Assay of Amoxicillin and Clavulanic Acid, the Components of Augmentin, in Biological Fluids with High-Performance Liquid Chromatography

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Augmentin is a new antibacterial formulation comprised of amoxicillin and the β-lactamase inhibitor clavulanic acid. In the present paper, the use of high-performance liquid chromatography (HPLC) to provide a rapid assay of the components of Augmentin in body fluids is described. Clavulanic acid was assayed by reacting the sample with imidazole, which readily produces a derivative absorbing at 311 nm. This derivative chromatographs on reverse-phase HPLC columns clear of interfering components in both human serum and urine. Concentrations of clavulanic acid as low as 0.1 μg/ml were readily detectable in human serum with this procedure. There was no interference from amoxicillin, amoxicillin penicilloic acid, or the acid and alkali degradation products of clavulanic acid when this assay system was used. Amoxicillin in body fluids was assayed directly by HPLC without derivatization. The same chromatographic conditions were employed for the assay of amoxicillin and the clavulanic acid derivative, simplifying the methodology. Amoxicillin, however, was determined by monitoring at 227 nm, and the limits of detection in human serum were 0.5 μg of the antibiotic per ml. An alkali blanking procedure for amoxicillin and clavulanic acid is also described which allows the detection of any underlying peaks which may cochromatograph. The use of ultrafiltration to remove protein from serum samples before HPLC was successfully applied to the assay of clavulanic acid and amoxicillin. Ultrafiltration is not an essential procedure for these assays, but it prolongs column life and reduces interference in the amoxicillin assay. Results obtained by HPLC were compared with those obtained by using microbiological assays.

Clavulanic acid is a novel β-lactam compound which was isolated from the culture fluid of Streptomyces clavuligerus (8). The compound is a potent inhibitor of a large number of β-lactamase enzymes which are responsible for the resistance of many bacteria to β-lactam antibiotics. In the presence of clavulanic acid, β-lactamase-labile penicillins are protected from degradation by cell-free β-lactamase preparations (8) and by whole bacterial cultures (7). One such penicillin-clavulanic acid formulation is Augmentin, which is comprised of amoxicillin and clavulanic acid (Fig. 1) and which has been reported (1, 5) to give good results in clinical use.

Clavulanic acid may be detected by using a special microbiological assay method (2) which has been adapted for routine assay purposes (1). It may also be determined biochemically by using its enzyme inhibitory properties against a suitable β-lactamase preparation (9). In the present paper, the use of high-performance liquid chromatography (HPLC) to rapidly determine low concentrations of clavulanic acid in biological fluids is described, and the assay of amoxicillin by using the same chromatographic system is also reported. These methods may be of use in the study of the pharmacokinetics of formulations of penicillins and clavulanic acid, such as Augmentin, and for rapidly determining body fluid concentrations of the components of these formulations during clinical use.

MATERIALS AND METHODS

HPLC equipment. A Waters M6000A pump was used, and injections were made with a Hamilton 25-μl syringe via a U6K injector (Waters Associates, Northwich, England). The column eluant was monitored by using a Cecil 2012 variable-wavelength UV detector with a 1-cm 8-μl flow cell (Cecil Instruments Ltd., Cambridge, England). The HPLC column (25 cm; inner diameter, 4.6 mm) contained a C18 μBondapak support prepacked by Waters Associates. A guard column (Whatman column survival kit) was used throughout these studies and was frequently repacked.

**Ultrafiltration equipment.** Serum samples and standards in serum may be ultrafiltered before HPLC by using the Amicon MPS-1 micropartition system with Amicon YMB membranes (Amicon Ltd., Woking, Surrey, England) and a standard, refrigerated, angle-head centrifuge.

**Materials.** Imidazole, free of UV-absorbing impurities, and mercuric chloride were of analytical grade and were supplied by BDH Ltd., Poole, Dorset, England. Clavulanic acid (Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey, England) was used as its potassium or lithium salt and amoxicillin (Beecham) as its trihydrate. The penicilloic acid of amoxicillin was prepared by using a TEM-1 β-lactamase solution prepared as described previously (8) from cells of *Escherichia coli* J74 and had an activity of 500 µg of amoxicillin hydrolyzed/ml per min at pH 7.3 and 37°C. This solution (0.1 ml) was added to 10 ml of amoxicillin trihydrate as a mg/ml in 0.05 M sodium phosphate buffer (pH 7.3). Complete loss of amoxicillin and the generation of penicilloic acid was followed by HPLC under the appropriate conditions (Table 1).

**Derivatization procedure for clavulanic acid assays.** The imidazole reagent was prepared by dissolving 8.25 g of imidazole in 24 ml of distilled water plus 2 ml of 5 M HCl. The solution was adjusted to pH 6.8 by the addition of 5 M HCl, and the volume was made up to 40 ml with distilled water. This reagent was routinely used for the derivatization of clavulanic acid in buffer, serum, ultrafiltered serum, and urine. The sample to be assayed (4 parts) was added to the imidazole reagent (1 part), and, after a 10-min reaction period at room temperature, 25 to 50 µl was loaded onto the HPLC column. The imidazole reagent was stored at room temperature and was stable over a period of 2 to 3 months.

The reagent used for reactions with amoxicillin penicilloic acid was more concentrated. Imidazole (16.5 g) was dissolved in 24 ml of distilled water plus 4 ml of 5 M HCl, and 4 ml of 1.35% (wt/vol) mercuric chloride was also added before adjustment to pH 6.8. The volume was finally adjusted to 40 ml with distilled water. Even with this more concentrated reagent, the formation of the reaction product with penicilloic acid (0.4 ml of sample plus 0.1 ml of reagent) proceeded slowly; therefore, a 20-min reaction period at room temperature was used. A 25-µl sample was loaded onto the HPLC column.

**Standards.** For serum assays, amoxicillin and clavulanic acid standards were freshly prepared in pooled human serum which was adjusted to pH 6.8 with CO2, which was added as dry ice granules to the serum. Amoxicillin (10 µg/ml) in serum was serially diluted 1/2 with serum to provide five standards (10 to 0.625 µg/ml). Clavulanic acid was prepared similarly to give five standard solutions in the pH-adjusted serum (5 to 0.3125 µg of clavulanate per ml as the pure free acid).

For the assay of amoxicillin in urine, standard solutions were prepared in either 0.1 M sodium phosphate buffer (pH 7.0) or control urine which had been diluted 1/10 into the buffer. The five standard concentrations were 100 to 6.25 µg of amoxicillin per ml prepared by serial dilution 1/2.

Similarly, clavulanic acid standards were prepared in buffer or in diluted (1/10) control urine to provide five concentrations (50 to 3.125 µg of clavulanic acid per ml). Where concentrations of clavulanic acid in urine below 2 µg/ml were to be assayed, standards were prepared in neat control urine (5 to 0.3125 µg of clavulanate pure free acid).

**Ultrafiltration of serum samples and standards.** The Amicon YMB membranes were washed before use by stirring gently for 30 min in 200 ml of 0.1 M sodium phosphate buffer (pH 7.0). This step removed glycerol, which can interfere in subsequent chromatography. The membranes were blotted dry with filter paper and assembled in the Amicon MPS-1 filtration unit following the manufacturer’s instructions. The serum samples or serum standards were diluted with an equal volume of 0.1 M sodium phosphate buffer (pH 7.0), and a standard volume (0.5 to 1 ml) was placed in the filtration unit, which was capped and then centrifuged at 5°C in a 35° angle-head rotor at 1,000 x g for 15 min. The ultrafiltrate collection cup was removed, the cap was applied, and the filtrate sample was stored at 5°C until assay.

**Urine samples and blanks.** Samples for amoxicillin assay were diluted 1/10 into 0.1 M sodium phosphate buffer (pH 7.0). To 0.6 ml of the diluted sample, an additional 0.2 ml of buffer was added, and 25 µl then applied to the HPLC column.

A blank of each sample was also prepared by adding 0.6 ml of the diluted sample to 0.1 ml of 1 M NaOH. After 30 min at room temperature, 0.1 ml of 1 M HCl

### TABLE 1. HPLC on C18 µ-Bondapak reverse-phase columns

<table>
<thead>
<tr>
<th>Compound to be assayed</th>
<th>Solvent system</th>
<th>Detection wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clavulanic acid</td>
<td>Buffer</td>
<td>220–227</td>
</tr>
<tr>
<td>Amoxicillin in serum or pure solution</td>
<td>Buffer + 6% methanol</td>
<td>227</td>
</tr>
<tr>
<td>Amoxicillin in urine</td>
<td>Buffer + 4% methanol</td>
<td>227</td>
</tr>
<tr>
<td>Amoxicillin peniciloate in pure solution</td>
<td>Buffer + 4% methanol</td>
<td>227</td>
</tr>
<tr>
<td>Derivatized clavulanic acid in serum</td>
<td>Buffer + 6% methanol</td>
<td>311</td>
</tr>
<tr>
<td>Derivatized clavulanic acid in urine (&gt;2 µg/ml)</td>
<td>Buffer + 6% methanol</td>
<td>311</td>
</tr>
<tr>
<td>Low concentrations (&lt;2 µg/ml) of clavulanic acid derivatized in urine</td>
<td>Buffer + 4% methanol</td>
<td>311</td>
</tr>
<tr>
<td>Amoxicillin peniciloate-imidazole-HgCl2 product in pure solution</td>
<td>Buffer + 6% methanol</td>
<td>311</td>
</tr>
</tbody>
</table>

* Buffer eluant in all cases was 0.1 M potassium dihydrogen orthophosphate adjusted to pH 3.2 with phosphoric acid. The flow rate was 2.5 ml/min, and chromatography was carried out at room temperature.

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was added to neutralize the sample, and 25 μl was then applied to the HPLC column. Amoxicillin standards in diluted control urine or buffer were treated identically to the samples to provide test and blanks when necessary.

For the routine assay of clavulanic acid in urine, the samples were diluted 1/10 into the phosphate buffer, and samples were then derivatized and applied to the HPLC column. Blanking procedures were not normally required for clavulanic acid assay. However, when concentrations of clavulanic acid of less than 2 μg/ml in neat urine were required to be assayed, test and blanked samples were prepared. The sample in neat urine (0.6 ml) was diluted with 0.2 ml of phosphate buffer and then derivatized by adding 0.2 ml of the imidazole reagent. After 10 min at room temperature, 25 μl of the reaction mixture was applied to the HPLC column. The blanked sample was prepared by adding 0.1-ml portions of alkali and acid to 0.6 ml of the sample, as described for the amoxicillin blanks. This was then derivatized by the addition of 0.2 ml of imidazole reagent, and after 10 min at room temperature, 25 μl was applied to the HPLC column.

**HPLC conditions.** Table 1 summarizes the chromatographic and detection conditions used for the determination of clavulanic acid, amoxicillin, and amoxicillin penicilloate without reaction with the imidazole reagent, as well as for the imidazole reaction products of clavulanic acid and amoxicillin penicilloate.

**Assay of a sample for both clavulanic acid and amoxicillin.** Where assays of both components in a sample were required, the clavulanic acid content was determined first. This order was used because clavulanic acid was less stable than amoxicillin when stored in solution in body fluids. After removal of sample for derivatization and clavulanic acid assay, serum, ultrafiltered serum, or urine samples were stored in closed vessels at 5°C if amoxicillin assays were to be performed on the same day; they were stored at −40 to −70°C if the assays were to be performed at a later time. Amoxicillin assays were carried out on the stored samples that had not been reacted with imidazole. The buffer eluants shown in Table 1 were used for amoxicillin assay, and the eluant was monitored at 227 nm; the earlier assays of the clavulanate-imidazole derivative were monitored at 311 nm under the conditions given in Table 1.

**Microbiological assay methods.** Amoxicillin was assayed by a hole-in-plate method with *Sarcina lutea* as the test organism. Clavulanic acid was determined by using the special synergistic assay system devised in these laboratories (2) and adapted for the assay of clavulanic acid in the presence of amoxicillin (R. Horton, unpublished work) as described by Ball et al. (1). This method used agar which contained benzyl penicillin and *Klebsiella pneumoniae* ATCC 29665 as the test organism. Clavulanic acid in the samples inhibited the β-lactamase produced by this organism and so allowed the penicillin in the agar to produce a zone of inhibition.

**RESULTS**

HPLC of underivatized clavulanic acid and amoxicillin. Clavulanic acid is poorly retained on C18 reverse-phase columns. Retention times can be lengthened by using buffer eluants at acid pH, but even under these conditions it was not possible to obtain clavulanic acid peaks distinct
FIG. 3. Amoxicillin (Amox.) in human serum. Samples were chromatographed in 0.1 M potassium phosphate (pH 3.2)-6% (vol/vol) methanol, and the eluant was monitored at 227 nm and 0.01 AUFS. (A) Serum (25 μl). (B) Serum containing 2.5 μg of amoxicillin per ml (25 μl injected [Inj.]). (C) Serum diluted 1/2 into 0.1 M sodium phosphate buffer (pH 7.0) and ultrafiltered (50 μl injected). (D) Serum containing 2.5 μg of amoxicillin per ml diluted and ultrafiltered (50 μl injected).

FIG. 4. Amoxicillin (Amox.) in urine. Samples were chromatographed in 0.1 M potassium phosphate (pH 3.2)-4% (vol/vol) methanol. The eluant was monitored at 227 nm and 0.01 AUFS. Amoxicillin (200 μg/ml) in urine was diluted 1/10 into 0.1 M sodium phosphate buffer (pH 7.0). (A) 0.6 ml of diluted urine plus 0.2 ml buffer (25 μl injected [Inj.]). (B) 0.6 ml of diluted urine blanked with 0.1 ml 1 M NaOH (30 min at room temperature) neutralized with 0.1 ml of 1 M HCl (25 μl injected).

from interfering components in human serum or plasma samples (Fig. 2). Clavulanic acid, like the penicillins, has no specific chromophore, and to obtain reasonable detection limits, eluants must be monitored at low wavelengths (<230 nm). In this end-absorbance region, many compounds in biological fluids interfere.

Amoxicillin, however, gives longer retention times on C18 columns and can be distinguished from serum components, as shown in Fig. 2, by using a phosphate buffer eluant at pH 3.2. With the same buffer system but with added methanol (6% [vol/vol]), retention times can be shortened without deleterious effect, as shown in Fig. 3. With this eluant and a 25 μl injection of serum, amoxicillin can be detected down to 0.5 μg/ml when monitored at 227 nm and 0.005 absorbance...
FIG. 5. HPLC of the imidazole-clavulanic acid reaction product in human serum. The column was eluted with 0.1 M potassium phosphate buffer (pH 3.2)-6% (vol/vol) methanol at 2.5 ml/min and monitored at 311 nm and 0.01 AUFS. Samples (25 μl) were (A) serum plus imidazole reagent after 10 min at room temperature and (B) 2 μg of clavulanic acid per ml in human serum plus imidazole reagent after 10 min at room temperature.

FIG. 6. Effect of reagent concentration on the rate of derivatization of clavulanic acid. Clavulanic acid (100 μg/ml) in phosphate buffer (pH 7.3) was added (0.5 ml) to (●) 0.4 ml, (○) 0.2 ml, (■) 0.1 ml, and (□) 0.05 ml of imidazole reagent. Reaction volumes were made up to 1 ml with 0.05 M potassium phosphate buffer (pH 7.3). Formation of the clavulanic acid derivative at room temperature was followed with time by loading 25-μl samples onto the HPLC column under the conditions described in the legend to Fig. 4. Peak height (millimeters) was measured at 0.5 AUFS. On the basis of these data, a 1:4 addition of reagent to sample (○) was chosen for routine use with a 10-min reaction period.

units full scale (AUFS). The ultrafiltration of serum samples and standards containing amoxicillin was not absolutely essential. This procedure did, however, reduce the level of high-molecular-weight material that was applied and so greatly extended the efficient working life of the column. Ultrafiltration also removed interfering peaks on the HPLC trace, as illustrated in Fig. 3, making measurements of peak height easier and more accurate. This procedure can be used for both amoxicillin and clavulanic acid because both these β-lactams are not highly serum bound (4); thus, ultrafiltration does not greatly decrease the sensitivity of the assay by leaving the majority of the antibiotic behind in the protein-rich retentate. The dilution of the serum sample with 0.1 M sodium phosphate buffer (pH 7.0) before filtration served two purposes. The percentage binding was decreased by dilution, but the main reason for diluting with buffer was to maintain the pH of the filtrate at neutrality. It was found that the pH of the serum after ultrafiltration was in excess of 8.0 when unbuffered serum was used. This actually had little effect on the stability of amoxicillin, but clavulanic acid was relatively unstable at alkaline pH. Since these ultrafiltered samples were to be assayed for both components of Augmentin, the
buffer dilution stage was routinely adopted when ultrafiltration was used. For assays of amoxicillin in serum, a range of standard concentrations in serum was used, and standard lines of peak height versus concentration were plotted from which the concentration of amoxicillin in the serum sample was determined. If the serum samples were to be assayed after ultrafiltration, then the standards in serum were similarly diluted and ultrafiltered. With ultrafiltered samples and standards, the injection volume was increased from 25 to 50 µl to allow for the dilution step. Standard lines for amoxicillin in serum and ultrafiltered serum were linear up to at least 20 µg/ml.

When amoxicillin in urine was assayed, the samples were initially diluted 1/10 into phosphate buffer to reduce the level of extraneous material that was loaded onto the column. In urine containing low concentrations of amoxicillin, interference from urine components can occur. For this reason, such urine samples must be blanked by the addition of alkali followed by neutralization, as described in Materials and Methods. In this way, the absorbance of minor peaks which cochromatograph with amoxicillin may be subtracted from the peak heights obtained for the untreated urine samples containing amoxicillin. The alkali treatment generates the faster-running penicilloic acid well separated from the original amoxicillin position (Fig. 4). The addition to urine of alkali followed by HCl does not appear to affect the chromatographic results for other urine components. Even with the use of blanked samples, it proved difficult to assay amoxicillin in concentrated urine at less than 100 µg/ml. After oral administration of amoxicillin, urine concentrations of the antibiotic can, however, be very high. For assays of amoxicillin in urine, the methanol content of the eluant was 4% (vol/vol). Standard lines for amoxicillin in urine were linear up to at least 1 mg/ml.

**Assay of derivatized clavulanic acid.** The generation of a more suitable chromophore by derivatizing clavulanic acid before chromatography was considered as a means of overcoming the interference problems discussed earlier. The reaction of imidazole with penicillins in the presence of mercuric chloride to form penicillenic acid mercuric mercaptides was first described by Bundgaard and Ilver (3). The reaction of imidazole with clavulanic acid in the absence of mercuric chloride occurs readily at room temperature to form a relatively stable product (Fig. 1) absorbing at 311 nm (A. E. Bird, J. M. Bellis, and B. C. Gasson, The Analyst, in press). HPLC examination of this product by using C18 reverse-phase columns revealed a single peak when chromatographed in the phosphate buffer (pH 3.2) containing 6% methanol (Fig. 5).
Materials and Methods). The sample (0.4 ml) was added to 0.1 ml of the reagent, and after 10 min at room temperature, it was injected (25 µl) onto the column. The rate of formation of the clavulanate-imidazole product was limited by reagent concentration (Fig. 6). A 1:4 addition of reagent to sample and a 10-min reaction at ambient temperature were selected as routine procedure, although shorter reaction times may be used if a more concentrated reagent is employed or if larger reagent volumes are added. As can be seen in Fig. 6, the reaction product was stable over a period of at least 2 h. With the routine procedure, linearity was obtained for plots of peak height versus concentration up to at least 200 µg/ml of clavulanic acid. With a 25-µl injection volume, the detection limit was 0.1 µg/ml at 311 nm and 0.005 AUFS.

In serum samples, clavulanic acid was readily derivatized, and there was no interference, even at 0.005 AUFS, during HPLC when the buffer (pH 3.2) plus 6% methanol was used (Fig. 7). Ultrafiltration of serum samples for the assay of derivatized clavulanate had no benefit in terms of reducing interference from serum components because at 311 nm very few of these other components were detectable (Fig. 7). The ultrafiltration step in this instance therefore served merely to prolong column life by reducing the amount of extraneous material that was loaded onto the column. If only a few samples are to be assayed on an occasional basis, then ultrafiltration can probably be avoided. As for the assay of amoxicillin in serum, standards of clavulanate were prepared in serum (see Materials and Methods), and a standard line of peak height versus concentration was used to determine clavulanate concentration in the serum samples.

For the assay of clavulanic acid in urine after derivatization, the methanol level in the buffer eluant was normally 6% (vol/vol). However, the selection of suitable methanol concentrations and the correct buffer pH can be used to optimize separation conditions on a particular column, as these appear to vary from column to column and individual columns also become less efficient with use. In the assay of urine samples after derivatization (Fig. 8), problems only occurred with minor cochromatographing peaks when assays of clavulanic acid at concentrations of less than 2 µg/ml in concentrated urine at 0.02 AUFS were attempted. In this situation, the alkali blanking procedure described in Materials and Methods was used. For the assay of low concentrations of clavulanic acid (<2 µg/ml) in urine, the methanol content was reduced to 4% (vol/vol). Standards for urine assays were prepared as described in Materials and Methods.

**Interference from other β-lactams and degradation products.** Penicillins do not readily react

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**FIG. 8.** Clavulanic acid (Clav.) (derivatized) in urine. Samples were chromatographed with 0.1 M potassium phosphate (pH 3.2)-6% (vol/vol) methanol, and the eluant was monitored at 311 nm and 0.1 AUFS. (A) Neat urine plus imidazole (25 µl injected [Inj.]). (B) Clavulanic acid (10 µg/ml) in neat urine was derivatized and 25 µl was injected.

For the routine assay of clavulanic acid, the imidazole reagent was used at a concentration 2.5 times greater than that described originally (3) and without added mercuric chloride (see
examination when monitored at 311 nm. The presence of 1 mg of penicilloic acid per ml had no effect when standard solutions of clavulanic acid were derivatized and chromatographed. If, however, the reagent included mercuric chloride, the penicilloic acid did indeed produce a reaction product after the 10-min reaction at room temperature. Figure 9 shows that penicilloic acid produces one main component, distinguishable from the clavulanate derivative. This penicilloate product was not produced when penicilloic acid was reacted with mercuric chloride alone.

The absorbance at 311 nm for the major product of the penicilloic acid-imidazole-mercuric chloride reaction was significantly lower than that generated by clavulanic acid with either reagent. As shown in Fig. 9, even after optimizing the reaction with penicilloic acid by using a more concentrated reagent (see Materials and Methods) and reacting for 20 min at room temperature, the assay of the penicilloic acid derivative was 100-fold less sensitive than that achieved with clavulanic acid. Although the two derivatives were readily separated on fresh C18 Bondapak columns, it was noted that column performance rapidly decreased in terms of retaining the penicilloic acid derivative. This occurred even though retention times for derivatized clavulanic acid remained fairly constant. This problem, combined with poor sensitivity, did not allow the use of the imidazole reaction for penicilloic acid determinations. For the routine determination of clavulanic acid, therefore, a mercuric chloride-free reagent was used, totally eliminating the possibility of interference from amoxicillin penicilloate in assay samples.

Comparison of HPLC and microbiological assays for Augmentin. Human serum and urine were spiked with mixtures of amoxicillin and potassium clavulanate and were assayed by using both HPLC and microbiological assay methods. The results of this comparison are shown in Tables 2 and 3.

Samples from healthy human volunteers who had been given Augmentin were also assayed by using both methods. A typical result for serum concentrations obtained from a single male volunteer given 500 mg of amoxicillin and 125 mg of potassium clavulanate is shown in Fig. 10.

Assay of amoxicillin and clavulanate in uremic serum. Sera from uremic patients may contain high concentrations of various components not present in normal serum. The direct HPLC assay of amoxicillin in such sera proved difficult, even after ultrafiltration of the samples. Under these circumstances, the alkali blanking procedure described for the assay of amoxicillin in urine can be used to reveal interfering components, and the methanol concentration in the
eluant may be adjusted to provide better separations. Some interference from a component in uremic serum also occurred when low concentrations of clavulanate were assayed with the precolumn derivitization assay. This was overcome by reducing the methanol level in the eluant to 4% (vol/vol), thus improving the separation of the derivative from the interfering peak.

**DISCUSSION**

The use of HPLC and precolumn derivitization for the determination of clavulanic acid, described in the present paper, provides a simple rapid assay method. The optional use of ultrafiltration, combined with the addition of a single reagent and a short reaction period before HPLC, is in contrast to many procedures for the HPLC assay of clinical samples, for which complex extraction and precipitation procedures are required. The reaction of the imidazole reagent with clavulanic acid and the generation of a product absorbing at 311 nm considerably reduces interference from UV-absorbing components in human serum and urine. The high sensitivity obtained for serum samples, with 0.1 μg of clavulanic acid per ml being readily detectable with a 25-μl sample, is similar to that obtained with special microbiological assays for this compound. Further increases in sensitivity may be achieved by using larger sample volumes. The HPLC assay is highly specific for clavulanic acid, and under the routine conditions used for derivitization, penicillins, penicilloic acids, and clavulanic acid degradation products do not interfere with the assay. Although HPLC assays provide rapid results, in contrast to biological methods, for which overnight incubation is required, there is often a limitation on sample throughput. With serum samples for clavulanic acid assay, the short retention time for the derivative peak allows one assay every 5 to 6 min, providing reagent addition is timed in sequence to allow a 10-min reaction period before injection. With urine samples which contain slower-running components, the assay time is somewhat longer. The assay of clavulanic acid in urine by using HPLC has been previously described by Haginaka et al. (6). This ion pair HPLC method had a detection limit of 5 μg/ml for clavulanic acid and was not suitable for the assay of clavulanate in serum.

Amoxicillin determination by direct UV monitoring of HPLC column eluants has been described previously (10). In the present study, amoxicillin was assayed in column eluants at 227 nm with chromatographic conditions similar to those used for the clavulanic acid-imidazole derivative. Ultrafiltration of serum samples has been introduced for amoxicillin assay, which markedly reduces interference from high-molec-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amoxicillin (μg/ml)</th>
<th>Clavulanic acid (μg/ml)</th>
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<tr>
<td></td>
<td>Actual concn</td>
<td>Microbiological assay</td>
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<tr>
<td>1</td>
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**TABLE 3.** Comparison of microbiological and HPLC assays of human urine spiked with both amoxicillin and potassium clavulanate
ular-weight serum compounds and prolongs column life. Amoxicillin concentrations down to 0.5 μg/ml were assayed by using these procedures. The use of alkali treatment to provide blanks when assaying urine containing amoxicillin has been used, and this improves the reliability of HPLC determinations by revealing any components which may cochromatograph with amoxicillin in these complex and variable samples.

Comparison of HPLC and microbiological assays for serum and urine samples which were spiked with amoxicillin plus clavulanic acid showed that both methods gave reliable results. Good correlation was also obtained when microbiological and HPLC systems were compared for samples from human volunteers given amoxicillin plus clavulanic acid.

It is hoped that the HPLC assay of clavulanic acid and amoxicillin will provide a useful method for pharmacokinetic studies on Augmentin, as well as providing the clinical laboratory with a rapid and reliable assay of body fluid concentrations in patients under treatment.

ACKNOWLEDGMENTS

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LITERATURE CITED