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A sensitive assay for clavulanic acid and sulbactam in biological fluids by high-performance liquid chromatography and precolumn derivatization

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Abstract: Precolumn derivatization procedures using 1,2,4-triazole for the detection and quantitation of sulbactam and clavulanic acid spiked into urine and blood serum at trace levels have been developed. Sulbactam and clavulanic acid produced derivatives which absorbed maximally at 325 and 315 nm, respectively. The methods allow the detection of clavulanic acid and sulbactam down to 0.05 $\mu g\ ml^{-1}$ in serum and 0.5 $\mu g\ ml^{-1}$ in urine. The relative standard deviation for five replicate analyses of sulbactam and clavulanic acid at a concentration of 20 $\mu g\ ml^{-1}$ in serum and urine ranged from 2–6%. In further HPLC experiments with sulbactam in phosphate buffer solution, ampicillin was found as a contaminant (0.5% by mass) in the sulbactam sample provided. The significance of this finding is discussed.

Keywords: *Clavulanic acid; sulbactam; ampicillin; derivatization; 1,2,4-triazole; reversed-phase chromatography; serum; urine.*

Introduction

Sulbactam and clavulanic acid are potent inhibitors of β-lactamase enzymes which are responsible for the protection of microorganisms against β-lactam antibiotics. Clavulanic acid is a natural β-lactam antibiotic produced by *Streptomyces clavuligerus* [1, 2]. Sulbactam, a semisynthetic β-lactam, is a penicillanic acid sulphone. Both of these β-lactams are prescribed clinically in combination with β-lactamase-labile β-lactams.

Clavulanic acid can be detected using microbiological plate assay [3, 4] or biochemically by exploiting its enzyme inhibitory properties against β-lactamase enzymes [5]. Sulbactam and clavulanic acid can be detected after separation by high-performance liquid chromatography (HPLC), by direct absorption of ultraviolet (UV) light at 220 [6] or 225 nm [7]. However, measurement at trace levels in biological fluids by HPLC with direct UV detection is not feasible due to chromatographic interference by other UV-absorbing components in the matrices. This problem can be overcome by the use of precolumn or post-column derivatizing reagents. For example, imidazole and 1,2,4-triazole are two such reagents which have been used to measure clavulanic acid in body fluids [8–10]. In this paper we report the first application of 1,2,4-triazole to the analysis of sulbactam in blood serum and urine, and lower detection limits for a similar analysis of clavulanic acid in the same fluids.

Experimental

Materials

Clavulanic acid (potassium salt), penicillin G (potassium salt) and 6-aminopenicillanic acid (6-APA) were kindly donated by Beecham Pharmaceuticals (Brockham Park, UK). Sulbactam (sodium salt) and ampicillin trihydrate were acquired from Pfizer (Sandwich, UK). 1,2,4-Triazole was purchased from Aldrich Chemicals (Gillingham, UK). Potassium dihydrogen orthophosphate and mercury(II) chloride were supplied by Fisons (Loughborough, UK). Sodium thiosulphate (AR grade) was purchased from BDH Chemicals (Poole, UK). Acetonitrile (HPLC grade) was supplied by Rathburn Chemicals (Peebleshire, UK).

Preparation of derivatizing reagent

1,2,4-Triazole (3.45 g) was dissolved in 15 ml of water. The pH of the solution was adjusted to 6.0 for sulbactam and 7.0 for clavulanic acid, using 4 M aqueous sodium

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hydroxide. The volume was then made up to 25 ml to give a final reagent concentration of 2 M.

Preparation and derivatization of standards

(i) *In serum.* Separate 100 μg ml^{-1} stock solutions of sulbactam and clavulanic acid in 0.02 M phosphate buffer pH 7.0 were prepared. Separate series of standards (0.1, 1.0, 2.0, 4.0, 10.0 and 20.0 μg ml^{-1}) of clavulanic acid and sulbactam in human blood serum were prepared by diluting the appropriate volumes of stock solution to 1.0 ml with serum. Each standard was shaken with acetonitrile (1.0 ml) for 15 min at 0°C. The precipitated protein was removed by centrifugation for 10 min at 8000*g*. The supernatant was transferred to a clean centrifuge tube and treated with methylene chloride (10 ml). The mixture was again shaken (15 min at 0°C) and centrifuged (10 min at 8000*g*). The organic layer was discarded and a portion (0.2 ml) of the aqueous layer was incubated with the appropriate 1,2,4-triazole reagent solution (0.4 ml) for 5 min at room temperature for clavulanic acid standards and for 40 min at 40°C for sulbactam standards.

(ii) *In urine.* Series of standards (1.0, 2.0, 4.0, 10.0 and 20.0 μg ml^{-1}) of clavulanic acid and sulbactam in urine which had been filtered through a 0.22-μm Millipore filter were prepared by diluting the appropriate volumes of stock solution to 1.0 ml with the filtered urine. The resulting solutions were diluted 10-fold. 0.2-Millilitre portions of the diluted solutions were then derivatized with the appropriate 1,2,4-triazole reagent solution (0.4 ml) for 5 min at room temperature for clavulanic acid standards and for 40 min at 40°C for sulbactam standards.

(iii) *In aqueous buffer.* A solution of sulbactam (100 μg ml^{-1}) in 20 mM phosphate buffer pH 7.0 was prepared. 0.2-Millilitre portions were treated with 0.4 ml of 1,2,4-triazole derivatizing reagent containing mercury(II) chloride at a final concentration of 10 mM. The mixtures were incubated at 40°C for 40 min.

(iv) *Ampicillin standards.* A stock solution of ampicillin (10 μg ml^{-1}) in 20 mM phosphate buffer pH 7.0 was prepared. Mixtures of sulbactam and ampicillin were prepared as shown in Table 1. These mixtures were incubated with 1,2,4-triazole containing mercury(II) chloride as described in section (iii) above.

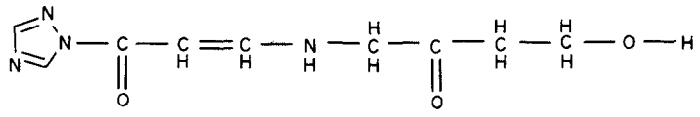
High-performance liquid chromatography

A Perkin Elmer Series 4 system consisting of a microprocessor controlled solvent delivery system, an ISS-100 autoinjector, an LCI-100 laboratory computing integrator and an LC-235 diode array detector was employed. Chromatographic separation was performed on a 25 × 0.46 cm i.d. analytical column packed with 5 μm Spherisorb C-18 (Hichrom, Reading, UK). A guard column 5 × 0.46 cm i.d. (Thames Chromatography, Maidenhead, UK) containing identical packing material to that in the analytical column was used. Aliquots (40 μl) of the derivatized antibiotic standard solutions were injected onto the guard column. Separations of sulbactam in serum and urine were effected with 20 mM phosphate buffer–acetonitrile (99.8:0.2%, v/v) at 0.5 ml min^{-1}. Clavulanic acid in urine and blood serum was analysed using a linear gradient of phosphate buffer–acetonitrile from 98:2% (v/v) to 75:25% (v/v) over 25 min. The ampicillin-spiked sulbactam samples in phosphate buffer were separated by using the above-mentioned isocratic conditions for elution of derivatized sulbactam, followed immediately by application of a linear gradient of phosphate buffer–acetonitrile from 99.8: 0.2% (v/v) to 75:25% (v/v) over 25 min.

Our studies indicate that the derivatives formed by both sulbactam and clavulanic acid with 1,2,4-triazole are stable in aqueous sol-

Table 1
Standard mixtures of sulbactam and ampicillin in 20 mM phosphate buffer

Volume (μl) of sulbactam solution (100 μg ml^{-1})	Volume (μl) of ampicillin solution (10 μg ml^{-1})	Concentration of added ampicillin (μg ml^{-1})
(a) 190	10	0.50
(b) 185	15	0.75
(c) 180	20	1.00



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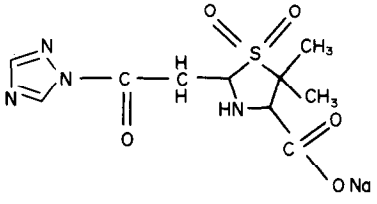

II

ution at neutral pH and room temperature for at least 24 h. By analogy with the structure reported for the product of clavulanic acid with imidazole [11], we suggest that the compounds formed by clavulanic and sulbactam with 1,2,4-triazole are (**I**) and (**II**), respectively, although structural studies are in progress. The corresponding molar absorptivities (calculated on the basis of structures **I** and **II**) are 5.58×10^5 mol^{-1} cm^{-1} and 8.37×10^5 mol^{-1} cm^{-1}, respectively.

Diode array detection of derivatized clavulanic acid and sulbactam, following HPLC separation, gave the absorption maxima of the derivatives at 315 and 325 nm, respectively. The detector was set at these wavelengths for quantitative measurements in the subsequent calibrations.

Results and Discussion

The incubation times and temperatures cited above were chosen as optimal from studies in which times were varied from 1–60 min and temperatures from 20–60°C. The pH of the derivatizing reagent solution was also varied from 6 to 9. Again, the pH values selected (6.0 for sulbactam and 7.0 for clavulanic acid) were optimal in terms of detection sensitivity.

Typical chromatograms obtained are shown in Figs 1 and 2, from which it can be seen that suitable chromatographic windows were found for each antibiotic in each medium. The regression data obtained with the clavulanic acid and sulbactam standards are summarized in Table 2. Since peak areas were in the range of 10^5–10^7 arbitary units, it is clear that the intercepts are negligibly small. The correlation coefficients (0.87–0.98) are satisfactory.

The detection limits for clavulanic acid and sulbactam are virtually identical in a given medium. The difference in detection limit for either compound in serum and urine are functions of the relative complexities of the matrices. It will be recalled that the urine-containing standards were diluted 10-fold prior to derivatization. The dilution of the urine standards was carried out because the column would otherwise have been over-loaded with other components in the urine.

The precision of the analysis of each antibiotic in each matrix was evaluated by carrying out five sets of replicate experiments using 20 $\mu g\ ml^{-1}$ in urine and (2 $\mu g\ ml^{-1}$) in serum. These levels correspond to those commonly encountered in patient samples. In urine, the relative standard deviations (RSD) for sulbactam and clavulanic acid were 6.2 and 1.9%, respectively, whilst for serum the RSDs were 6.4 and 2.9%, respectively. Recovery values are given in Table 3.

Experiments in which penicillin G, penicillin V and 6-APA were subjected to derivatization and HPLC analysis under the same conditions as those specified for clavulanic acid, yielded no identifiable peaks. Previous work [12] has established the need for metal ions, e.g. Hg(II) or Ag(I) or Au(I), for the successful formation of stable chromophoric derivatives of these compounds.

In preliminary HPLC experiments with underivatized sulbactam in phosphate buffer, a number of peaks attributable to UV-absorbing impurities were noted with detection at 325 nm. In further HPLC experiments with derivatized sulbactam in phosphate buffer, a substantial late eluting peak was observed with a retention time of 38 min (Fig. 3). The area of this peak changed in proportion to that of sulbactam with variation in concentration of the latter, prior to derivatization. Spiking experiments indicated that this peak was due to ampicillin. The peak areas resulting from standard additions of ampicillin to sulbactam,

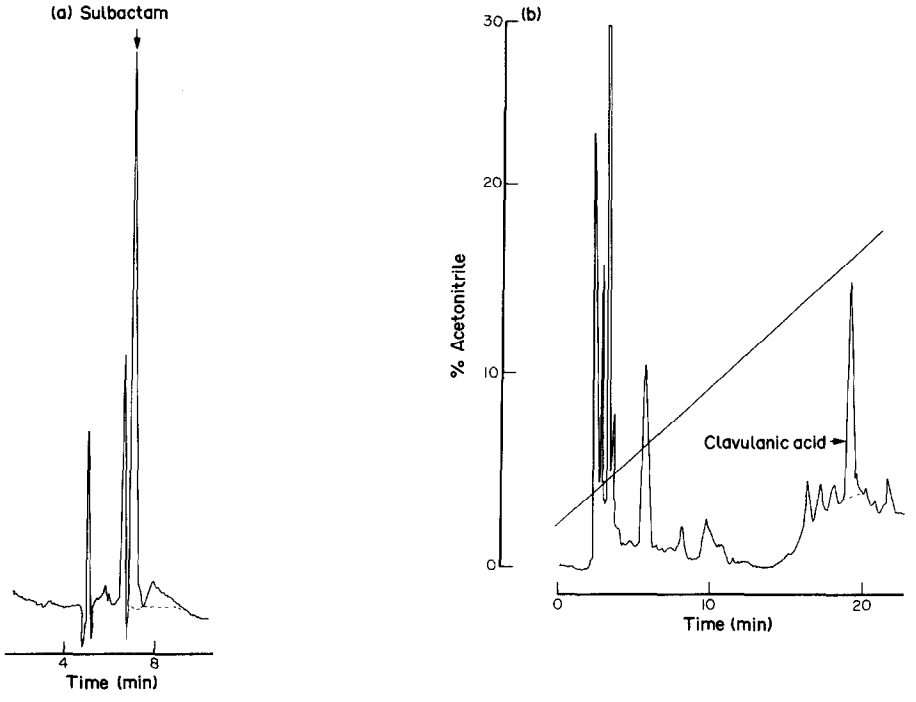

Figure 1
Chromatograms of (a) sulbactam and (b) clavulanic acid in serum at 1 μg ml^{-1}. Serum samples were pretreated and derivatized as described under Experimental, prior to HPLC separation. Sensitivity, 0.005 AUFS.

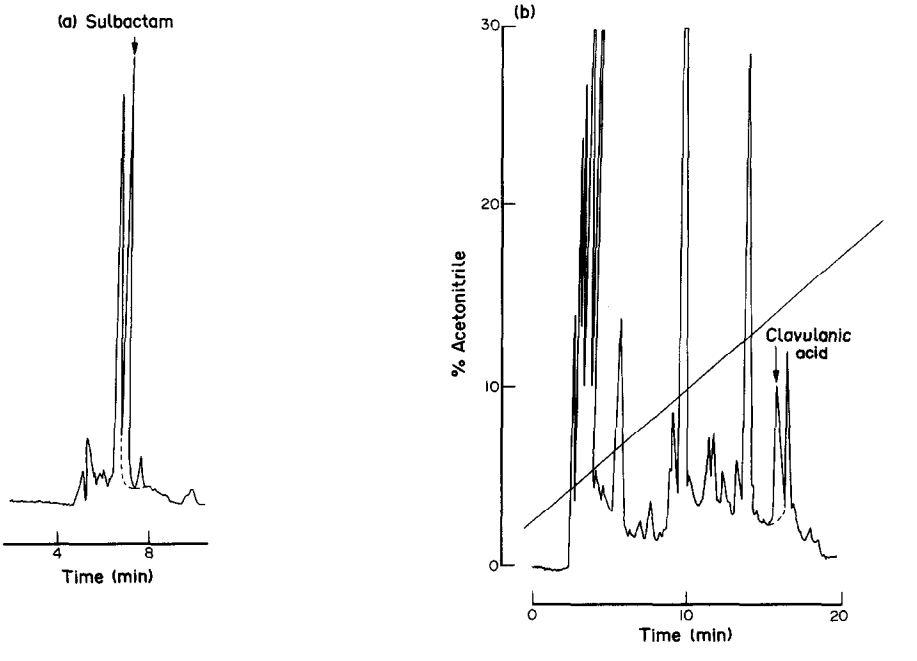

Figure 2
Chromatograms of (a) sulbactam and (b) clavulanic acid in urine at 10 μg ml^{-1}. Samples were derivatized as described under Experimental, prior to HPLC separation. Sensitivity, 0.005 AUFS.

the latter at nominal 20 μg ml^{-1} concentration, were determined (Table 4).

Graphical and calculation methods of treatment of these data indicated that the concentration of ampicillin in the sample was 0.1 μg ml^{-1}. This corresponds to the presence of 0.5% by mass of ampicillin in the sulbactam provided.

Table 2
Regression data for sulbactam and clavulanic acid spiked into (a) serum (b) urine. Slope and intercept values are related to antibiotic concentration by the equation: Peak area (arbitary units) = Slope × Concentration + Intercept

β-lactam	No. of samples	Concentration range ($\mu g\ ml^{-1}$)	Intercept	Slope × 10^{-5}	Standard deviation	Correlation coefficient	Detection limit ($\mu g\ ml^{-1}$)	S/N ratio
(a) Sulbactam	6	0.1–20.0	−4.2	1.8	2.6	0.98	0.05	10:1
Clavulanic acid	6	0.1–20.0	−0.3	1.3	0.4	0.94	0.05	10:1
(b) Sulbactam	5	1–20	−13.8	8.9	0.81	0.87	0.5	5:1
Clavulanic acid	5	1–20	−7.5	7.8	0.5	0.94	0.5	5:1

S/N = Signal to noise ratio.

Table 3
Recovery of sulbactam and clavulanic acid from (a) urine and (b) serum

β-lactam	Concentration (μg ml^{-1})	Recovery (%)
(a) Sulbactam	20.0	97.5
Clavulanic acid	20.0	99.2
(b) Sulbactam	2.0	100.1
Clavulanic acid	2.0	109.0

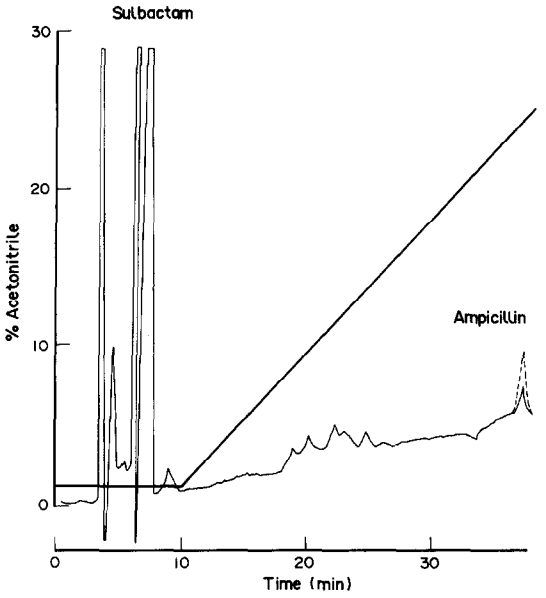

Figure 3
Chromatogram of sulbactam in phosphate buffer at 20 μg ml^{-1}. The chromatogram also shows ampicillin standard added to the sulbactam sample at a final concentration of 0.3 μg ml^{-1} prior to derivatization. Samples were derivatized as described under Experimental, prior to HPLC separation. Sensitivity, 0.005 AUFS.

Table 4
Effect of ampicillin addition to area of peak at 38 min, in Fig. 3

Concentration of added ampicillin (μg ml^{-1})	Peak area (arbitary units)
0.00	1986
0.50	8065
0.75	12216
1.00	16080

We have been informed by the suppliers that ampicillin was the starting compound for the manufacture of sulbactam and remained as the major impurity. Furthermore, ampicillin is one of a range of β-lactam antibiotics which is used clinically in combination with sulbactam. HPLC analysis of ampicillin in physiological media, using 1,2,4-triazole and mercury(II) chloride, with prior acylation of the free amino group, has been reported previously [13]. In our experiments, the acylation stage was omitted because it interferred with the sulbactam assay and the ampicillin measurement was achieved without the use of mercury(II) chloride with improved resolution and a small loss in sensitivity. The derivatization procedure was not optimized for ampicillin detection. Nevertheless, a detection limit for ampicillin of 0.01 μg ml^{-1} with a signal to noise ratio of 5:1 was achieved. Work in our laboratories is now directed towards simultaneous determination of sulbactam and other β-lactams, including ampicillin, in complex physiological media.

We believe that this is the first reported precolumn derivatization-HPLC based analytical procedure for the quantitation of sulbactam at low levels in biological fluids. Our method is substantially more sensitive than a previously reported postcolumn method [14] for the analysis of sulbactam in urine and comparable in sensitivity to that for a similar analysis in blood samples [14]. Although our method for the trace analysis of clavulanic acid is a variation on a procedure previously reported [10], the detection limits achieved in our laboratory are significantly lower with a lower injection volume. The procedures for both clavulanic acid and sulbactam do not lend themselves to the automation required for routine multiple sample analyses. Nevertheless, we feel they are of use where analysis of trace residues in biological matrices is required.

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