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Concentration of amoxycillin and clavulanate in lung compartments in adults without pulmonary infection

P J Cook, J M Andrews, J Woodcock, R Wise, D Honeybourne

Abstract

Background **– The efficacy of an antibiotic is usually predicted from serum levels and MIC_{90} values for likely pathogens, but in the lung tissue concentrations may be more informative. This study compares concentrations of amoxycillin and clavulanate in serum, epithelial lining fluid (ELF), alveolar macrophages, and bronchial mucosa in 15 adults.**

Methods **– Amoxycillin 500 mg and clavulanic acid 250 mg were given 1–2 hours before diagnostic bronchoscopy for haemoptysis or radiological abnormality. Mucosal biopsy samples were taken from macroscopically normal sites, alveolar macrophages harvested by lavage, and ELF volume derived from urea concentrations in bronchial lavage fluid and blood. Amoxycillin was assayed by inhibition of growth of *Micrococcus lutea*, and clavulanate (in serum, ELF, and bronchial mucosa) by inhibition of growth of *Klebsiella pneumoniae*; in macrophages clavulanate was measured by high performance liquid chromatography.**

Results **– The median concentrations in serum were 6·90 mg/l for amoxycillin and 5·25 mg/l for clavulanate. The median bronchial mucosal concentration of amoxycillin was 2·99 mg/l and of clavulanate was 1·65 mg/l; the median concentrations in ELF were 0·89 and 0·96 mg/l, and in macrophages 0 and 0·76 mg/l, respectively. In macrophages amoxycillin levels were undetectable in 10 of 14 subjects (71%); by contrast, only 6 of 14 subjects (43%) had no detectable clavulanate.**

Conclusions **– Clavulanate levels exceeded quoted MIC_{90} values (around 0·25 mg/l) for *Legionella pneumophila* both in ELF and in macrophages. Amoxycillin-clavulanate may therefore have a clinical role in infections with *Legionella pneumophila*.**

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During the last decade 20–32% of *Haemophilus influenzae* serotype b and about 15% of non-encapsulated *H influenzae* isolates have been resistant to ampicillin, mainly as a consequence of the non-inducible β-lactamase TEM-1.[1] A similar increase in the production of β-lactamases has been observed, particularly in the hospital setting, among clinical isolates of several other organisms including enterobacteria (especially *Escherichia coli*), *Moraxella catarrhalis*,[2] and some Gram positive cocci.[3] These enzymes now provide the commonest mechanism of resistance to β-lactam antibiotics among clinical bacterial isolates. A number of β-lactam compounds, including the oxapenam clavulanic acid and the penicillanic sulphones tazobactam and sulbactam, inhibit a wide range of β-lactamases by binding to their active sites, thus increasing the efficacy of β-lactam antibiotics. Amoxycillin-clavulanate, a broad spectrum antibiotic comprising mixtures of clavulanic acid with the modified aminopenicillin amoxycillin is used in both lower and upper respiratory tract infections,[4] particularly community acquired pneumonias and acute purulent exacerbations of chronic bronchitis or bronchiectasis.[5,6]

To be effective against respiratory tract pathogens an antibiotic must achieve bactericidal concentrations at sites of infection. The present study compares concentrations of amoxycillin and clavulanate in serum, bronchial mucosa, epithelial lining fluid (ELF), and alveolar macrophages in adults undergoing diagnostic bronchoscopy for chest radiographic abnormalities or haemoptysis.

Methods

PATIENTS

Fifteen patients (12 men and three postmenopausal women) were recruited into the study. These patients had a mean age of 58·7 years (range 24–75) and mean weight of 67·0 kg (range 47·2–97·0). None had significant renal or hepatic impairment and all were free of lung infection. Thirteen patients had abnormal chest radiographs; the remaining two presented with haemoptyses. Each gave informed consent before entry into the study, the protocol of which had been approved by the hospital ethical committee.

BRONCHOSCOPY

One to two hours before bronchoscopy each patient (having fasted for the previous eight hours) swallowed 750 mg amoxycillin-clavulanate (comprising amoxycillin 500 mg and clavulanic acid 250 mg) with 200 ml water. A standard premedication of 600 μg atropine was given by intramuscular injection 60 minutes before bronchoscopy, 4 ml of 4% lignocaine by nebuliser 40 minutes later, and up to 5 mg midazolam intravenously at the time of bronchoscopy. At that time up to 4 ml of 4% lignocaine was administered to the pharynx and

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vocal cords and 6 ml of 2% lignocaine to the carina and main bronchi.

All antibiotic assays, except those in the ELF, were performed on the day of bronchoscopy. Venous blood was collected before the administration of amoxycillin-clavulanate and at bronchoscopy, and centrifuged at 1500 *g* for 10 minutes at 4°C.

Bronchoalveolar lavage (BAL) was performed in the right middle lobe or the lingula. The first 50 ml of BAL fluid was discarded to avoid contamination with cells and secretions from the proximal airways, and the next three 50 ml aliquots were collected into a Teflon container (thus preventing adherence of aspirated cells). Lavage time was reduced to a minimum to limit the concentration-dependent efflux of solutes into BAL fluid. The total leucocyte count was estimated immediately with an improved Neubauer haemocytometer. Within five minutes of lavage the pooled BAL fluid was centrifuged at 400 *g* for five minutes: the supernatant was then freeze dried and reconstituted in one tenth of the original volume of distilled water, making a tenfold concentration.

Assuming that urea is present at the same concentration in ELF and blood, the volume of ELF in bronchoalveolar lavage aspirate can be calculated from urea concentrations in BAL fluid and serum,[7] and concentrations of antibiotics derived as follows:

$$\begin{aligned}\text{Vol}_{\text{ELF}} &= \text{Vol}_{\text{BAL}} \times [\text{urea}]_{\text{BAL}}/[\text{urea}]_{\text{ELF}} \\ &= \text{Vol}_{\text{BAL}} \times [\text{urea}]_{\text{BAL}}/[\text{urea}]_{\text{SERUM}}\end{aligned}$$

$$\begin{aligned}[\text{antibiotic}]_{\text{ELF}} &= [\text{antibiotic}]_{\text{BAL}} \\ &\times [\text{urea}]_{\text{SERUM}}/[\text{urea}]_{\text{BAL}}\end{aligned}$$

In health approximately 95% of cells in the alveoli are alveolar macrophages[8] whose mean (SE) volume has previously been estimated[9] by velocity gradient centrifugation[10] at 2·42 (0·41) µl/10^6 cells. The total volume of macrophages in the BAL aspirate was calculated, assuming that the leucocyte count and macrophage count were equal, as follows:

$$\begin{aligned}\text{Vol}_{\text{Mo}} &= 2{\cdot}42 \times 10^{-6} \times \text{leucocyte} \\ &\text{count per ml} \times \text{Vol}_{\text{BAL}}\end{aligned}$$

The cell pellet was suspended in a measured volume of cold phosphate buffer (pH 7·0) and homogenised by ultrasonication on ice before antimicrobial concentrations were assayed. Concentrations of antimicrobials in macrophages were therefore calculated from the formula:

$$[\text{A}]_{\text{Mo}} = \frac{[\text{A}]_{\text{Mo+pb}} \times \text{Vol}_{\text{Mo+pb}}}{\text{Vol}_{\text{Mo}}}$$

where $[\text{A}]_{\text{Mo+pb}}$ is the concentration measured and $\text{Vol}_{\text{Mo+pb}}$ is the volume of macrophages and buffer.

Superficial biopsy samples for antimicrobial assay were taken from macroscopically normal subcarinal sites – that is, without erythema, oedema, induration, or abnormal friability – before diagnostic biopsy samples. Visibly bloodstained specimens were discarded. The tissue was transferred immediately to a humidity chamber, weighed, and homogenised by ultrasonication in a known volume of phosphate buffer (pH 7·0) on ice.

MICROBIOLOGICAL ASSAYS

Amoxycillin was measured by inhibition of growth in an overnight broth culture of *Micrococcus lutea* (ATCC 9341) diluted to an OD_{630} of 0·05 and poured over a 200 ml No. 1 agar plate (Unipath, UK). Aliquots of all serum samples, concentrated ELF, mucosal tissue, and macrophages were placed in 5 mm wells cut in this agar and the plate was incubated for 24 hours at 37°C. Zones of inhibition of bacterial growth around patient serum samples were compared with zones produced by standard amoxycillin concentrations in human serum (pH 7·0): zones around mucosal tissue and macrophage samples were compared with those produced by amoxycillin in phosphate buffer (pH 6·6). For assays in concentrated ELF standard amoxycillin preparations were made in 9% sodium chloride. The lower limit of sensitivity for these assays was 0·03 mg/l.

Assay for clavulanic acid in serum, ELF, and mucosa was performed in a similar manner; 16 ml of an overnight broth culture of *Klebsiella pneumoniae* (ATCC 29665), diluted to an OD_{630} of 0·8, was incorporated into a 200 ml No. 2 agar plate (Oxoid, Basingstoke, UK) containing piperacillin at a concentration of 80 mg/l. Samples were incubated in 5 mm wells for 24 hours at 37°C. As described above, zones of inhibition were compared with those produced by standard clavulanate concentrations in human serum (pH 7·0) and 9% sodium chloride. The lower limit of sensitivity for these assays was 0·02 mg/l.

In these systems negative control preparations had no inhibitory effect on the assay organisms. The between assay coefficients of variation were 11·4% for both assays.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Clavulanic acid was assayed in alveolar macrophages by high performance liquid chromatography (HPLC), after reaction with imidazole, which produces a derivative absorbing at 311 nm.[11,12] The mobile phase was composed of 10 mM disodium hydrogen orthophosphate (BDH), titrated to pH 2·5 with orthophosphoric acid (BDH), 15 mM octanesulphonic acid (BDH) as pairing ion, 23 mM tetraethylammonium bromide (Sigma) as organic counter ion, and 5% acetonitrile (Rathburn Chemicals) passed through a 0·22 µm filter: this was pumped at 0·4 ml/min. All compounds were analytical grade or higher. 500 µl of each alveolar macrophage homogenate or 500 µl of phosphate buffer standard (pH 6·6) was mixed with 50 µl imidazole (BDH ≥ 99% pure) in 5 M hydrochloric acid (pH 6·8) and incubated in a light proof cabinet at room temperature for 60 minutes. 200 µl of

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Table 1 Calculations of amoxycillin and clavulanic acid concentrations (in mg/l) in epithelial lining fluid

Patient no	[Urea]SERUM	[Urea]BAL	[Amox]10 × BAL	[Amox]ELF = 0·1 × [A]10 × BAL × [U]SERUM/[U]BAL	[Clav]10 × BAL	[Clav]ELF = 0·1 × [C]10 × BAL × [U]SERUM/[U]BAL
1	6·5	0·028	0·15	3·48	0·36	8·36
2	3·7	0·035	0·08	0·85	0·10	1·06
3	7·0	0·035	0·06	1·20	0·11	2·20
4	5·5	0·060	0·30	2·75	0·40	3·67
5	6·0	0·080	0·13	0·98	0·32	2·40
6	3·7	0·014	NDL	0	NDL	0
7	7·5	0·036	NDL	0	NDL	0
8	3·4	0·016	0·07	1·50	0·13	2·70
9	4·4	0·029	0·06	0·93	0·06	0·85
10	9·8	0·036	0·02	0·55	0·02	0·49
11				Unsuitable for processing		
12	4·9	0·050	0·09	0·98	0·11	1·14
13	8·1	0·097	NDL	0	NDL	0
14	4·5	0·016	NDL	0	0·05	1·08
15	7·1	0·081	NDL	0	NDL	0

NDL = no detectable level.

Table 2 Concentrations (in mg/l) of amoxycillin and clavulanic acid in lung compartments

Patient no	Serum		Bronchial mucosa		Epithelial lining fluid		Alveolar macrophages	
	[Amox]SERUM	[Clav]SERUM	[Amox]MUC	[Clav]MUC	[Amox]ELF	[Clav]ELF	[Amox]Mo	[Clav]Mo
1	6·50	6·50	3·84	3·26	3·48	8·36	0	4·28
2	8·60	3·65	2·83	1·24	0·85	1·06	3·18	1·03
3	9·80	9·45	2·99	2·99	1·20	2·20	0	2·58
4	9·50	6·50	3·53	1·77	2·75	3·67	1·64	2·74
5	7·20	6·60	3·36	3·73	0·98	2·40	0	5·81
6	6·60	9·95	3·66	3·26	0	0	0	0
7	9·00	5·25	3·57	2·50	0	0	0	0
8	8·20	7·30	2·57	2·38	1·50	0·48	0	2·70
9	9·10	3·40	3·30	1·10	0·93	0·85	0·78	0·49
10	6·90	5·40	2·70	1·20	0·55	0·49	0	0
11	6·00	2·30	2·55	0·89		Lavage unsuitable for processing		
12	6·60	5·20	3·51	1·65	0·98	1·14	0	0
13	1·70	1·75	0·57	0·57	0	0	0	0
14	2·20	3·10	0·05	0·04	0	1·08	0	2·90
15	1·20	0·70	0·77	0·46	0	0	0	0
No.	15	15	15	15	14	14	14	14
Median	6·90	5·25	2·99	1·65	0·89	0·96	0	0·76
Range	8·60	9·25	3·79	3·69	3·48	8·36	3·18	5·81

a 2 mg/l solution of salicylamide (Sigma) was added to provide an internal standard immediately before solid phase extraction. Extraction was carried out using 100 mg (1 ml) C-18 Bond Elut cartridges (Varian), preconditioned with 1 ml methanol, followed by 1·5 ml water. Samples were added to the preconditioned cartridges which were then washed with 1·5 ml water and allowed to dry. The adsorbed compounds (clavulanate derivative and salicylamide) were eluted into glass tubes with 500 µl methanol, dried under a stream of air, and reconstituted in 50 µl HPLC grade water by vortex mixing. 25 µl of the eluate was then injected into a C_{18} Hypersil-ODS (100 × 2·1 mm, 3 µm) reverse phase steel chromatographic column (HPLC Technology Ltd, UK). Samples were read with ultraviolet light at 311 nm using a Kontron 432 detector with an 8 µl flow cell.

In this system the amoxycillin does not interfere with the assay for clavulanic acid. Recovered salicylamide gave a coefficient of variation of 10·0% and clavulanate 12·5%, with satisfactory resolution of the two compounds. The between-run coefficient of variation was 6·8% (at 0·25 and 0·1 mg/l) and the lower limit of sensitivity was 0·0125 mg/l.

Results

Table 1 shows the urea concentrations measured in BAL fluid and serum, assayed concentrations of amoxycillin and clavulanic acid in the lavage fluid (10 × concentrated), and calculated ELF concentrations for each of the patients studied. The BAL fluid aspirated from one patient (no. 11) was unsuitable for analysis so that ELF and alveolar macrophage concentrations of these antibiotics could be determined in only 14 patients.

Table 2 shows concentrations of amoxycillin and clavulanic acid (in mg/l) measured in each lung compartment for each subject.

The calculation of mean values quoted takes account of those subjects in whom antibiotics were undetectable in one or more compartments; in macrophages, for example, amoxycillin levels were not detected in 11 of 14 subjects (79%), while six of 14 subjects (43%) had no detectable clavulanate.

Discussion

Methods for the measurement of total antibiotic concentrations in biopsy specimens are now well established.[13,14] The concentrations of antibiotics at the site of infection may be more informative than serum concentrations[15] – for example, antibiotics may be concentrated in the urine and hence effective in urinary infections even where serum concentrations are so low as to predict clinical failure[16] but renal tissue concentrations have been shown to be good predictors of efficacy in ascending urinary infections.[17,18] Differences between serum and tissue concentrations of antibiotics occur in discrete compartments of the lung (mucosa and submucosa, alveolar macrophages, epithelial lining fluid, and sputum). The alveolar membrane is thus relatively impermeable to antibiotics owing to the presence of many tight junctions (zonulae occludentes)[19–21] and the

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capillary endothelium which separates the submucosa from blood, being non-fenestrated,[22 23] is also less permeable than the fenestrated capillaries of other organs.[24] Measured concentrations in each compartment depend on the rates of penetration and clearance: the lipophilicity, pH, molecular weight, and protein binding of the drug also influence these processes.[25]

In acute bronchitis and acute exacerbations of chronic obstructive airways disease and bronchiectasis bacteria are found within the lumen of the airways, at the mucosal cell surface, and within bronchial mucosal tissue.[26] In pneumonia they are also chiefly intraluminal: the ELF and alveolar macrophages appear to be important locations.[27] These organisms are therefore separated from blood by significant barriers to antibiotic diffusion but, in some cases, antibiotics may be secreted into the bronchial lumen by epithelial cells. Measurement of antibiotic concentrations in sputum is technically difficult – for example, sputum is frequently contaminated by saliva into which antibiotics such as β-lactams penetrate only poorly, or by blood.[28] Bronchial secretions collected by direct aspiration via an endotracheal tube or fibreoptic bronchoscope may have been pooling for some hours, allowing time for degradation by the β-lactamases of commensal bacteria;[29] and concentrations of clavulanic acid, which is unstable at physiological temperatures, are particularly unreliable.[30] Nevertheless the reported concentrations of β-lactams in directly harvested sputum samples are only 5–25% of serum levels.[31] This may increase somewhat in the presence of active inflammation. By contrast, in all β-lactam antibiotics studied to date penetration into the bronchial mucosa is 35–50%[32 33] whether multiple or single dosing is used.

Bronchoalveolar lavage allows the measurement of antibiotic levels in ELF and alveolar macrophages, as well as in bronchial secretions, sputum, and lung tissue.[34 35] For practical purposes the ELF is defined as the fluid that lines the small airways distal to the point of impaction of the tip of the bronchoscope that is recovered by BAL. It is a complex mixture of solutes and anti-inflammatory cells, present in both healthy and infected people, bathing the terminal bronchioles, alveoli, and alveolar macrophages.

According to our data serum antibiotic concentrations show a partial correlation with those in bronchial mucosa, but not with ELF or macrophage levels. There is no reason, as far as we know, to expect a correlation between serum and macrophage concentrations, but in the case of ELF such a relationship might certainly be predicted. However, technical problems in processing BAL fluid cause a significant variation in antibiotic levels, particularly resulting from the movement of solute and solvent across the alveolar-capillary membrane. For example, vigorous suction during lavage promotes the efflux of urea into the aspirate, artificially increasing the estimated volume of ELF and thus depressing the derived drug concentrations; the antibiotics, especially clavulanic acid, are unstable at physiological temperatures. Despite the care that we have taken to standardise the procedures used in this study to keep dwell times to a minimum and to freeze dry all specimens (or store them at −70°C) before assay, we therefore believe that a correlation would always be difficult to demonstrate in ELF and macrophages.

However, the other drugs used during bronchoscopy (atropine, lignocaine and midazolam) are unlikely to have influenced the distribution or activity of the antibiotics. Atropine certainly reduces the volume of bronchial secretions but is not known to have any effect on ELF, while lignocaine, though exerting its pharmacological effects on cell membranes, has not been reported to alter antibiotic uptake by pulmonary tissues.

There is now considerable interest in the importance of intracellular infections in the lung with such pathogens as *Legionella pneumophila*, *Chlamydia pneumoniae*, and atypical mycobacteria, and in the special problems of antimicrobial delivery to these sites of infection. In such infections penetration of macrophages is an important property of antibiotics, but it is not clear whether this penetration occurs in the alveolar lumen, or whether circulating monocyte-derived macrophages acquire the drug before migrating to the alveolar compartment. Beta-lactams have generally been shown to be taken up only poorly by phagocytic cells compared with macrolides and quinolones. If particulate carriers such as liposomes and polyisohexylcyanoacrylate nanoparticles are used to transport β-lactam agents into macrophages, their bactericidal action is significantly enhanced: thus nanoparticle-bound ampicillin is more effective than free ampicillin against *Listeria monocytogenes* in mouse peritoneal macrophages.[36] In infections with *Legionella pneumophila* clavulanate and β-lactam-clavulanate combinations are both bactericidal in vitro and in animal models.[37] The present study indicates that, at least in some patients, clavulanate is taken up to a significant degree by bronchial mucosa, ELF, and macrophages, and exceeds quoted MIC_{90} values (around 0·25 mg/l) for *Legionella pneumophila*[38 39] in these compartments. Its results are therefore consistent with those of the animal experiments mentioned above.

Interest in the treatment of *Mycobacterium tuberculosis* infection with β-lactam antibiotics has also been aroused recently by the discovery of an inducible, clavulanate-sensitive β-lactamase produced by this bacterium, active against penicillins and cephalosporins, and of a synergistic bactericidal effect of penicillin-clavulanic acid combinations against *M tuberculosis*.[40]

A potential clinical role for amoxycillin-clavulanate in a variety of infections with intracellular pathogens is suggested. Further clinical studies to evaluate such a role may be warranted.

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