was fully loaded with the drug–HSA solution and connected to the mobile phase flow (0.3 ml/min) for a certain period by switching the injector valve, which resulted in a sample injection of desired volume. The injector valve was then reswitched to the load position, and the loop was detached from the mobile phase flow. Using this technique, the diffused tail of the samples was cut off and the sample input could be regarded as an ideal rectangular shape. It is well known that when an equilibrated solution of drug–protein is injected onto an ISRP column, drug is released from protein in the mobile phase. When the injected sample volume is small, the released drug is eluted as a sharp peak. When the sample injection volume is large enough a trapezoidal peak with a plateau region appears for the elution of drug. The plateau region extends with increasing injection volume, but the peak height remains constant, which is dependent on the free drug concentration in the original sample solution. Fig. 5 shows the effect of sample injection volume upon the elution profile of 20 μ*M* oxacillin and 42 μ*M* HSA mixed solution. When 200-μl sample solution was injected, oxacillin was eluted as an ordinary HPLC peak. When the injection volume of sample solution was increased to 500 μl, oxacillin was eluted as a zonal peak with a plateau region. Further increasing the injection volume up to 975 μl, the plateau height was hardly changed. Fig. 6 shows the relationship between the injection volume and the peak height of oxacillin.

For a quantitative determination of the unbound drug concentration, the height of trapezoidal peak was measured. A calibration was carried out under the same chromatographic conditions as in HPFA, but standard samples with no protein were injected, which were made up in phosphate buffer (67 m*M*, pH 7.4) at concentrations of 0.41, 0.82, 3.28, 6.56, and 13.12 μ*M* oxacillin. Oxacillin was eluted as an ordinary HPLC peak when the sample volumes were small. When the injection volumes were large enough, it was eluted as a zonal peak, and the peak height was proportional to oxacillin concentration. A typical regression equation for oxacillin was $y =$

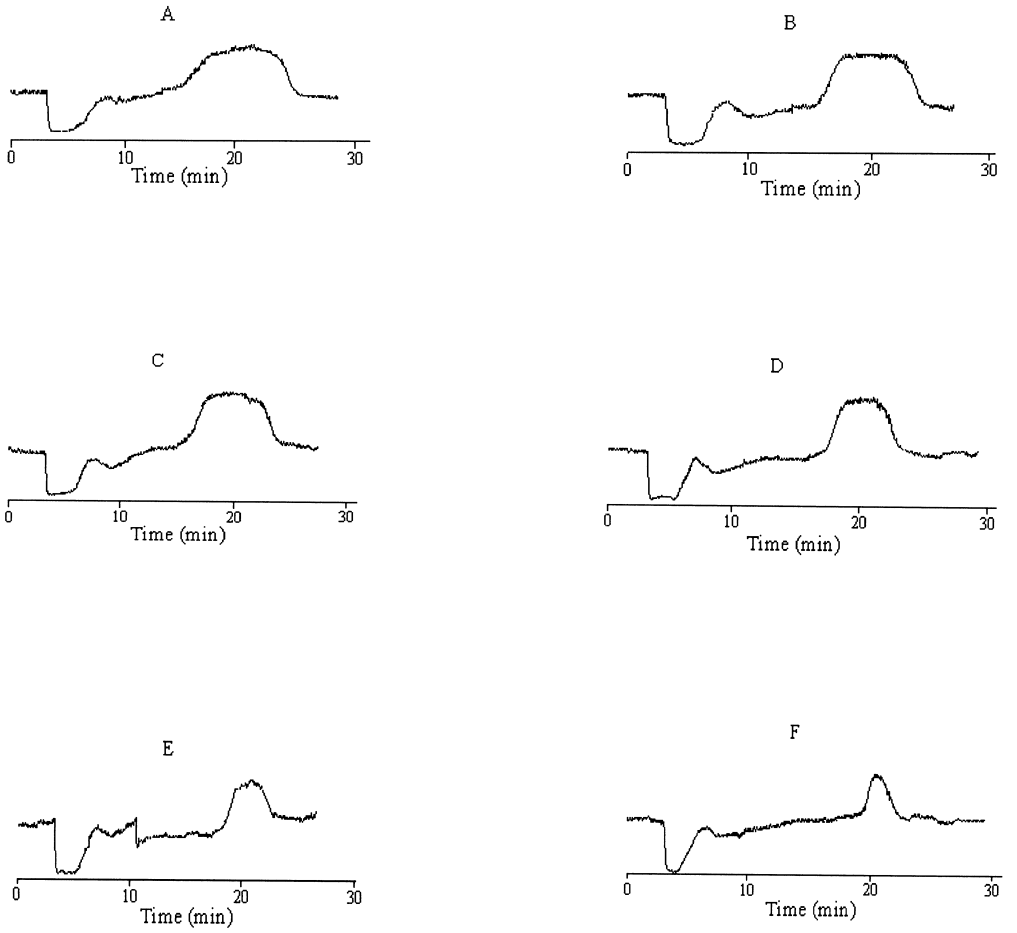

Fig. 5. HPFA–CL elution profiles of 20 μ*M* oxacillin and 42 μ*M* HSA mixed solutions. Injection volumes: (A) 975 μl; (B) 900 μl; (C) 700 μl; (D) 500 μl; (E) 400 μl; (F) 200 μl. For HPFA conditions, see Table 1.

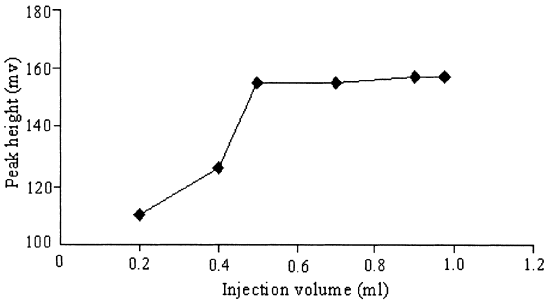

Fig. 6. Relationship between injection volume of oxacillin–HSA solution and peak height.

$2.26 \times 10^4 x - 2.58 \times 10^3$ with a correlation coefficient of 0.9993. The linear range was 0.41–13.12 μ*M* and the limit of detection at a *S*/*N* ratio of 2/1 was 0.12 μ*M*. A concentration of 0.41 μ*M* was accurately determined with this chemiluminescence coupled HPFA, which is adequate for monitoring unbound oxacillin level in human serum at a clinical therapeutic dose.

The most significant characteristic of this HPFA coupled with chemiluminescence is its high selectivity. Those drugs not affecting the chemiluminescence of luminol system would not interfere with the detection of oxacillin, even though they might be coeluted with oxacillin. Drugs tested in this study included cefoperazone, ketoprofen and cefozolin.

Table 2
Unbound concentrations of oxacillin in HSA solutions determined by HPFA–CL and ultrafiltration–HPLC

Sample solution	Unbound concentration (μM)			
	HPFA–CL		Ultrafiltration–HPLC	
	Within run	Day to day	Within run	Day to day
20 μM oxacillin–42 μM HSA	6.12±0.05	6.08±0.09	6.28±0.16	6.12±0.11
14 μM oxacillin–42 μM HSA	3.74±0.09	3.79±0.09	3.70±0.16	3.74±0.29
20 μM oxacillin–550 μM HSA	0.52±0.03	0.54±0.04	0.52±0.04	0.54±0.05

All data are expressed as means±SD ($n=4$).

Therefore, free oxacillin concentration in protein-equilibrated solution or serum containing those drugs could be determined directly. HPFA–CL could be used for studying drug interactions in protein binding which requires a column switching technique to connect HPFA with nonselective detection to an HPLC for a further separation. Furthermore only a negative peak for HSA appeared in the elution profile (Fig. 5), which did not interfere with the measurement of oxacillin peak height. In contrast, in HPFA with UV detection the protein forms a large positive peak, which often interferes with measurement of drug. Some other drugs, such as penicillin G and penicillin V, were examined, they could also enhance the luminescence of the luminol system. This method may be employed in the determination of their unbound concentration in HSA solution.

The developed method in this study was compared with ultrafiltration–HPLC. Table 2 lists the unbound oxacillin concentrations determined by HPFA–CL and by the ultrafiltration–HPLC method. The unbound oxacillin concentrations calculated from trapezoidal peak heights agree well with those determined by ultrafiltration method. A good reproducibility was indicated from both within-run and day-to-day relative standard deviation of $\leq$7.4% ($n=4$) for HPFA–CL.

4. Conclusion

Chemiluminescence detection was successfully coupled with high-performance frontal analysis. Unbound oxacillin concentrations in the HSA solution were determined using this HPFA–CL system with good reproducibility. The comparison of the analytical data with those obtained by using conventional ultrafiltration–HPLC method supported the reliability of the present system.

Acknowledgements

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