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Analytica Chimica Acta 414 (2000) 15–23

ANALYTICA CHIMICA ACTA

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Sensitive assay for clavulanic acid and sulbactam in pharmaceuticals and blood serum using a flow-injection chemiluminometric method

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Received 3 November 1999; received in revised form 4 February 2000; accepted 9 February 2000

Abstract

A sensitive, simple and inexpensive chemiluminescence (CL) method using flow injection is described for the determination of two beta-lactamase inhibitors sulbactam sodium and clavulanic acid (potassium salt) in their pure form, in pharmaceutical preparations and added to blood serum. The method is based on the enhancing effect of these compounds on the CL generated by the oxidation of luminol with hydrogen peroxide in alkaline medium. The CL intensity is a linear function of sulbactam sodium concentration over the range 0.1–150 $\mu g\,ml^{-1}$ with a detection limit (2×noise) of 0.05 $\mu g\,ml^{-1}$, and for clavulanic acid over the range 0.01–12 $\mu g\,ml^{-1}$ with a detection limit of 0.01 $\mu g\,ml^{-1}$. The method is applied successfully to the determination of the drugs in their dosage forms without interference from co-formulated drugs. The method is specific for the intact drugs in the presence of their degradation products.

Keywords: Sulbactam sodium; Clavulanic acid; Luminol; Chemiluminescence detection; Flow injection; Pharmaceutical preparations; Biological fluids

1. Introduction

Sulbactam and clavulanic acid are potent inhibitors of β-lactamase enzymes which are responsible for the protection of micro-organisms against β-lactam antibiotics. Clavulanic acid is a natural β-lactam antibiotic produced by *Streptomyces clavuligerus* [1]. Sulbactam, a semisynthetic β-lactam, is a penicillanic acid sulphone which is somewhat less potent in inhibitory activity, but is much more stable, than clavulanic acid [2]. Both compounds are prescribed clinically in combination with β-lactamase-labile β-lactams. Because of the therapeutic importance and widespread use of these compounds, the literature contains many publications dealing with their determination either in pure forms, in dosage forms or in biological fluids.

The B.P. recommends a liquid chromatography (LC) method for the determination of potassium clavulanate in raw material and a spectrophotometric method for its determination in co-amoxiclav tablets [3]. However, the USP recommends LC methods for the determination of sulbactam sodium and potassium clavulanate in raw materials and in their dosage forms [4].

Most of the methods employed for the determination of sulbactam and clavulanic acid in biological fluids are based on LC with UV detection [5–13]. These methods involve direct absorption of UV radiation at 220 [5] or 225 nm [6]. However, precolumn or postcolumn derivatizing reagents (for example pyrazole, 1,2,4-triazole and imidazole [7–11].) are used

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0003-2670/00/$ – see front matter
PII: S0003-2670(00)00800-X

for measurement at trace levels in biological fluids. Also, postcolumn alkaline degradation is reported [12,13]. Other reported methods for determination of sulbactam in serum in the presence of ampicillin involve synergic bioassay, gas chromatography–mass spectrometry and LC [14].

For dosage forms, potassium clavulanate is formulated with amoxicillin, but sodium sulbactam is formulated with ampicillin or cefoperazone. A few methods are reported for the simultaneous determination of these three binary mixtures, viz. LC [15–17] and spectrophotometry [18–20].

During the preparation of this manuscript, a paper dealing with the determination of β-lactam ring compounds using LC with postcolumn chemiluminescence (CL) detection appeared [21]. Luminol and hydrogen peroxide were used as postcolumn derivatizing reagents. A wide range of β-lactam ring compounds were screened for enhancement of luminol CL, among them sulbactam and clavulanic acid. The method was only used for the quantitation of penicillin G, penicillin V and dicloxacillin.

CL analysis has advantages such as sensitivity, ease of use and simple instrumentation. It has been actively investigated for the highly sensitive detection of small amounts of chemical species at ultra-trace levels [22,,23]. The reproducibility and selectivity of the CL analysis can be improved by combination with flow injection (FI).

In this paper, sulbactam sodium and potassium clavulanate enhance the CL emission from luminol oxidation by hydrogen peroxide in alkaline solution. If sulbactam sodium or potassium clavulanate is arranged to be the rate-limiting reagent, the enhanced emission is proportional to the concentration of the studied compounds; thus their amount can be determined by measuring the increase in CL. Based on these findings, a new FI–CL method is developed for the determination of sulbactam sodium and potassium clavulanate in pharmaceuticals and biological fluids.

2. Experimental

2.1. Instruments and flow system

The flow system used for determination and CL detection of sulbactam sodium and potassium clavulanate is shown schematically in Fig. 1. An Ismatec peristaltic pump was used to drive the carrier and CL reagent streams through the flow system. Each stream was pumped at a constant flow rate of 1.9 ml min^{-1}, using PTFE tubing (1.4 mm i.d.). The sample solution was inserted into the carrier stream of luminol using an injection valve from Rheodyne. The emitted light intensity was measured by a photomultiplier tube (THORN EMI, 9789 QB). The signal was recorded by a Kipp and Zonen BD112 recorder. Peak heights were measured.

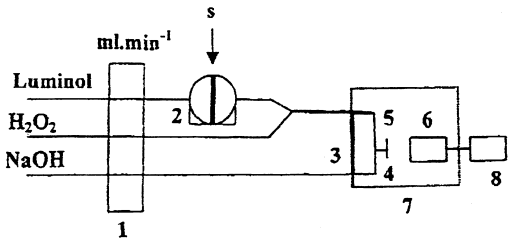

Fig. 1. Flow-injection manifold used for the CL determination of potassium clavulanate and sulbactam sodium; (1) peristaltic pump; (2) sample injection; (3) Perspex T-piece; (4) waste; (5) coiled flow cell; (6) photomultiplier tube (PMT); (7) housing; (8) recorder.

2.2. Reagents and materials

Analytical reagent grade chemicals and distilled water were used throughout. Pure drug samples were kindly provided by pharmaceutical companies: sulbactam sodium (Pfizer) and potassium clavulanate (Pharco Pharmaceuticals). Dosage forms were obtained from commercial sources. Luminol (Sigma Chemical Co.) 5×10^{-4} M solution, was prepared in 1.0 M sodium carbonate solution (BDH). Hydrogen peroxide solution, 5×10^{-3} M, was prepared daily by diluting a measured amount of 30% (w/v) solution (BDH) with degassed water. Sodium hydroxide (Merck) was 0.2 or 0.05 M solution. Other reagents used were methylene chloride (Winlab) and acetonitrile (Merck).

2.3. Preparation of standard solutions

A 1 mg ml^{-1} stock solution of each drug was prepared in distilled water. Serial dilutions with distilled water were made to cover the working ranges (Table 1).

Table 1
Performance data for chemiluminometric determination of the studied β-lactams

Compound	Linear calibration range ($\mu g\,ml^{-1}$)	Limit of detection[a] ($\mu g\,ml^{-1}$)	Regression equation[b] ($I=a+bC$) [c]	Correlation coefficient[c]
Sulbactam sodium	0.1–4.0	0.05	$I=1.08+67.10C$	0.99999
	4.0–150		$I=-57.34+85.79C$	0.99991
Potassium clavulanate	0.01–0.05	0.01	$I=-3.29+398.40C$	0.99999
	0.5–12		$I=-73.48+558.98C$	0.99997

[a] Signal/noise=2.
[b] Intensity (mV); concentration in $\mu g\,ml^{-1}$.
[c] 5,7 data points for potassium clavulanate for the two ranges, respectively and 5,12 data points for sulbactam sodium for the two ranges, respectively.

2.4. Procedures

2.4.1. General procedure

Working solutions of sulbactam sodium and potassium clavulanate in the range cited in Table 1 were prepared from stock solutions. A 250 μl portion of sulbactam or clavulanate were injected into a stream of 5×10^{-4} M luminol which was then combined with a stream of 5×10^{-3} M hydrogen peroxide and then mixed with a stream of 0.2 or 0.05 M sodium hydroxide for sulbactam and potassium clavulanate, respectively. The resulting peak heights were measured. Calibration graphs were prepared by plotting the peak heights against the drug concentration over the ranges cited in Table 1.

2.4.2. Procedure for tablets and injections

An accurately weighed amount of 10 powdered tablets or mixed contents of 10 vials equivalent to 10.0 mg of the drug, was transferred into a 50 ml calibrated flask and completed to the mark with distilled water. The flask with its contents was sonicated for 5 min and filtered, and the measurement carried out as described earlier under general procedure. The nominal content was calculated from the corresponding calibration graph or using the regression equation (Table 1).

2.4.3. Procedure for spiked serum

An aliquot of serum (1.0 ml) in a 15 ml centrifuge tube was spiked with an aliquot of aqueous solution containing 0.5 mg (for potassium clavulanate) or 2.0 mg (for sulbactam sodium) of the drug. Acetonitrile (2.0 ml) was added and the solution was shaken for 5 min. The precipitated protein was removed by centrifugation for 10 min at 8000 rpm. The supernatant was transferred to a clean centrifuge tube and treated with 10.0 ml of methylene chloride. The mixture was again shaken and centrifuged for 10 min at 8000 rpm. The lower organic layer was discarded and the aqueous upper layer was transferred into a 25 ml calibrated flask and completed to volume with distilled water. The general procedure was then followed. The absolute recovery was determined for each drug by comparing the representative peak height of extracted serum with the peak height of the standard drug at the same concentration.

3. Results and discussion

The factors affecting the enhancing effect of the studied β-lactams on the CL generated by the oxidation of luminol with hydrogen peroxide in alkaline medium were carefully studied. Different oxidants such as potassium hexacyanoferrate(III), hydrogen peroxide and *N*-bromosuccinimide were used. Maximum CL intensity was obtained only when hydrogen peroxide was used as an oxidant in alkaline medium. In the absence of hydrogen peroxide, no CL signals were obtained.

3.1. Configuration designs

The FI configuration used for the determination of the studied β-lactams was designed so as to provide different reaction conditions for magnifying their enhancing effect on the CL generated by the reaction of

luminol and hydrogen peroxide. Two different configurations were tested for this purpose.

In the first configuration, the sample was mixed with the luminol solution, after which it mixed with the hydrogen peroxide and sodium hydroxide solutions before it reached the detector, as shown in Fig. 1. In the second, the sample was mixed with the hydrogen peroxide solution, then mixed with the luminol and sodium hydroxide solutions before it reached the detector.

The first configuration was found to give a greater CL signal, approximately three and five times greater than the signal obtained using the other configuration for clavulanate and sulbactam, respectively. Therefore it was chosen for the determination of the studied β-lactams as it provided the greatest sensitivity and the fastest restoration of the baseline.

3.2. Optimization of reagent concentrations

The effects of varying the concentrations of luminol, sodium carbonate as a diluent for luminol, hydrogen peroxide and sodium hydroxide were tested.

The effect of luminol concentration was studied in the range 1.0×10^{-5}–1.0×10^{-2} M. The peak height increased with increasing luminol concentration up to 5.0×10^{-4} M, but decreased at higher concentration (Fig. 2); and a 5.0×10^{-4} M luminol solution was selected.

The effect of sodium carbonate concentration as a diluent for luminol was studied in the range 0.05–2.0 M. The greatest CL intensity was obtained with 1.0 M Na_2CO_3 for both drugs.

Peak height increased with increasing hydrogen peroxide concentration up to 5.0×10^{-3} M, above which it decreased, as shown in Fig. 3. The working solution chosen was 5.0×10^{-3} M.

The effect of sodium hydroxide on the CL reaction is shown in Fig. 4. A strongly basic solution was required for maximum development of the CL reaction in the case of sulbactam (0.2 M) and a less strongly basic solution was required in case of potassium clavulanate determination (0.05 M).

3.3. Optimization of manifold parameters

The variables studied under the optimized reagent concentrations were the injected sample volume and the flow rate.

The volume injected was varied between 10 and 600 μl. The resulting peak height increased with increasing volumes injected up to 250 μl for both potas-

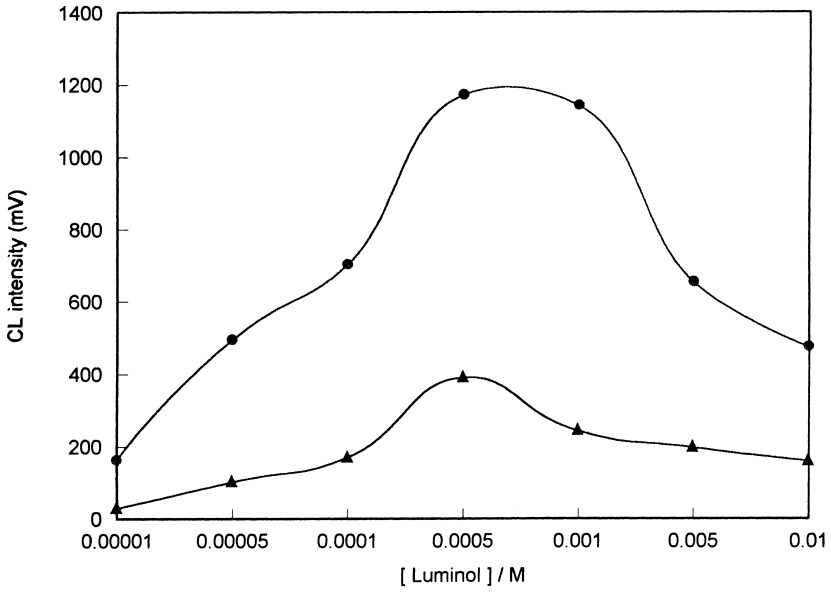

Fig. 2. Effect of luminol concentration on CL intensity of potassium clavulanate (5 μg ml^{-1}; ●) and sulbactam sodium (10 μg ml^{-1}; ▲). H_2O_2 5×10^{-3} M, NaOH 0.05 and 0.2 M, respectively, flow rate 1.9 ml min^{-1}.

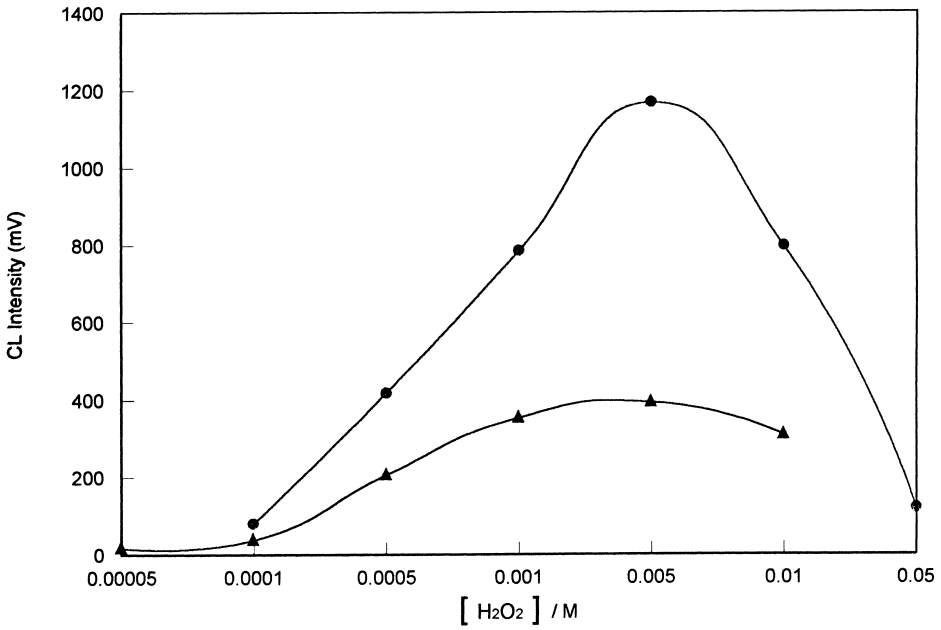

Fig. 3. Effect of hydrogen peroxide concentration on CL intensity of potassium clavulanate (5 μg ml^{-1}; ●) and sulbactam sodium (10 μg ml^{-1}; ▲). Luminol 5×10^{-4} M, NaOH 0.05 and 0.2 M, respectively, flow rate 1.9 ml min^{-1}.

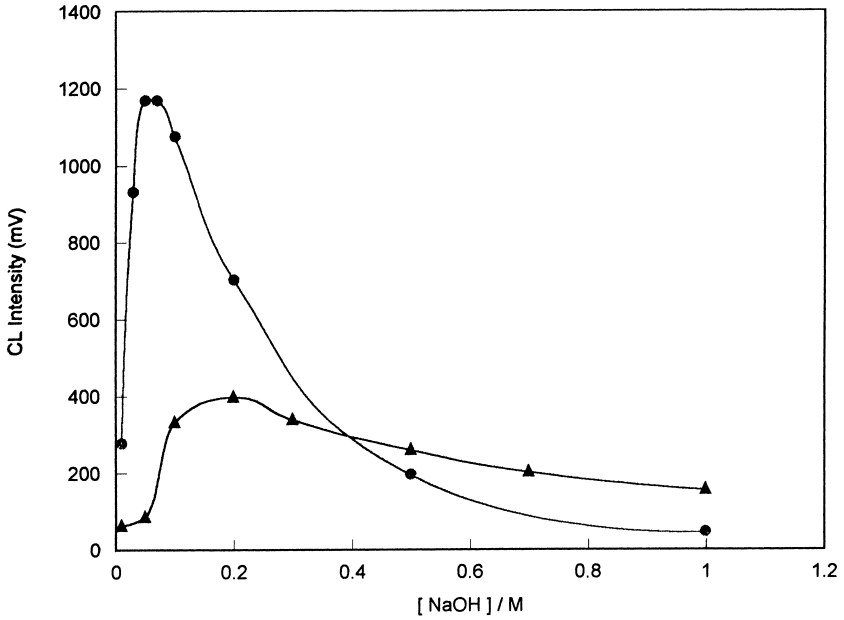

Fig. 4. Effect of sodium hydroxide concentration on CL intensity of potassium clavulanate (5 μg ml^{-1}; ●) and sulbactam sodium (10 μg ml^{-1}; ▲). Luminol 5×10^{-4} M, H_2O_2 5×10^{-3} M, flow rate 1.9 ml min^{-1}.

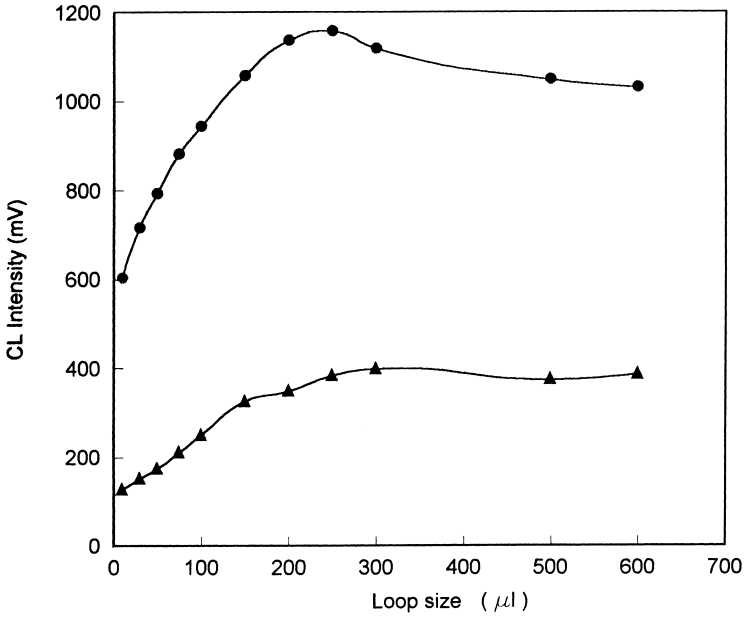

Fig. 5. Effect of sample volume on CL intensity of potassium clavulanate (5 μg ml^{-1}; ●) and sulbactam sodium (10 μg ml^{-1}; ▲). Luminol 5×10^{-4} M, H_2O_2 5×10^{-3} M, NaOH 0.05 and 0.2 M, respectively, flow rate 1.9 ml min^{-1}.

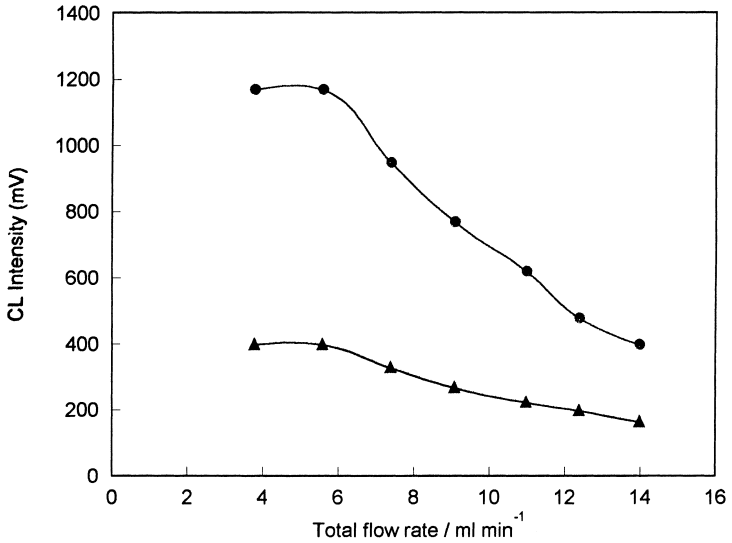

Fig. 6. Effect of total flow-rate on CL intensity of potassium clavulanate (5 μg ml^{-1}; ●) and sulbactam sodium (10 μg ml^{-1}; ▲). Luminol 5×10^{-4} M, H_2O_2 5×10^{-3} M, NaOH 0.05 and 0.2 M, respectively.

sium clavulanate and sulbactam, respectively, above which it was constant. The volume chosen was 250 μl for both drugs (Fig. 5).

The total flow rates of the luminol, hydrogen peroxide and sodium hydroxide streams were varied over the range 3.8–14.0 ml min^{-1}. The signal was maximal at 5.6 ml min^{-1} for clavulanate and sulbactam (1.9 ml min^{-1} for each channel), above which it decreased continuously (Fig. 6).

3.4. Effect of some micellar solutions and sensitizers

The effect of some organized systems, including neutral surfactants (Triton X–100), cationic surfactants (cetyltrimethylammonium bromide, cetylpyridinium bromide and cetylpyridinium chloride), and anionic surfactants (sodium dodecylsulphate) on the CL reaction were investigated. All these surfactants had no effect on the CL intensity.

The effect of some sensitizers, including quinine sulphate, rhodamine B and fluoresceine on the CL reaction were also investigated. All these sensitizers gave no enhancement on the CL intensity.

3.5. Determination of the studied β-lactams

Under the optimum operating conditions, a series of standard solutions over the concentration ranges cited in Table 1 was pumped, each as three replicates, to test for calibration linearity. A plot of the CL intensity versus concentration of the studied β-lactams was linear over the ranges given in Table 1. The accuracy and

Table 2
Analysis of the studied β-lactams in pharmaceutical preparations by the proposed and official methods

Preparation	Concentration taken (μg ml^{-1})	Recovery (%)	
		Proposed method	Official method [3,4]
Unasyn injection[a] (500 mg sulbactam+1000 mg ampicillin/vial)	1.0	100.0	
	10	100.0	
	50	99.9	
	100	99.5	
	150	100.0	
Mean±S.D.		99.9±0.22	100.2±0.44
Sulperazone injection[b] (500 mg sulbactam+1000 mg cefoperazone/vial)	5	98.9	
	10	98.9	
	40	100.0	
	80	99.9	
Mean±S.D.		99.4±0.61	99.0±1.48
Augmantin injection[c] (200 mg pot. Clavulanate+1000 mg amoxycillin/vial)	1.0	99.1	
	2.0	100.0	
	4.0	100.0	
	8.0	99.8	
	10.0	100.0	
Mean±S.D.		99.8±0.39	99.7±0.29
Augmantin tablets[c] (125 mg pot. Clavulanate+250 mg amoxycillin/tablet)	4.0	118.5	
	5.0	114.7	
	8.0	114.8	
	10.0	117.1	
Mean±S.D.		116.3±1.85	115.8±0.75

[a] Product of Pfizer, Egypt.
[b] Product of Pfizer, Germany.
[c] Product of SPIMACO Beecham, England.

precision of the method was evaluated by analyzing pure samples of the studied β-lactams. The average % recoveries were 100.7±1.05 for potassium clavulanate and 100.7±1.67 for sulbactam sodium (n=7, mean±standard deviation).

3.6. Application of the method

3.6.1. Analysis of pharmaceutical preparations

In order to evaluate the analytical usefulness of the chemiluminescent method, the studied β-lactams were determined in pharmaceutical preparations. The results in Table 2 are in accordance with those obtained by the official methods [3,4]. The results indicate that ampicillin did not interfere during the determination of sulbactam sodium in unasyn injection. For sulperazone injection, sulbactam sodium can be determined in the presence of cefoperazone up to 80 μg ml^{-1}, above which cefoperazone caused slight suppression of the CL signal. Also, amoxicillin did not interfere during the determination of potassium clavulanate in its dosage forms. Statistical analysis [24] of the results by using a *t*-test and *F*-test showed no significant difference between the two methods as regards to accuracy and precision.

It was reported earlier [12,13] that potassium clavulanate was degraded under alkaline conditions to give methyl-8-hydroxy-6-oxo-4-azaoct-2-enoate. Similarly, sodium sulbactam was degraded to methyl-5- carboxy -6 - methyl-6-sulphino-4-azahept-2-enoate. When both drugs are degraded by dissolution in sodium hydroxide, no CL signals were obtained. Thus the proposed method can be considered for a stability indicating assay.

3.6.2. Analysis of spiked serum samples

The high sensitivity attained by the proposed method allows the determination of the studied β-lactams in blood serum. For potassium clavulanate peak concentrations of about 2–4 μg ml^{-1} are obtained 1–2 h after administration of 125 mg by mouth [25]. For sulbactam sodium the peak serum level was 58 μg ml^{-1} after intravenous administration of 1 g [26]. Thus the proposed method proved to be satisfactory for kinetic studies and routine estimation of potassium clavulanate and sulbactam sodium in human serum.

Table 3
Determination of the studied β-lactams in spiked serum

Compound	Concentration taken (μg ml^{-1})	Found (%)
Sulbactam sodium	5.0	95.4
	10.0	92.7
	20.0	91.5
	30.0	88.4
	40.0	87.8
Mean±S.D.		91.2±3.14
Potassium Clavulanate	1.0	100.7
	2.0	100.0
	3.0	97.9
	7.0	94.9
	10.0	94.5
Mean±S.D.		98.6±2.8

The spiked serum sample was deproteinized with acetonitrile and extracted with methylene chloride to return the drug back to aqueous phase, as reported by Shah et al. [11]. Table 3 shows the results of determination of the studied β-lactams added to serum. The results show that sulbactam sodium can be determined in spiked serum up to 40 μg ml^{-1}, above which the CL signal decreases continuously. The proposed method is simple, sensitive, accurate and precise. It allows the determination of the studied drugs in the presence of co-formulated β-lactam antibiotics without interference and allows their determination in serum samples. Solutions can be analyzed at a rate of about 80 samples per hour for either drug.

The proposed method is characterized by its simplicity compared with the official methods. The USP [4] recommends a LC method for both sulbactam sodium and potassium clavulanate, that requires special skill to obtain reliable results. The BP [3] recommends a spectrophotometric method using imidazole solution for determination of potassium clavulanate in co-amoxiclav tablets. The method is tedious requiring immersion in a water bath at 30°C for exactly 12 min and then cooling to 20°C before immediate measurement of absorbance.

Acknowledgements

The authors gratefully acknowledge King Abdul Aziz City for Science and Technology (KACST) for the grant supporting this project (AT-17-T16).

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