Use of an Enzyme-Linked Immunosorbent Assay To Assess Penetration of Amoxicillin into Lung Secretions

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An enzyme-linked immunosorbent assay (ELISA) was developed to measure total amoxicillin concentrations penetrating lung secretions, which were compared with “active” concentrations measured by conventional bioassay. An antibody was raised in rabbits to amoxicillin conjugated to bovine serum albumin and used in a competitive binding ELISA (sensitivity, 10 ng/ml; precision [coefficient of variation], 9%). The measurement of amoxicillin in lung secretions by using the ELISA method was verified by high-performance liquid chromatography. Amoxicillin concentrations were found to be similar in both whole sonicated sputum and sol-phase sputum obtained by ultracentrifugation following single oral doses of 3 g (4.6 mg/liter for sonicated and 4.7 mg/liter for sol-phase preparations) and 250 mg (0.23 mg/liter for both preparations). Eight patients with bronchiectasis received 500 mg of amoxicillin three times daily. On the second day of therapy (4 h after the morning dose), the mean concentration of amoxicillin in sputum was 0.88 mg/liter (standard error of the mean [SEM], 0.11) by ELISA and 0.40 mg/liter (SEM, 0.05) by bioassay, suggesting a significant degree of local inactivation. This difference between total and active amoxicillin levels was found to correlate significantly (r = 0.693; P < 0.05) with β-lactamase levels (mean, 29.5 mU/ml; SEM, 9.4). A pharmacokinetic study on day 3 revealed maximum levels in secretions 2 to 4 h after dosing (mean, 1.36 mg/liter; SEM, 0.26). At the end of successful therapy (day 14), total and active levels were lower (mean, 0.48 mg/liter; SEM, 0.11 [total]; mean, 0.21 mg/liter; SEM, 0.06 [active]); this result was associated with a reduction in lung inflammation (decreased serum-derived albumin in the lung secretions). In conclusion, antibiotic penetration is partly dependent on the degree of lung inflammation. The differences observed in total and active levels of amoxicillin and the relationship to β-lactamase activity in sputum suggest why higher doses of antibiotic may be required to produce a therapeutic response in some patients.

Conventional doses of β-lactam antibiotics often fail to elicit a clinical response in patients with chronic bronchial sepsis associated with the chronic lung disease bronchiectasis (2, 10, 13, 20). Recent studies have shown that these patients require higher doses of oral β-lactam antibiotics or even direct airway delivery of the drug to clear their purulent secretions (3, 4, 5, 10, 29), suggesting that factors governing local antibiotic concentrations are critical in determining the clinical efficacy.

In order for antibiotic therapy to be effective, the concentrations of the antibiotic in lung secretions should, theoretically, exceed the MIC or MBC for the colonizing bacteria (19, 23). Conventional doses of antibiotics used in the treatment of patients with chronic bronchial sepsis associated with bronchiectasis frequently fail to achieve these concentrations (2, 3). Effective antibiotic concentrations in lung secretions are dependent on several factors, including the degree of tissue penetration (23) and, in the case of β-lactam antibiotics, inactivation by β-lactamases (22). For measurement of antibiotic concentrations, the conventional bioassay measures active drug alone (31). Therefore, although such assays may indicate that antibiotic levels within lung secretions have not achieved the MIC or MBC for identified pathogens, bioassays cannot establish whether this is due to poor penetration of the drug into the affected airways or its inactivation by β-lactamases. Consequently, the reasons for success or failure of antibiotic treatment often remain uncertain.

The present study was designed to develop an immunobioassay to measure total amoxicillin and to verify the results by using conventional high-performance liquid chromatography (HPLC). Following the development of the immunoassay, amoxicillin penetration into lung secretions was assessed and the results were compared with measurements by bioassay to estimate the amount of inactive amoxicillin present. In addition, the β-lactamase activity of the sputum was determined in order to assess the relationship of inactive antibiotic concentrations to the activity of this enzyme in the lung secretions.

MATERIALS AND METHODS
Preparation of antiserum to amoxicillin. (i) Preparation of alpha-amino-linked amoxicillin to bovine serum albumin (BSA). The method was based on that described previously for ampicillin (12).

(a) Acylation of amoxicillin. Ten milliliters of 0.05 mol of phosphate buffer, pH 7.0, per liter containing 150 μmol (58 mg) of sodium amoxicillin (SmithKline Beecham Research Laboratories) was added to 150 μmol (47 mg) of N-[maleimidobenzoxyloxy]-succinimide (MBS; Pierce Chemicals). The mixture was incubated for 3 h at 37°C and then freeze-dried overnight. The solid product was washed five times with a 2:1 (vol/vol) mixture of diethyl ether and dichloromethane (Fisons), both of which had been dried over a molecular sieve. The solid was then placed in a vacuum.
Absence of desiccator over phosphorus pentoxide (Fisons) for 4 h. Absence of free MBS was confirmed by silica plate thin-layer chromatography run in 1 vol of methanol–9 vol of dichloromethane (Fisons).

(b) Reductive cleavage of BSA disulfide bonds. BSA (1.5 μmol [100 mg]) (Sigma Chemical Co.) was dissolved in 5 ml of 0.2 mol of Tris-HCl buffer, pH 8.6, per liter containing 2.4 g of 8 mol of urea (BDH Ltd.) per liter. To this solution a 100 M excess (21 mg) of dithiothreitol (Sigma Chemical Co.) was added. The mixture was stirred for 2 h at 20°C. The reduced BSA was precipitated by adding 5 ml of 5% trichloroacetic acid (BDH Ltd.). The suspension was centrifuged (400 × g), the supernatant was discarded, and the precipitated BSA was washed with distilled water and resuspended in 5 ml of 0.05 mol of phosphate buffer, pH 7.0, per liter containing 8 mol of urea per liter.

(c) Conjugation of acetylated amoxicillin to reduced BSA. The solution of reduced BSA was added to the acetylated amoxicillin, and the mixture was stirred continually for 2 h at 20°C. The resultant suspension was dialyzed for 24 h in visking tubing (1/32 to 8/32 in. [1 in. = 2.54 cm]; Meditel) with four changes of 10 liters of distilled water. The dialyzed suspension was removed from the tubing and centrifuged at 400 × g to obtain the soluble fraction, which was freeze-dried. Approximately 3 mg of the preparation was tested by infrared scanning (Perkin-Elmer 298) in order to confirm the presence of an intact β-lactam ring in the conjugated amoxicillin.

(ii) Preparation of rabbit antiserum to amoxicillin-BSA conjugate. New Zealand White rabbits were used for the preparation of antiserum. On each occasion, the rabbits were immunized with 1 mg of the conjugate dissolved in 0.1 ml of sterile water and emulsified in 1 ml of Freund's complete adjuvant (supplied by Immunodiagnostic Research Laboratories, University of Birmingham, Birmingham, United Kingdom). The first immunization consisted of two intramuscular (i.m.) and one subcutaneous (s.c.) injection. The rabbits were immunized again at 2 weeks (three s.c. injections), 1 month (one i.m. and one s.c. injection), 3 months (three s.c. injections), 4 months (one i.m. and one s.c. injection), and 6 months (three i.m. injections). Trial blood samples were taken at regular intervals during the immunization schedule and tested by Ouchterlony immunodiffusion and enzyme-linked immunosorbent assay (ELISA) (diffusion by the method of Cieplak and Grabarczyk). Test blood samples showing suitable titers of amoxicillin (as determined by ELISA) were pooled and the immunoglobulin fraction was precipitated with ammonium sulfate (final concentration, 30% saturated). The precipitate was redissolved in phosphate-buffered saline (PBS) and dialyzed against PBS (4°C) to remove residual ammonium sulfate. The final concentration of immunoglobulin was 5.6 mg/ml.

ELISA for amoxicillin. An indirect (inhibition) ELISA was developed for the measurement of amoxicillin and was performed as follows.

(i) General procedure. Microtiter plate (Nunc Immunoplate-Maxisorb; Inter Med) wells were each filled with 200 μl of a 100 mg/liter solution of sodium amoxicillin dissolved in 0.05 mol of sodium carbonate-sodium bicarbonate buffer, pH 9.6, per liter and incubated at room temperature for 2 h. The microtiter plate wells were washed three times with PBS containing 1% (wt/vol) BSA and 0.1% (vol/vol) Tween 20 (PBS-BSA-T). Samples containing amoxicillin were diluted appropriately with PBS-BSA-T and mixed with an equal volume of rabbit antiamoxicillin diluted 1/400 (giving a final dilution of 1/800) in PBS-BSA-T. The mixtures of sample and antibody (200 μl) were applied immediately to the coated microtiter wells and incubated at 4°C for 1.5 h. The plates were calibrated with a series of concentrations of sodium amoxicillin dissolved in PBS-BSA-T. Blanks consisted of wells which were coated but from which the antigen-antibody stage was omitted. Maximum (100%) binding of antibody was determined by adding antibody alone to coated wells. For control wells, nonimmune rabbit serum was substituted for the antibody.

After incubation, the plates were washed three times with PBS-BSA-T, and 200 μl of a 1/800 dilution of sheep anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (supplied by the Immunodiagnostic Research Laboratories, University of Birmingham) was applied to each well and incubated at 4°C for 2 h. The plates were again washed three times with PBS-BSA-T and filled with 200 μl of the peroxidase substrate, 10 mg of o-phenylenediamine hydrochloride (Sigma Chemical Co.) dissolved in 20 ml of 0.05 mol of citrate-phosphate buffer, pH 5.0, per liter to which was added 20 μl of hydrogen peroxide (BDH Ltd.). The color development was monitored with a Titertek Unispec reader, and, when appropriate, the reaction was stopped by the addition of 50 μl of 0.5 mol of citric acid per liter. The A492 of each well was measured.

The percent inhibition of binding of rabbit antiamoxicillin to the coated plates by each antigen sample was calculated with reference to the 100% binding wells (no antigen). A calibration line was constructed by using the results from the standard dilutions of amoxicillin by plotting percent binding against the log10 of known amoxicillin concentrations. The concentration of amoxicillin in each test sample was determined by interpolation from the linear portion of the calibration line and corrected for any dilution that had been made.

(ii) Precision of assay. The precision of the assay (within batch) was calculated by measuring two concentrations of amoxicillin (50 and 200 ng/ml) dissolved in PBS-BSA-T 12 times on one occasion.

(iii) Cross-reactive. Sodium amoxicillin, temocillin (Smith-Kline Beecham), and Kefamandol (Dista Products Ltd.) were diluted separately in PBS-BSA-T to concentrations ranging from 100 to 1,000 ng/ml. Amoxicillic acid (supplied by SmithKline Beecham Research Laboratories) was diluted in PBS-BSA-T to give final concentrations of 50 to 500 ng/ml. The samples were then assayed immediately by ELISA, with reference to a calibration line constructed by the measurement of sodium amoxicillin diluted in PBS-BSA-T.

In addition, amoxicillin (100 ng/ml) was diluted in PBS-BSA-T containing β-lactamase (Bacillus cereus type I, Sigma Chemical Co.) at concentrations of 0.00025 to 2.5 U/ml. The amoxicillin β-lactamase mixtures were incubated for 15 min at room temperature, and the concentrations of antibiotic were measured by ELISA by using sodium amoxicillin diluted in PBS-BSA-T (no β-lactamase) to construct a calibration line.

(iv) Measurement of amoxicillin in sputum samples. Sodium amoxicillin was added to a pool of either mucoid or purulent sputa to give final concentrations of between 25 and 250 ng/ml. The amoxicillin concentrations were measured immediately by ELISA, with reference to a calibration line constructed from dilutions of sodium amoxicillin in PBS-BSA-T.

From these experiments, it became apparent that amoxicillin concentrations in sputa were overestimated by the ELISA (see Results). In order to overcome this problem, the following procedure was employed.
Each sputum sample and the standard dilutions of amoxicillin in PBS-BSA-T were preincubated with 0.25 U of β-lactamase per ml for 15 min before addition to the microtiter wells. Bioassays were performed to ensure complete conversion of all amoxicillin present to the inactive form. All sputum specimens were assayed in duplicate; to one of these duplicate samples, 50 ng of amoxicillin per ml was added. After the concentrations of amoxicillin in each duplicate well had been measured, the value obtained from the native sample was deducted from that to which 50 ng of amoxicillin per ml had been added. The differences in these values represented the measurement contributed by the additional 50 ng of antibiotic per ml. The value was compared with that of the 50-ng/ml standard dilution of amoxicillin and the degree of overestimation in the sputum was calculated. This index of overestimation was used to correct the measurement of amoxicillin in the native sample to obtain the true value. The accuracy of the method was confirmed by dissolving amoxicillin in mucoid or purulent sol-phase sputum (each diluted 1/20 or 1/40) to give a final concentration of 50 ng of amoxicillin per ml in each sample. The spiked sputum samples were assayed for the presence of amoxicillin by using the modified ELISA described above and by an HPLC method based on that of Lovering et al. (17).

In brief, chromatography was performed with a model M45 pump, a model 481 detector (Waters Associates, Harrow, United Kingdom), a Gilson model 231 autoinjector (Anachem Ltd., Luton, United Kingdom), a computing integrator (Vinton Instruments, Sandy, United Kingdom), and a Hypersil 5 ODS column (150 by 4 mm; HPLC Technology, Macclesfield, United Kingdom). The mobile phase was pumped at a flow rate of 2 ml/min and was composed of methanol, water, and phosphoric acid (25:74:1) with the addition of 2 g of octane sulphonic acid per liter and with the pH adjusted to 4.5. Detection was by UV A241 with quantitation by the external standard method. Sputum samples were diluted with an equal volume of ice-cold perchloric acid (3.3%) stored at 4°C for 3 min while protein precipitation occurred and then were centrifuged at 5,000 × g for 5 min. A 100-μl volume of the resultant supernatant was then injected immediately into the chromatograph. In a previous study using this method, approximately 100% of known concentrations of amoxicillin were recovered in spiked sputum samples (17) and the coefficient of variation of intrasay precision was shown to be in the range of 3 to 6%.

The amoxicillin concentrations measured by ELISA and HPLC were compared and the correlation with target concentrations was obtained.

Measurement of amoxicillin in sputa from patients receiving oral antibiotic treatment. (i) Partitioning of amoxicillin in sputum samples. Two patients with bronchiectasis, both producing purulent secretions, were studied. One patient received 250 mg of amoxicillin orally and the other received 3 g of amoxicillin orally, as a single dose. Both patients performed their usual postural drainage routine prior to receiving the antibiotic. Two hours after taking the antibiotic, each patient expectorated a sputum sample which was divided into two aliquots. One aliquot of each sample was centrifuged at 50,000 × g (4°C) for 90 min to obtain the sol phase. The second aliquot was liquefied by sonication with high frequency (22-μ amplitude) for 12 30-s bursts (MSE Soniprep 150).

The amoxicillin concentrations in the sol-phase and whole sonicated samples were measured by the ELISA by using the modified procedure detailed above for sputum samples. (ii) Amoxicillin and β-lactamase in sputum. Eight patients with bronchiectasis who produced secretions which fluctuated between mucopurulent and purulent, assessed as described previously (10), received 500 mg of amoxicillin orally three times daily for 14 days. After a 1-day lead-in, sputum was collected for 4 h after the morning dose on the second day and the sol phase was obtained. In addition, a 10-ml venous blood sample was taken at the end of the sputum collection period and the serum was obtained. Further sputum and blood samples were obtained on the 14th day of treatment, again following the morning dose. On both study occasions, patients performed their usual postural drainage routines before taking the antibiotic. The following investigations were performed.

(a) Albumin concentrations in sputum and serum. The albumin concentrations in the sputum and paired serum samples were measured by rocket immunoelectrophoresis and expressed as a ratio as previously described (29). Since albumin enters the secretions by passive diffusion, this concentration ratio provides a guide to protein transudation into the lung and can therefore be used to determine the degree of lung inflammation (27). Differences in ratios of albumin in sputum and serum on the 2nd and 14th days of therapy were tested by the Wilcoxon test for paired data.

(b) β-Lactamase activity in sputum. The β-lactamase activity in each sputum sample was measured by using the chromogenic substrate nitrocefin as described previously (8). Briefly, 200 μg of nitrocefin (supplied by SmithKline Beecham Research Laboratories) per ml was dissolved in 0.05 mol of phosphate buffer, pH 7.2, per liter containing 0.1% (vol/vol) Triton X-100 (Fisons). A volume of 100 μl of the substrate solution was pipetted into microtitrator plate wells, and to this was added 100 μl of each sputum sample. The mixtures were incubated for 2 h at 37°C. Standard solutions for calibration purposes were prepared from dilutions of β-lactamase (Bacillus cereus type I, obtained from Sigma Chemical Co.). The presence of β-lactamase activity was associated with a color change of nitrocefin substrate from yellow to red, which was measured by spectrophotometry at 492 nm with a Titertek Uniscan reader. The concentrations of β-lactamase activity in test samples (sputum) were calculated by interpolation from a calibration line derived from values obtained with the standard solutions of β-lactamase.

(c) Amoxicillin measurements. Concentrations of amoxicillin in sputum were measured by using the modified ELISA and also by conventional bioassay (31) by using the test organism Sarcina lutea NCTC 8340.

The amoxicillin concentrations measured by ELISA and bioassay were compared and the differences in values obtained were related to the β-lactamase activity in each sputum sample, by using Kendall's Tau correlation for non-parametric data. Changes in total (ELISA) and active (bioassay) amoxicillin concentrations in sputum from the 2nd and 14th days of therapy were tested by Student's t test for paired data.

(iii) Pharmacokinetic study. A pharmacokinetic study was performed on the third day of therapy for the eight patients described above. All subjects performed postural drainage (under the supervision of a physiotherapist), and a sputum sample was collected before the morning dose (oral) of 500 mg of amoxicillin was taken. Each subject collected all spuata expectorated at consecutive 2-h intervals for 6 h after taking...
the antibiotic. The amoxicillin concentrations in the sputum samples were measured by ELISA.

RESULTS

Preparation of antiserum to amoxicillin. The yield of BSA-conjugated amoxicillin was 25 mg. Infrared scanning of the material revealed a peak at 1,780 cm, confirming that the amoxicillin β-lactam ring was intact.

Ouchterlony immunodiffusion confirmed the presence of antiserum to the BSA-amoxicillin conjugate. No immunoprecipitate was seen with unconjugated amoxicillin. The indirect ELISA, however (described above), did detect the amoxicillin activity and was used to monitor antibody titers during the immunization schedule.

ELISA for amoxicillin measurement. (i) Precision of assay. Figure 1 shows a calibration line obtained with the indirect ELISA by using dilutions of sodium amoxicillin in PBS-BSA-T. The assay was linear for concentrations of amoxicillin between 10 and 500 ng/ml. The precision of the assay (within batch coefficient of variation) was 10% at 50 and 200 ng/ml (n = 12).

(ii) Cross-reactivity. The antibody did react with another penicillin β-lactam antibiotic, temocillin, but the cross-reactivity to this compound was approximately 50% (wt/wt) (Table 1). The assay also detected the cephalosporin Kefamandol, but the cross-reactivity was very low (Table 1), indicating poor recognition by the antiserum.

Amoxylloic acid was detected by the assay, but with a high degree of overestimation (Table 2). This effect was also demonstrated by the addition of β-lactamase to sodium amoxicillin at concentrations in excess of $2.5 \times 10^{-4}$ U/ml (Table 2).

(iii) Measurement of amoxicillin in sputum samples. Table 3 shows the results of measuring amoxicillin that had been diluted in mucoid or purulent sputum. The concentrations of amoxicillin diluted in mucoid sputum were overestimated.

The degree of overestimation of amoxicillin diluted in purulent sputum was on average even greater, being approximately three times the expected value.

Since the overestimation was caused, in part, by the conversion of amoxicillin to amoxylloic acid by β-lactamases present in sputum, all samples and reference solutions for calibration were preincubated with β-lactamase. This ensured complete conversion of all amoxicillin to amoxylloic acid and was confirmed by the absence of active antibiotic zones on bioassay. Nevertheless, measurements of amoxicillin in sputum continued to result in an overestimation. The cause was not identified. It was not attributable to cross-

TABLE 1. Estimations of various concentrations of amoxicillin, temocillin, and Kefamandol by using the amoxicillin antibody

<table>
<thead>
<tr>
<th>Amt of drug added to serum (ng/ml)</th>
<th>Concentration by ELISA (ng/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td></td>
</tr>
<tr>
<td>1,000..............................</td>
<td>980</td>
</tr>
<tr>
<td>500..................................</td>
<td>490</td>
</tr>
<tr>
<td>250..................................</td>
<td>220</td>
</tr>
<tr>
<td>100..................................</td>
<td>70</td>
</tr>
<tr>
<td>Temocillin</td>
<td></td>
</tr>
<tr>
<td>1,000..............................</td>
<td>520</td>
</tr>
<tr>
<td>500..................................</td>
<td>310</td>
</tr>
<tr>
<td>250..................................</td>
<td>150</td>
</tr>
<tr>
<td>100..................................</td>
<td>74</td>
</tr>
<tr>
<td>Kefamandol</td>
<td></td>
</tr>
<tr>
<td>1,000..............................</td>
<td>58</td>
</tr>
<tr>
<td>500..................................</td>
<td>15</td>
</tr>
<tr>
<td>250..................................</td>
<td>4</td>
</tr>
<tr>
<td>100..................................</td>
<td>Not detectable</td>
</tr>
</tbody>
</table>

* Concentrations were measured by ELISA with reference to a calibration line constructed from amoxicillin diluted in PBS-BSA-T.
TABLE 2. Estimation of concentrations of amoxylloic acid and amoxicillin treated with β-lactamase, as shown by ELISA

<table>
<thead>
<tr>
<th>Substance and amount</th>
<th>Concentration by ELISA* (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxylloic acid added to PBS-BSA-T (ng/ml)</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>740</td>
</tr>
<tr>
<td>250</td>
<td>420</td>
</tr>
<tr>
<td>100</td>
<td>260</td>
</tr>
<tr>
<td>50</td>
<td>120</td>
</tr>
<tr>
<td>β-lactamase (U/ml) added to 100 ng of amoxicillin per ml</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>275</td>
</tr>
<tr>
<td>1.25</td>
<td>280</td>
</tr>
<tr>
<td>0.625</td>
<td>270</td>
</tr>
<tr>
<td>0.25</td>
<td>260</td>
</tr>
<tr>
<td>0.025</td>
<td>220</td>
</tr>
<tr>
<td>0.00025</td>
<td>130</td>
</tr>
</tbody>
</table>

* Determined with reference to a calibration line constructed by diluting sodium amoxicillin in PBS-BSA-T.

reactivity with sputum components since no signal was obtained from control wells containing sputum with no amoxicillin present, nor was it attributable to the presence of serine proteinases, mucopolysaccharides, or large quantities of DNA (results not shown).

Table 4 shows the results of measuring amoxicillin in mucoid and purulent sputa with correction for overestimation determined by the addition of 50 ng of standard amoxicillin solution per ml as described in Materials and Methods. The results confirmed that the concentrations of amoxicillin in sputum samples were overestimated and related to the degree of sputum purulence. The addition of 50 ng of amoxicillin per ml allowed the degree of overestimation to be determined and hence confirmed that the original concentration of amoxicillin in the sputum sample to which amoxicillin had been added could be assessed accurately.

(iv) HPLC verification of modified ELISA for the measurement of amoxicillin in lung secretions. Figure 2 shows the results of measuring known concentrations of amoxicillin diluted in mucopurulent sol-phase sputum by ELISA and HPLC. Similar results were obtained by both methods, which gave correlation coefficients of 0.981 (HPLC) and 0.991 (ELISA) compared with target concentrations.

TABLE 3. Concentrations of amoxicillin in sputa from patients receiving oral antibiotic treatment. (i) Partitioning of amoxicillin in sputum. Following a single oral dose of 250 mg of amoxicillin, there was no difference between the amoxicillin concentrations measured by ELISA in the sol-phase and the whole sonicated preparations of the sputum sample (0.23 mg/liter). This was similar to the result following oral administration of 3 g of amoxicillin, for which the values were 4.6 mg/liter for the sonicated preparation and 4.7 mg/liter for the sol-phase preparation.

(ii) Amoxicillin and β-lactamase in sputum. On the second day of therapy, the mean amoxicillin concentrations measured by ELISA and bioassay in the sputum samples were 0.88 mg/liter (standard error of the mean [SEM], 0.11) and 0.40 mg/liter (SEM, 0.05), respectively. By the 14th day of therapy, these concentrations had fallen significantly to 0.48 mg/liter (SEM, 0.11) by ELISA (P < 0.05) and 0.21 mg/liter (SEM, 0.06) by bioassay (P < 0.001). These results are shown graphically in Fig. 3.

β-Lactamase activity was present in all but one of the sputum samples on the second day of treatment (mean, 29.5 mU/liter; SEM, 9.4). By the 14th day of treatment, the average levels of activity had fallen to 21.6 mU/liter (SEM, 2.7), although this change failed to reach statistical significance.

The differences between the ELISA and bioassay results (expressed as percent inactive amoxicillin) for the results on

TABLE 4. Results of experiments to determine the accuracy of measuring a known quantity of amoxicillin in sputum by modified ELISA

<table>
<thead>
<tr>
<th>Sputum sample</th>
<th>Native (A)</th>
<th>Native + 50 ng/ml (B)</th>
<th>Difference (B−A)</th>
<th>Correction factor</th>
<th>Corrected value of native</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucoid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/20 dilution</td>
<td>94</td>
<td>200</td>
<td>105</td>
<td>0.48</td>
<td>45.2</td>
</tr>
<tr>
<td>1/40 dilution</td>
<td>74</td>
<td>140</td>
<td>66</td>
<td>0.75</td>
<td>56.1</td>
</tr>
<tr>
<td>Purulent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/20 dilution</td>
<td>380</td>
<td>800</td>
<td>420</td>
<td>0.119</td>
<td>45.2</td>
</tr>
<tr>
<td>1/40 dilution</td>
<td>260</td>
<td>500</td>
<td>240</td>
<td>0.208</td>
<td>54.2</td>
</tr>
</tbody>
</table>

* Amoxicillin (50 ng/ml) was added to a native sample which already contained 50 ng/ml. The results show the values obtained in native samples (A) and those to which an additional 50 ng of amoxicillin per ml was added (B). The difference between these values was calculated, and this difference represented the measured value of the additional 50 ng/ml. A correction factor was obtained for B−A in order to obtain the original value for B (50/B−A). This correction factor was then applied to A to obtain a final result for the original sample.

FIG. 2. Assay by HPLC and ELISA of mucopurulent sputum sol phase spiked with known concentrations of amoxicillin.
the second day of treatment correlated with the β-lactamase activities of the sputum samples ($r = 0.693; P < 0.05$). The results are summarized in Fig. 4.

The mean ratio of albumin in sputum and serum on the second day of treatment for all the patients was $3.15 \times 10^{-2}$ (SEM, 1.1), and although this value fell by day 14 of treatment to $1.66 \times 10^{-2}$ (SEM, 0.8), this did not represent a significant reduction ($P < 0.1 > 0.05$).

Of the eight patients treated, six showed a macroscopic response to treatment (with their sputa becoming mucoid). The results for amoxicillin concentrations, β-lactamase activity, and ratios of albumin and sputum and serum are shown in Table 5. The amoxicillin concentrations on day 14 for these six subjects were significantly lower than those on day 2 (for ELISA, $P < 0.05$; for bioassay, $P < 0.001$), and the ratios of albumin in sputum and serum were also significantly reduced ($P < 0.05$).

### DISCUSSION

This article describes the development of an ELISA to measure total concentrations of amoxicillin in sputa of patients with bronchiectasis. Amoxicillin is a small molecule and under normal circumstances is a poor antigen. Khagawa and colleagues (12) described a method for conjugating ampicillin to BSA by using the bifunctional reagent $N$-$\text{maleimidobenzoyloxy}$-succinamide. The method proved to be successful in producing a BSA-amoxicillin conjugate in which the antibiotic β-lactam ring was shown to remain intact by infrared absorption spectrophotometry. The antibody cross-reacted with another β-lactam penicillin, temocillin, but only weakly with a cephalosporin; therefore, it had properties similar to those of the ampicillin antibody (12). Unlike the antibody of Khagawa and colleagues (12), however, the current antibody did show strong recognition of amoxylic acid. This form of the antibiotic was overestimated by the ELISA. Since amoxicillin in sputum may be at least partially hydrolyzed by β-lactamase produced by bacteria present in the airways (35), it was necessary to ensure that all of the antibiotic present in the samples was fully converted to amoxylic acid (confirmed by lack of active antibiotic zones on bioassay) by the addition of β-lactamase before measurement and that all standard solutions used for calibration were treated identically. Despite this modification, the measurement of the antibiotic levels in sputum samples continued to be overestimated by the ELISA. The reasons for this overestimation remain unknown, although the overestimation was related to sputum purulence. The addition of a serine proteinase inhibitor to sputum did not reduce the overestimation, suggesting that the overestimation did not result from proteolysis of the amoxicillin or the antiserum by serine proteinases in the sputum samples (results not shown). In addition, although cephalosporins are...
Sputum has been shown to contain large quantities of DNA (16) presumably derived from bacteria and damaged lung cells. The enzyme DNase was therefore added to the sputum samples in order to determine whether high levels of DNA were interfering with the antigen-antibody reaction, but no effect was observed (results not shown). Similarly, no effect was observed when N-acetyl-glucosamine, the terminal carbohydrate residue of mucopolysaccharides, was added to amoxicillin solutions. It nevertheless remains possible that other carbohydrates present in sputum may have interfered with the ELISA measurements of amoxicillin.

Since no cause for sputum amoxicillin ELISA overestimation could be identified, the method of adding a known quantity of amoxicillin to all samples was used throughout to estimate and correct for the degree of interference. This technique resulted in a reproducible assay of amoxicillin in lung secretions, which was verified by HPLC. In comparison to the HPLC technique, the ELISA is quick and easy to perform and has a much lower limit of detection.

No significant differences were observed in the amoxicillin concentrations measured in sputum that had been liquefied by sonication or the sol phase obtained by centrifugation. The results showed, therefore, that all the amoxicillin found in the secretions was reflected in the sol-phase measurement and that centrifugation to obtain the sol phase is an appropriate treatment of sputum for measurement of this antibiotic.

The average amoxicillin concentrations, measured by bioassay, in sputum samples collected from eight patients over the first 4 h following oral administration of 500 mg of amoxicillin on the second day of therapy was 0.4 mg/liter (range, 0.21 to 0.62). This value is in agreement with other studies of patients with chronic bronchitis (21, 26) and with a recent study performed with bronchiectatic patients (13) but is higher than that reported by Cole (2).

Amoxicillin concentrations, measured by ELISA, in sputum collected on the second day of treatment represent average values for the concentrations in the 4 h after patients received the drug. The collection of sputum at 2-h intervals during the pharmacokinetic study on day 3 of treatment showed that the peak concentration (mean, 1.4 mg/liter) was achieved in the period 2 to 4 h after administration of the drug. The levels at 4 to 6 h were lower than those seen in the first 2 h. The finding that peak concentrations in sputum were achieved at 2 to 4 h is similar to those of previous studies (7, 15, 17). Ingold, however (11), reported peak concentrations in sputum 4 to 6 h after a single dose of amoxicillin in a mixed group of subjects with bronchiectasis or chronic bronchitis who were producing purulent sputum. The reasons for the difference between results obtained by Ingold (11) and those of the other studies, including those described here, are not clear since the procedures used in all these investigations were similar, with the exception of the study by Lovering and colleagues (17), in which the peak levels were observed following intravenous administration of the dose. The sputum pharmacokinetic data suggest that the delay in amoxicillin penetration into the lung secretions is about 0.5 to 2.5 h, since previous studies report that the peak level in serum in healthy fasting subjects is achieved 1.5 h after patients receive the drug (25, 33). The predose sputum collection was made 8 h after the previous dose of amoxicillin. The predose samples contained, on average, 0.57 mg of the antibiotic per liter (measured by ELISA), indicating a half-life of amoxicillin in the lung secretions of about 4 h, which is longer than that in serum (34). The decrease in antibiotic concentrations in the lung secretions after the peak levels is partly due to losses in expectorated sputum. Previous studies have shown that 4 h after dosing, the antibiotic concentrations in the lung are higher than that in the serum (1). The decrease in amoxicillin levels in sputum could also therefore be affected by reabsorption into the blood as levels in plasma decrease (6, 18, 30).

The sputum from a subject receiving 3 g of amoxicillin contained higher concentrations of the antibiotic measured by immunoassay (4.7 mg/liter) than those of patients taking 500 mg, whereas the sputum from the subject receiving 250 mg contained only 0.23 mg/ml. These results suggest that the penetration of antibiotic into the lung secretions is related to dose. This is consistent with the observations of other workers for patients with chronic bronchitis (11, 21) and bronchiectasis (2, 3).

The amoxicillin concentrations in sputum, measured both by ELISA and by bioassay, in the patients receiving 500 mg of amoxicillin three times daily were significantly lower after 14 days of treatment than at the start of therapy. This was associated with successful response to treatment (decreased sputum purulence and decreased ratios of albumin in sputum and serum) in six of the subjects. The results suggest that amoxicillin penetration is related to the permeability of the blood-lung barrier to solutes, with the concentrations in lung secretions decreasing as lung inflammation is reduced and the secretions become less purulent. Some previous studies have also reported a relationship between sputum purulence and amoxicillin penetration (9, 14, 26), although others failed to confirm this observation (11, 20, 21, 24). The reasons for this disparity are uncertain.

The immunological measurements of amoxicillin by ELISA were consistently higher than those obtained by bioassay. Whereas the ELISA measured total antibiotic, the bioassay measured only that which had not been inactivated by β-lactamases. This concept is supported by the observation that the differences between ELISA and bioassay results were related to β-lactamase activity in sputum. Indeed, all but one of the sputum samples contained detectable β-lactamase activity. The results are consistent with those of May and Ingold (20), who found only low levels of active amoxicillin in sputum containing β-lactamase activity. The regression line relating our inactive amoxicillin concentrations to β-lactamase activity did not pass through the origin but intercepted the ordinate. This indicates that a proportion of inactive amoxicillin was not attributable to β-lactamase activity. This may indicate the amount of protein-bound antibiotic which is not detected by bioassay (31).

In conclusion, an immunoassay was developed for the measurement of amoxicillin in sputum, which was verified by HPLC analysis. The ELISA, however, was much easier and quicker to perform than HPLC analysis and had a much greater sensitivity. By using the ELISA, it was shown that the penetration of total drug following oral administration of 500 mg of amoxicillin was greater than active levels determined by bioassay, indicating inactivation of the antibiotic, probably by β-lactamases in the secretions. In addition, amoxicillin penetration into lung secretions appeared to be related to lung inflammation as indicated by sputum purulence and the transudation of albumin from the plasma into the lung secretions.
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