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Postcolumn Chemiluminescence as a Detection Technique in the Liquid Chromatographic Analysis of Beta-lactam Ring Containing Compounds

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A broad range of compounds containing the beta-lactam ring were screened for enhancement of luminol chemiluminescence. A selection of these compounds were found to enhance the chemiluminescence of luminol. A high-performance liquid chromatographic method for the analysis of some beta-lactams employing postcolumn chemiluminescence was developed via flow injection analysis studies © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

Numerous quantitation methods for the penicillins have been reported in the literature, a collection of which have been documented as analytical profiles for the respective compounds (Florey; Aszalos, 1986).

The analysis of beta-lactam compounds in biological fluids has commonly involved a chromatographic separation with ultraviolet detection at approximately 254 nm. The mobile phase employed has typically been an aqueous buffered solution with or without a water miscible organic modifier; either acetonitrile or an alcohol. A limitation is that the beta-lactam antibiotics, especially those lacking an aromatic ring, typically have poor chromophores for UV detection at 254 nm which has the effect of compromising the sensitivity and detection limits of the analysis. This has limited the usefulness of UV spectrophotometry as a liquid chromatographic detection method for the beta-lactam compounds. The quantitative analysis of these compounds usually involves degradation or derivatization of the intact penicillin to a derivative that possesses a chromophore that can be more easily quantitated by some other means.

The development of methods for directly quantitating the intact beta-lactam ring would prove valuable to the analysis of the compounds in 'dirty' matrices such as biological fluids where the potential for interference is high. The penicillins as a group possess poor chromophores. There is room for improvement of sensitivity and limit of quantitation, especially with regard to analysis in biological samples such as meat products.

The luminol/hydrogen peroxide/metallic ion system, or variations thereof, have been used for the analysis of chemical and biological samples such as hydrogen peroxide, metal ions and a variety of compounds able to influence the chemiluminescence (Townshend, 1990; Maynoerd *et al*., 1955; Zhou *et al*., 1991; Sato and Tanaka 1990; Jones *et al*., 1989; Sakai *et al*., 1989; Lowery *et al*., 1977). More recently it has been applied to the analysis of selected penicillins and cephalosporins following the observation that some were able to enhance the intensity of luminol chemiluminescence (Milbrath, 1986; Schulman *et al*., 1991; Chen *et al*., 1991; Nakashima *et al*., 1993). In this report, using a modified luminescence enhancement system, a wider range of beta-lactam ring containing compounds is examined and the results of applying the technique to both flow injection analysis and liquid chromatography for postcolumn chemiluminescence detection is reported.

EXPERIMENTAL

Reagents. Hydrogen peroxide (30%) was supplied by Fisher Scientific Co., sodium hypochlorite and 3-aminophthalhydrazide (Luminol) were purchased from Aldrich Chemical Co. Inc, (Milwaukee, WI, USA). The antibiotics were obtained from various sources. Acetidinone from Aldrich Chemical Co. Ampicillin (ACS DOBFAR) from Interchem Corporation (Paramus, NJ, USA), Hetacillin was provided by Beecham Research Laboratories (Syracruse, NY, USA), *N*-formimidoyl thienamycin (Imipenem) was a gift from Merck & Co (Rahway, NJ, USA), Methicillin and Phenethicillin from Bristol Laboratories (Syracuse, NY, USA), Lithium clavulanate a gift from Smith Kline Beecham Pharmaceuticals (Philadelphia, PA, USA), Sulbactam from Pfizer Groton, CT, USA, Cephalothin from Eli Lilly Labs (Indianapolis, IN, USA). The other antibiotics were either purchased from Sigma Chemical Co. (St Louis, MO, USA) or Aldrich Chemical Co. Water was deionized by means of a Milli-Q50 water filtration apparatus from Millipore Corp. (Bedford, MA, USA).

All other chemicals and reagents were used as provided without further purification.

Preparation of luminol solutions. Solutions of 0.001 M luminol were prepared by dissolving 88.6 mg 3-aminophthalhydrazide in a mixture of about 25 mL deionized water and 2 mL sodium

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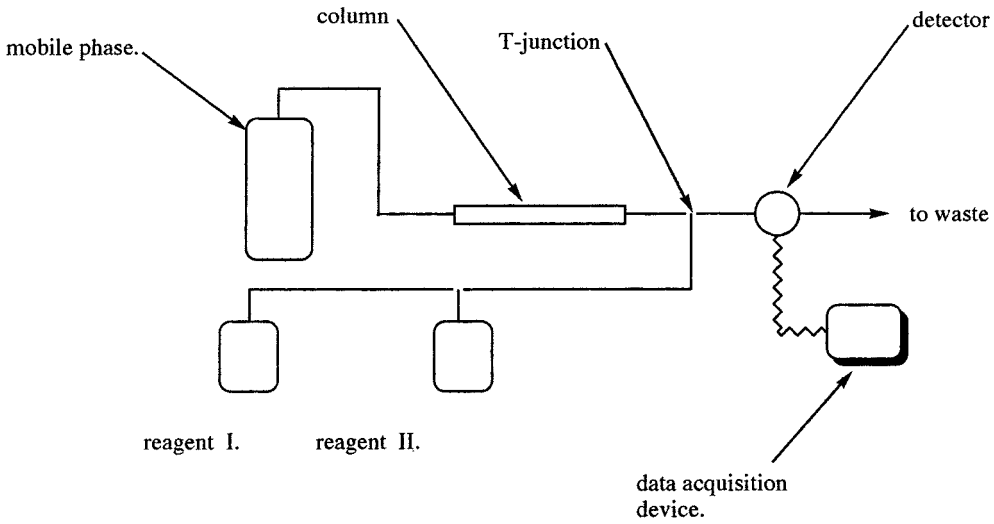

Figure 1. Schematic diagram of setup for chromatographic studies.

hydroxide with stirring, then adjusting to volume with water in a 500 mL volumetric flask. The solutions were employed for 48 h, following which they were discarded.

Preparation of hydrogen peroxide solutions. The hydrogen peroxide solutions were prepared by pipetting 2.0 mL of a 30% hydrogen peroxide solution into a 500 mL volumetric flask containing 2.0 mL of 2 M sodium hydroxide in about 25 mL deionized water, then made to volume with deionized water.

Preparation of stock sodium dihydrogen phosphate solution. A stock solution of monobasic sodium phosphate (NaH_2PO_4) 0.01 M was prepared by dissolving 690 mg of the anhydrous salt in 500 mL deionized water. From the stock solution 50 mL aliquots were diluted to 500 mL to obtain the 0.001 M solutions.

Preparation of mobile phase. To 35.0 mL methanol in a measuring cylinder was added the 0.001 M sodium dihydrogen phosphate solution to obtain a final volume of 100 mL. The solution was filtered through a 0.45 μm membrane filter and degassed by stirring the mobile phase under vacuum/suction.

Preparation of calibration series. Stock solutions of the beta-lactam compounds were prepared in deionized water prior to each analysis. Appropriate volumes of the solutions were then diluted with water to obtain the desired concentration ranges. Twenty-μL aliquots were injected into the chromatographic system. The mobile phase consisted of methanol (35%) in 0.001 M sodium phosphate pH 6.3.

Instrumentation. The separation was performed on an Exsil R ODS column (C_{18}) (150 × 4.6 mm, particle size 7 μm, pore size 100 Å, Keystone Scientific Inc.), employing methanol 35% in 0.001 M potassium hydrogen phosphate as the mobile phase with a flow-rate of 1.0 mL/min. The postcolumn reagent streams consisted of 1.0×10^{-4} M luminol in 0.0010 M sodium hydroxide (flow-rate 0.5 mL/min) and 0.030% hydrogen peroxide in 0.0010 M sodium hydroxide (flow-rate 0.5 mL/min. Twenty–μL samples of the beta-lactam antibiotic were injected using a Rheodyne (Cotati, CA, USA) loop injector fitted with a 20 μL loop. All reagent streams were served by pharmacia-LKB (Bromma, Sweden) model 2150 HPLC pumps. The ferrules, T-junctions and connectors were all made of stainless steel. Chemiluminescence was measured by a FL-750 spectro-fluorescence detector, McPherson instruments (Acton, MA, USA) hooked up to a IBM XT personal computer via an DT2811 analogue to digital I/O board and the data processed using the Spectracalc program from Galactic Industries (Salem, NH, USA). The results were also recorded on a Servogor 120 strip chart recorder (Fisher Scientific, Pittsburgh, PA, USA). The flow setup employed is as illustrated in Fig. 1.

Static experiments. The luminol solution was pipetted into a cuvette, the cuvette positioned in the cell holder of the detector (Fig. 2). The compartment was covered and a 5–50 μL aliquot of the beta-lactam antibiotic injected through a slit into the cuvette. The intensity–time profile was then obtained over a 3600 s time interval and either recorded on disk or a stripchart recorder. The peak height and/or area under the intensity–time curve was used to measure the sensitivity.

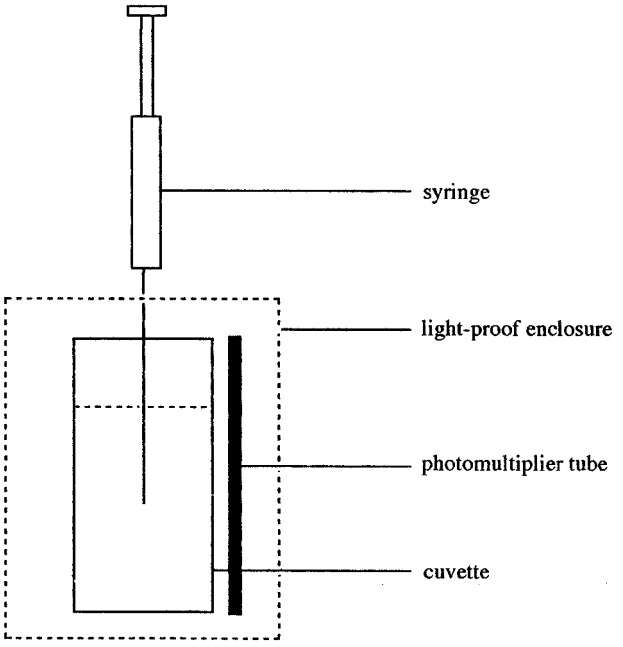

Figure 2. Schematic diagram of setup for static chemiluminescent measurements.

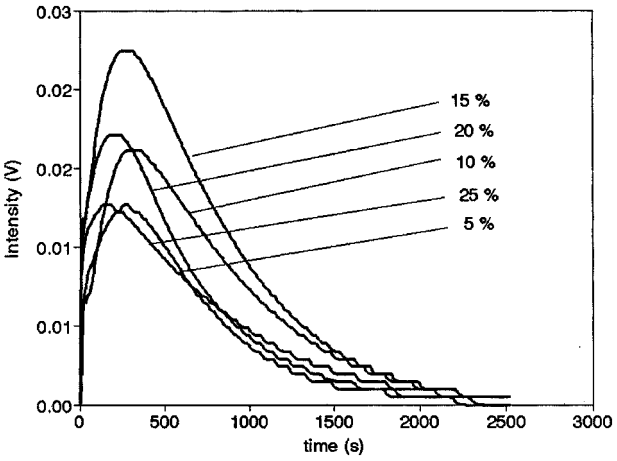

Figure 3. Chemiluminescent intensity–time profiles for dicloxacillin–luminol system on varying the proportion of methanol in analyte stream.

RESULTS AND DISCUSSION

Static studies

From the static studies it was possible to demonstrate that the methanol content of the analytical streams influences the extent of enhancement. Maximum enhancement was found to lie between 10 and 20% v/v methanol in water (Fig. 3).

The possibility that degradation products of penicillin hydrolysis could be responsible for the enhancement of chemiluminescence was also examined. However, both penicillamine and penicillenic acid were found not to enhance luminol chemiluminescence.

Flow studies

(i) Flow injection analysis. Preliminary work indicated that the enhancement has better analytical potential when applied to flowing streams than in a static setup, owing to the more consistent mixing. This advantage was considered to outweigh the loss of chemiluminescence associated with the stream flowing through the measuring cell. Quantitative signals could be obtained using either luminol or isoluminol solutions at concentrations as low as 10^{-5} to 10^{-6} M, metal ion solutions in the same concentration range and hydrogen peroxide solutions of concentration 9×10^{-3} M. The optimum pH was obtained using a 10^{-4} M solution of sodium hydroxide. This can be compared with the pH 11.7 employed by Yan (1991). However, owing to convenience of preparation, a 0.0010 M sodium hydroxide solution was employed as solvent for all the analytes (i.e. H_2O_2, luminol and cobalt/copper). If a metal ion was employed, its choice was noted to influence the shape of the peak qualitatively. Copper ions gave rise to less baseline noise than cobalt ions, at concentrations in the region of 10^{-6} M. Maynoerd *et al.* (1955) have reported that different concentrations of copper (Cu^{2+}) ions have yielded differing intensity–time profiles. Yan (1991) selected copper as opposed to cobalt, citing improved chromatograms obtained and the better signal-to-noise ratio as a result of the lower background noise. If the flow injection analysis (FIA) setup involved more than two streams, the order of mixing was found to influence the quality of the signal obtained, better results were obtained for streams in which the alkaline luminol solution was mixed with alkaline hydrogen peroxide prior to merging with the metal ion stream (Fig. 4). This order was consistent with the reasoning that the metal ion simply catalyses the emission arising from the reaction of luminol in alkaline peroxide. The beta-lactam analyte was then injected into the luminol stream prior to the merging of the stream with the metal ion stream. Optimal flow-rates for the setup were determined to be approximately 1.25 mL min per channel, resulting in a total output of 4.5 mL min flowing through the detector. A 24 μL cell permitted measurements in the millivolt range which were difficult to achieve with the 12 μL cell. This was probably due to longer residence time of the chemiluminescent stream within the larger volume detector.

It was also noted that the metal ion stream was not essential for chemiluminescence enhancement, and could in fact could be excluded altogether, the effect of which was a reduction in the intensity of light emission associated with removal of the catalytic influence of the metal ion. The reduction, however, does not significantly compromise the enhancement brought about by the beta-lactams. As a result all subsequent flow injection analyses excluded the metal ion stream. FIA studies revealed that solvents such as acetonitrile, tetrahydrofuran and butanol either alone or in mixtures with water did not support the chemiluminescence. It was observed that borate buffers did not support the chemiluminescence whereas phosphate buffers were able to support postcolumn chemiluminescence detection. This was found to be in agreement with reports by Nakashima *et al.* (1993) who found that borate as well as the imidazole buffer did support postcolumn chemiluminescence detection. Attempts to incorporate into the mobile phase an imidazole buffer pH 7.3 (British Pharmacopeia, 1988) in the mobile

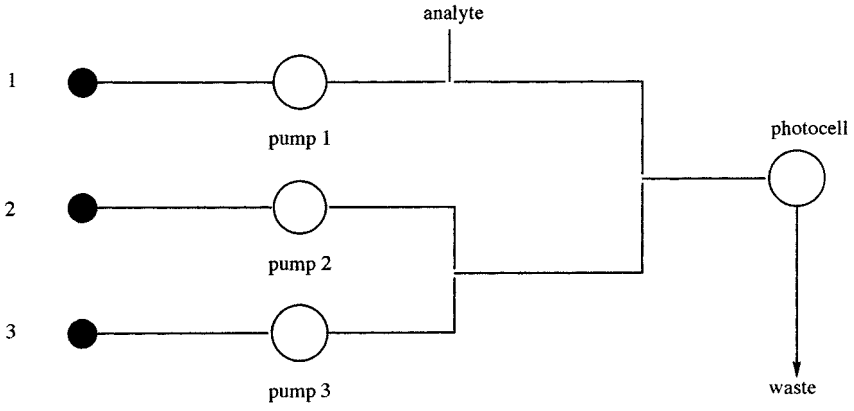

Figure 4. Schematic setup of three channel flow injection analysis setup.

1 6 aminopenicillanic acid
2 ampicillin
3 azetidinone
4 cefotaxine
5 cephalosporin C
6 cephalothin
7 clavulanic acid
8 dicloxacillin
9 penicillamine
10 hetacillin
11 imipenem
12 methicillin
13 penicillin G
14 penicillin V
15 phenethicillin
16 piperacillin
17 sulbactam

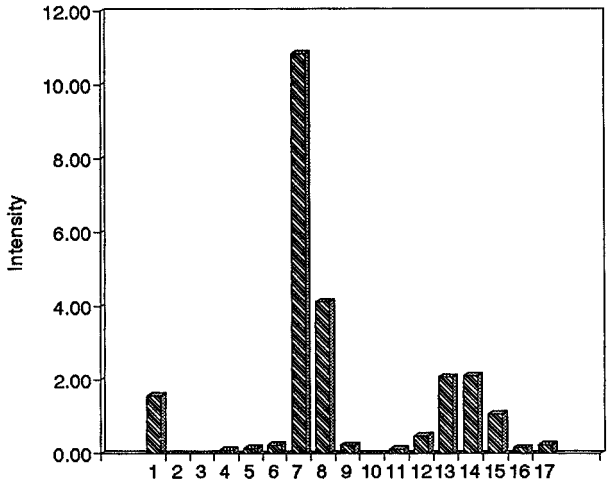

Figure 5. Histogram of relative enhancement obtained for selected penicillins in flow injection analysis setup.

phase did not yield a postcolumn detection system able to support chemiluminescence. The nature of the buffer used by Nakashima *et al* was not defined.

Employing the three channel setup consisting of a mobile phase (methanol (35%) in 0.001 M KH_2PO_4), an alkaline luminol stream (i.e. 1×10^{-4} M luminol in 0.0010 M NaOH) and an alkaline peroxide stream 0.03% in 0.0010 M NaOH) a number of penicillins, cephalosporins and related compounds were examined. The results indicate that on a molar basis dicloxacillin exhibited the best enhancement of chemiluminescence within the group examined, clavulanic acid also exhibited a comparable enhancement of chemiluminescence, then to a lower extent benzylpenicillin, penicillin V, phenethicillin, 6-aminopenicillanic acid, cephalothin, and piperacillin. A number of compounds exhibited weak enhancement while others did not afford a measureable enhancement of chemiluminescence (Fig. 5). Of significance was the fact that penicillamine and the isolated beta-lactam ring azetidinone did not significantly enhance the chemiluminescence. Using this setup the analytical parameters for selected compounds was obtained (Table 1).

(ii) Liquid chromatography. The objective was to develop a chromatographic system that would permit separation and quantitation of a mixture of at least two beta-lactam antibiotics and allow a postcolumn chemiluminescence detection system.

Mobile phase development was based on the findings made in both static studies and the flow injection analyses. Possible application of the enhancement as a postcolumn detection technique in the chromatographic analysis of beta-lactam antibiotics was also examined. Initial experiments indicated that separation with chemiluminescent detection is possible using silica columns. However, more recently Nakashima *et al*. (1993) carried out reverse-phase separation on octadecylsilane columns with postcolumn chemiluminescence detection. From the separation systems reported in the literature it was decided to confine the mobile phase choices to mainly aqueous-based mobile phases, simply to preclude compromising the chemiluminescent reaction by nonaqueous media or heats of solution arising from the mixing of two or more different streams. Thus, whereas acetonitrile and tetrahydrofuran were found to quench the luminescence, methanol generated a significant heat of solution on mixing with aqueous streams resulting in a significant baseline drift. From the initial experiments silica column based separations, with postcolumn chemiluminescence detection, were found to be feasible. However, emphasis was placed on developing a reverse-phase separation system owing to the attendant advantage of lower cost of analysis. The criteria applied to selecting the appropriate starting mobile phase were that the phase should be partly or entirely aqueous, permit reverse-phase separation, not contain known quenchers of the chemiluminescence and be easily adjusted to pH 10 by mixing the postcolumn alkaline streams. A number of the

Table 1. Analytical parameters for selected compounds from flow injection analysis

Compound	Sensitivity (V.l/m)	LOQ (m/L)	*R* squared	Precision (SD)
Benzylpenicillin	168.15	8.5E–06	0.986	11.61 (n=5)
Piperacillin	15.582	7.6E–05	0.98	3.59 (n=5)
Phenethicillin	14.479	8.8E–05	0.986	6.14 (n=5)
Penicillin V	7.643	0.00011	0.998	4.96 (n=4)
Ampicillin	7.098	0.00039	0.998	2.21 (n=4)
Cephalolthin	4.725	0.00082	0.97	15.84 (n=4)
Hetacillin	NS	–	–	–
Cephalosporin C	NS	–	–	–
Methicillin	NS	–	–	–

Table 2. Chromatographic retention times for selected compounds

Compound	Retention time (min)
6-Aminopenicillanic acid	1.35
Amoxicillin	NS
Ampicillin	NS
Azetidinone	NS
Cefotaxine	1.8
Cephalosporin C	1.2
Cephalothin	1.9
Clavulinic acid	1.2
d-Penicillamine	NS
Dicloxacillin	4.9
Hetacillin	NS
Imipenem	1.2
Methicillin	2
Penicillin G	3
Penicillin V	3.1
Phenethicillin	3.6
Piperacillin	1.2
Sulbactam	NS

NS, no measureable signal obtained.
Chromatographic system:

Column	Exsil R ODS	
Mobile phase	0.001 M KH_2PO_4: MeOH (65:35)	1.0 mL/min
Reagent streams	Alkaline luminol	0.5 mL/min
	Alkaline hydrogen peroxide	0.5 mL/min
Detection system	Chemiluminescence	

Table 3. Analytical parameters for selected penicillins obtained from Chromatographic studies

Compound	*X*-coefficient (mm.l/m)	*R* squared	Precision	Replicates
Penicillin G	4.3 exp(4)	0.99	5.11	6
Penicillin V	2.5 exp(4)	0.98	24.44	6
Dicloxacillin	4.9 exp(4)	0.98	7.6	6

chromatographic systems described in the literature for the separation of penicillins were not likely to support chemiluminescence, largely owing to the organic modifier or buffer employed in the chromatographic mobile phase.

Attempts to employ a methanol:water:acetonitrile combination without an inorganic salt failed to achieve separation of the penicillins with all eluting at the solvent front. Subsequent experimental runs were based on the separation reported by White and Zarembo (1981). These were initially carried out on an octadecylsilane (ODS) column using a 35% methanol in 0.01 M sodium dihydrogen phosphate NaH_2PO_4 mobile phase with a UV detector set at 254 nm. This system was able to separate dicloxacillin from penicillin G but did not support postcolumn chemiluminescence detection. Lowering the $Na_2H_2PO_4$ concentration to 0.001 M permitted postcolumn chemiluminescence detection, but significantly reduced the solute retention times. The reduced retention time(s) compromised the usefulness of postcolumn chemiluminescence detection as a reasonable different in retention times is necessary to counter the effects of band broadening arising from the postcolumn detection method. The use of 0.001 M potassium dihydrogen phosphate (KH_2PO_4) alone as the mobile phase for the elution of penicillin G gave rise to broad peaks on a noisy baseline. The addition of 10% methanol to the 0.001 M KH_2PO_4 significantly reduced the noise, but did not yield useful peaks. The use of 35% methanol in the 0.001 M KH_2PO_4 mobile phase eluted the penicillins but required the introduction of a delay coil. This was found to improve the quality of signal, both in terms of permitting the reaction to develop and improving the extent of mixing with the considerable improvement of the signal-to-noise ratio. For the chromatographic system a 35% methanol mobile phase in 0.001 M KH_2PO_4 was employed which on dilution by the postcolumn streams, would yield a final methanol concentration of approximately 15% v/v methanol.

With a 7.1 cm mixing loop the chemiluminescence enhancement was not detectable whereas lengthening the mixing loop to 47.0 cm afforded a measurable chemiluminescence enhancement signal.

Employing this setup the retention times for a number of penicillins as well as selected analytical parameters were obtained (Tables 2, 3) and calibration curves obtained for dicloxacillin, penicillin V and penicillin G (Figs. 6, 7, 8).

An estimate of the loss of resolution associated with the postcolumn chemiluminescence detection was also determined employing penicillin V and dicloxacillin as model compounds. The results exhibit a loss of resolution of 19.3% for the system employed. No literature values on the loss of resolution associated with postcolumn derivatization could be obtained.

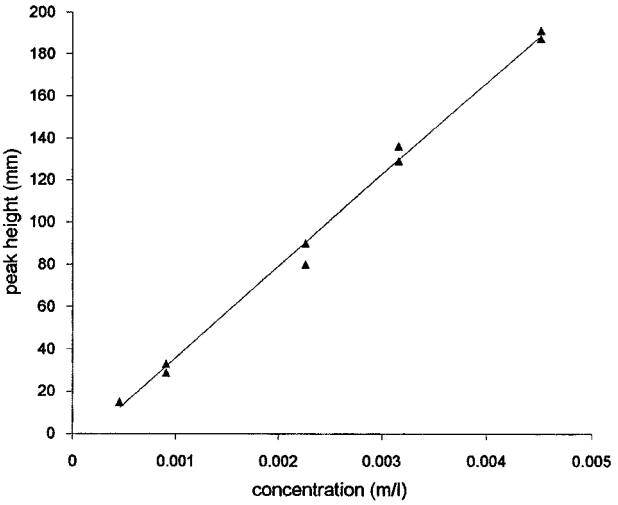

Figure 7. Calibration plot for penicillin G.

CONCLUSIONS

In addition to the beta-lactam antibiotics penicillin G, penicillin V, piperacillin and cephalothin, a broader range of beta-lactam ring containing compounds are able to enhance the chemiluminescence exhibited by luminol. It appears that a 'strained' beta-lactam ring is essential for the enhancement. The strain is achieved by fusion of the beta-lactam ring with the thiazolidine or the dihydrothiazine ring. However the presence of a beta-lactam ring alone does not assure enhancement of luminescence as other structural features in the molecule appear to be able to modulate this enhancement of luminol chemiluminescence.

The different penicillins enhance the chemiluminescence to varying degrees exhibiting differing intensity profiles. Of the beta-lactam compounds examined dicloxacillin and clavulanic acid exhibit the most profound enhancement of luminescence; more so than penicillin G and the phenoxyalkyl penicillins penicillin V and phenethicillin. Cephalothin, methicillin, aminopenicillanic acid, pipera-

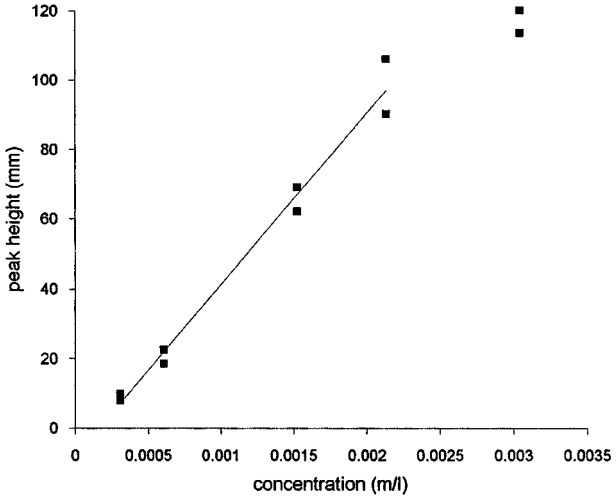

Figure 6. Calibration plot for dicloxacillin.

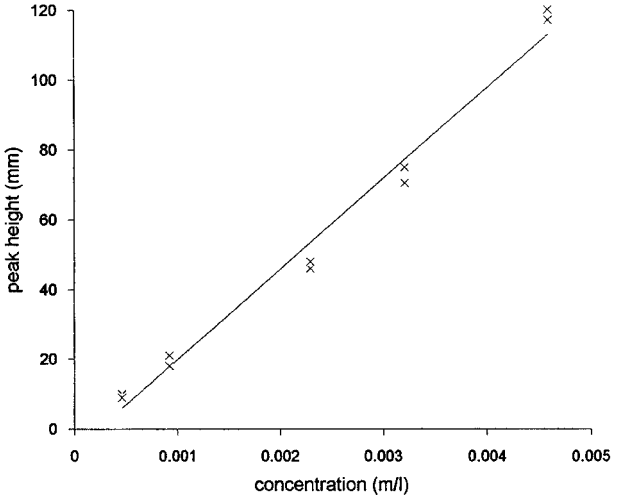

Figure 8. Calibration plot for penicillin V.

cillin and sulbactam exhibit much less enhancement. Ampicillin and its derivative hetacillin on the other hand do not enhance the chemiluminescence nor does the isolated beta-lactam ring, azetidinone. These differences are presumed to arise from differences in the accessibility of the nucleophilic oxidant to the electron deficient carbon of the beta-lactam ring. In the case of dicloxacillin the relative planarity of the side chain probably precludes the possibility of the side chain influencing nucleophilic accessibility to the beta-lactam carbonyl group. This would suggest that all isoxazolyl penicillins should be strong enhancers of the chemiluminescence. With regard to clavulanic acid, the enhancement was associated with the smaller molecule and the additional ring strain introduced by the double bond exocyclic to the furan ring, however no correlation was demonstrated between chemiluminescent enhancement and ring strain as measured by the position of the beta-lactam carbonyl infra red stretching frequency.

A metal ion stream was not found to be essential for chemiluminescence enhancement and could, in fact, be excluded altogether. The enhancement can be applied to the flow injection analyses of the beta-lactam antibiotics or as a postcolumn detection technique in liquid chromatographic analyses of penicillin mixtures whose retention times differ by more than 1 min.

In application of the technique to liquid chromatographic analyses the mobile phase combinations are limited to alcohol:water mixtures (primarily methanol:water) of not more than 50% alcohol. The enhancement was not supported by acetonitrile or tetrahydrofuran and is compromised by high (>0.01 M) concentrations of phosphate or borate buffer.

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