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CLAVULANATE-POTENTIATED TICARCILLIN: HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAYS FOR CLAVULANIC ACID AND TICARCILLIN ISOMERS IN SERUM AND URINE

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SUMMARY

High-performance liquid chromatographic assays for the determination of clavulanic acid and ticarcillin in biological fluids are described. The clavulanic acid assay uses serum ultrafiltrate and direct injection of diluted urine with reversed-phase ion-pair/counter-ion chromatography. The ticarcillin assay allows, for the first time, the separation and quantitation of two isomers of ticarcillin. The performance of these assays has been evaluated and found to be satisfactory for routine clinical use and thus the assays have been applied to the study of the pharmacokinetics of these analytes in a subject with renal failure.

INTRODUCTION

Clavulanate is a potent, irreversible, inhibitor of a number of bacterial β-lactamases [1]. Penicillins susceptible to lactamase attack have their activity enhanced in the presence of clavulanate and its combination with amoxicillin has been shown to be effective both in vitro [2, 3] and in vivo [3, 4].

Ticarcillin has a broader spectrum of activity than amoxicillin but is susceptible to the action of some β-lactamases; however, clavulanate potentiated inhibition has been shown to be effective in vitro and in vivo [2, 5, 6].

Typically, clavulanate and ticarcillin are assayed by microbiological assay [7, 8] which although more suited to large sample numbers may lack precision and also the turn round of such assays tends to be slow. High-performance liquid chromatography (HPLC) has recently been applied to the assay of clavulanate in urine [9]; an assay for serum has been described [10], but its performance characteristics were not validated. This paper describes simple, precise assays for clavulanate and ticarcillin in serum and urine.

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MATERIALS AND METHODS

HPLC equipment

A Gilson 302 B pump with a Series 5 head and an 802 manometric module were used with an Altex 160 fixed-wavelength filter detector (Scotlab Instruments Sales, Bellshill, U.K.). Injections were made with a Waters 710B WISP autosampler (Waters Assoc., Northwick, U.K.). A 25 cm × 4.6 mm I.D. Spherisorb 5-μm ODS column was used for the determination of clavulanate and a 10 cm × 5 mm I.D. Hypersil 3-μm ODS column was used for ticarcillin. Both columns were slurry packed in the laboratory using a Shandon column packer (Shandon Southern Products, Runcorn, U.K.). Results were integrated using a Shimadzu C-R2AX integrator (Dyson Instruments, Houghton-le-Spring, U.K.).

HPLC conditions

Ticarcillin. Methanol—water (30:70) was filtered through an 0.45-μm nylon filter in a glass filtration apparatus under vacuum supplied by a water-driven venturi pump. To the filtered solvent was added concentrated phosphoric acid to give a final aqueous concentration of 0.05 *M*. After gentle mixing the eluent was pumped at a flow-rate of 2 ml/min. A zinc lamp with a 214-nm filter was used to monitor the mobile phase at 0.01 a.u.f.s. Auto-zero was performed by the WISP prior to injection of 50 μl of sample at intervals of 15 min.

Clavulanate. The filtered, degassed mobile phase was methanol—0.1 *M* potassium dihydrogen phosphate (10:90) containing 0.05 *M* pentane sulphonic acid (HPLC grade, Fisons, Loughborough, U.K.) and 0.1 *M* ethanolamine at a flow-rate of 1.5 ml/min. A mercury lamp with a 313-nm filter was used to monitor the eluent at 0.01 a.u.f.s. Auto-zeroing of baseline prior to injection was again initiated by the WISP autosampler which injected 75-μl samples every 10 min.

Materials

Imidazole (BDH, for penicillin analysis), for the assay of clavulanate was prepared as described by Foulstone and Reading [10]. A 1 mg/l solution of thienylbutyric acid (Sigma, U.K.) in methanol was prepared for use as an internal standard in the ticarcillin assay. All other reagents were BDH AnalaR grade unless specified.

Sample collection

Both ticarcillin and clavulanate are labile, blood samples were allowed to clot for 1 h at room temperature, centrifuged and the serum was stored at −80°C prior to analysis. Urines were volumed and aliquotted prior to dilution 1:10 with 0.1 *M* citrate buffer, pH 6.5, to ensure analyte stability; the diluted urine samples were also stored at −80°C prior to analysis.

Calibration

Ticarcillin. From a freshly prepared stock of 1 g/l ticarcillin sodium (Beecham Pharmaceuticals, Brockham, U.K.) were prepared serum calibrators covering the range 0—500 mg/l using horse serum No. 3 (Wellcome Reagents,

U.K.). Urine calibrators were prepared in fresh human urine diluted 1:10 with 0.1 *M* citrate buffer and covering the range 0—2000 mg/l. The ratio of total peak area of ticarcillin to internal standard peak area was plotted against concentration to provide a calibration line for total ticarcillin. In the determination of ticarcillin isomers, peak height ratio of isomer 1 and isomer 2 to the internal standard was used. The relative contributions of the two isomers were calculated by proportion of peak height to the total ticarcillin concentration, as pure free acid, equal detector response being assumed on a weight for weight basis.

The sum of ticarcillin 1 and 2 compared well with the concentration of total ticarcillin calculated using peak area.

As batch analysis could extend to over 12 h an aqueous calibrator was also prepared in 0.1 *M* phosphate buffer, pH 7.0, with a value of 250 mg/l; during analysis this 'target' calibrator was assayed after every four injections; this allowed assessment and correction of any degradation of samples during sampling.

Clavulanate. In a similar approach to that outlined for ticarcillin, potassium clavulanate (Beecham Pharmaceuticals) 0.5 g/l was used to prepare serum calibrators over the range 0—50 mg/l and urine calibrators again diluted 1:10 with 0.1 *M* citrate buffer, pH 6.5, over the range 0—500 mg/l. The target calibrator was 20 mg/l in phosphate buffer, pH 7.0.

The procedure was externally calibrated, i.e. the peak areas were plotted against concentration and a calibration line produced. The measured product of clavulanate has a half-life in pH 7.0 buffer of around 15 h and the use of the target calibrator is of great importance.

In both the ticarcillin and clavulanate assays the results were subsequently converted to those of the pure free acid. From the material supplied the ticarcillin sodium was equivalent to 80.8% pure free acid and the potassium clavulanate was equivalent to 82.5% pure free acid.

Assay procedures

Ticarcillin. To 1 ml of sample in a Z10 plain tube (Brunswick, U.K.) were added 50 μl of 1 mg/l methanolic thienyl butyric acid and 6 ml of diethyl ether; to each tube were then added 100 μl of 3 *M* hydrochloric acid; the tube was immediately capped and inverted two or three times to ensure complete mixing. It was essential to perform this step immediately as ticarcillin was very labile in acid conditions. Once all the tubes were ready they were shaken for 5 min on a lateral shaker (Griffin and George, London, U.K.). The ether layer was transferred to a second Z10 tube containing 500 μl of 0.1 *M* phosphate buffer, pH 7.0, and the organic layer removed under a stream of air at 37°C. Of the remaining aqueous layer, 200 μl were transferred to a PRO tube (Sarstedt, U.K.) from which the upper 5 mm had been removed; these tubes were placed in the centre of a spring insert in the WISP autosampler vial and the septum screw caps applied; the carousel was then loaded and the samples injected. The procedures for serum and diluted urine were identical.

Clavulanate. Serum (500 μl) in a Z10 tube was diluted with 500 μl of 0.1 *M* phosphate buffer, pH 7.0, after mixing this was transferred to a Syva free drug assembly (Syva, Maidstone, U.K.). (The assembly is a pre-assembled version of

the Amicon MPS-1 ultrafiltration apparatus.) This was centrifuged for 20 min at 4°C at 1500 *g*. Ultrafiltrate (100 μl) was added to 100 μl imidazole reagent in a PRO tube, the tube was capped, mixed by flicking and inversion, the top 5 mm were removed and inserted as described above into the WISP autosampler. For urine the procedure is as described for ultrafiltrate.

Pharmacokinetics

The assays were applied to the study of clavulanate-potentiated ticarcillin in patients with renal failure. Informed consent and ethical permission were obtained and the individuals given an intravenous dose of the combination which comprised of 3 g of ticarcillin and 200 mg of clavulanate. Blood samples were drawn pre-dose and at 0.083, 0.25, 0.33, 1, 1.5, 2, 4, 8, 12 and 24 h post-dose. Urine was collected as passed and diluted with 0.1 *M* citrate buffer; 24-h collections were not made owing to analyte instability. Samples were stored as described above.

RESULTS

Clavulanate

A chromatogram from a pre-dose serum sample is shown in Fig. 1A. A patient's serum with a concentration of 2.5 mg/l clavulanate is illustrated in Fig. 1B. The chromatogram from a urine containing clavulanate is shown in Fig. 2.

Linearity. Linearity was established over the range 0—500 mg/l for serum assay and 0—2500 mg/l for the urine assay. This is equivalent to 0—20 μg on-column weight allowing for the differences in dilution.

Sensitivity. Sensitivity, defined as twice the signal-to-noise ratio, was equivalent to 0.1 mg/l, i.e. 2 ng on-column weight.

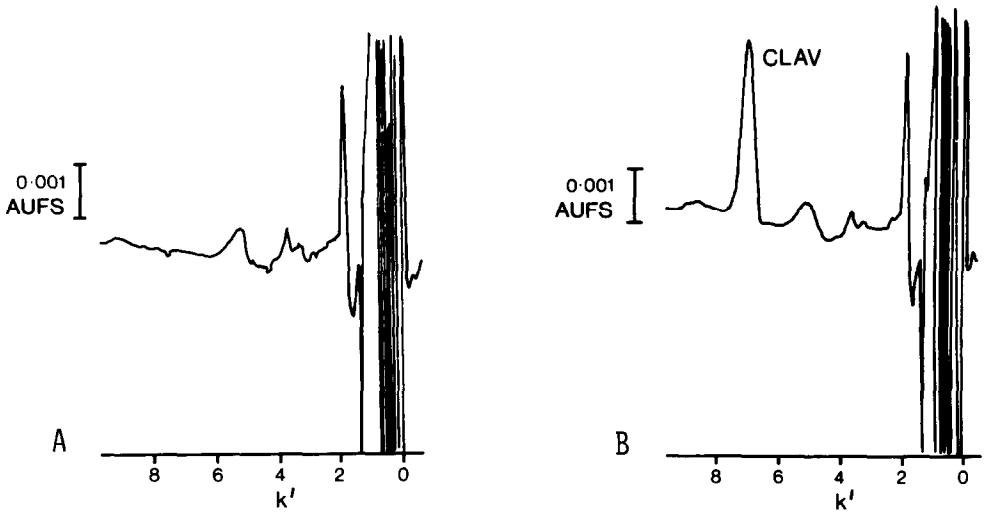

Fig. 1. Chromatograms for serum clavulanate assay. (A) Pre-dose sample; (B) 8-h post-dose serum sample, clavulanate concentration 2.5 mg/l. Chart speed 5 mm/min. Peak: CLAV = clavulanate.

Recovery. By comparison with directly injected calibrators at a concentration of 5 mg/l it was determined that the recovery from an aqueous standard was 93.8 ± 2.9% (n = 5) and for the serum standard was 79.2 ± 4.2% (n = 5). The average loss directly attributable to ultrafiltration was 6.2% and there was a further 12.6% loss associated with the ultrafiltration of serum owing to protein binding.

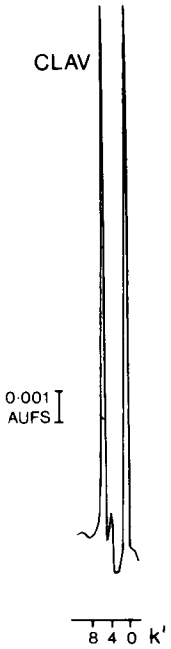

Fig. 2. Chromatogram following assay of clavulanate in urine. Chart speed 2 mm/min. Peak: CLAV = clavulanate.

Precision. The within-batch precision at 1 mg/l was 4.5% (n = 40) and at 8 mg/l 2.95% (n = 40). The between-batch precision at these levels was 6.6% (n = 44) and 4.3% (n = 48), respectively.

Accuracy. Samples from patients on a great variety of co-medication have been assayed with no apparent interference. However, the use of imidazole that is not free from ultraviolet-absorbing impurities results in a number of peaks which were found to interfere with the assay of clavulanate; the use of the recommended grade of reagent avoids this problem.

Ticarcillin

Ticarcillin is an isomeric mixture which chromatographs as two peaks the ratio of which (approx. 45:55) was the same in calibrators and patient samples (Fig. 3B); addition of a penicillinase to the sample resulted in the conversion to the corresponding penicillinoic acid(s). Typically the k' values for the two ticarcillin isomers were 4.5 and 6.1, and 1.6 for both penicillinoic acids. To maintain comparability with earlier work on ticarcillin pharmacokinetics, the total ticarcillin was calculated from the summed areas of the peaks; it was found that by decreasing the methanol content to 25% an endogenous

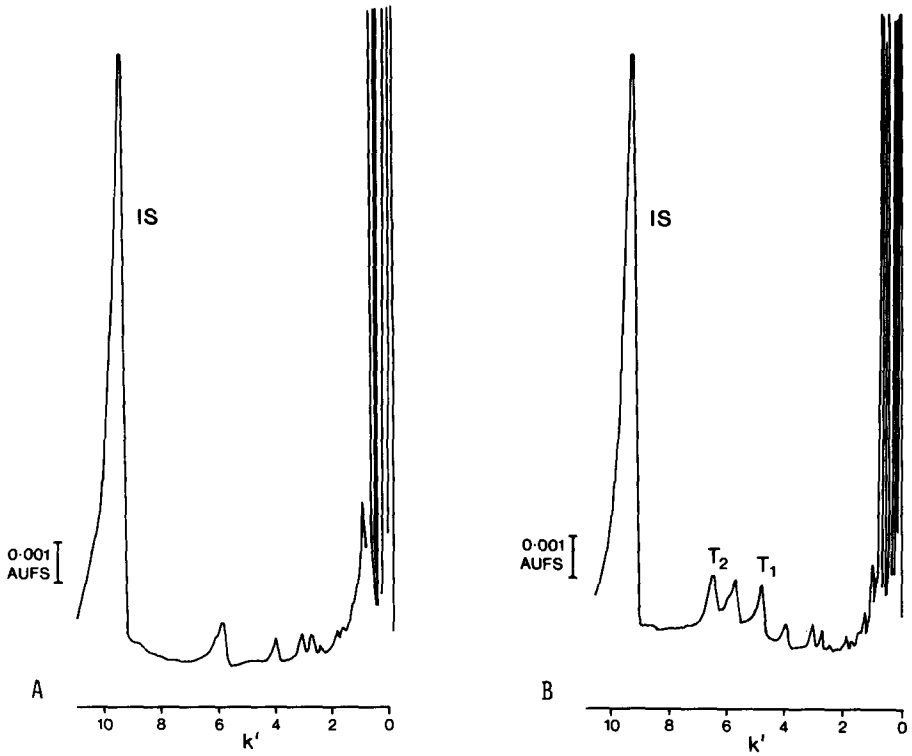

Fig. 3. Chromatgrams of pre-dose (A) and 8-h post-dose (B) sera for ticarcillin. Concentration in B is 10 mg/l. Eluent: methanol—0.05 *M* phosphoric acid (25:75); flow-rate 2 ml/min; chart speed 5 mm/min. Peaks: IS = internal standard, thienylbutyric acid; T_1 = ticarcillin isomer 1; T_2 = ticarcillin isomer 2.

interfering peak eluted between the ticarcillin peaks (Fig. 3A pre-dose, Fig. 3B post-dose total concentration 10 mg/l). In practise it was found that subtraction of the endogenous peak area from ticarcillin areas using the 30% methanol solvent yielded comparable results with a faster assay. The endogenous peak did not interfere with the quantitation of ticarcillin isomer 1 or isomer 2 if peak height measurements were used.

Fig. 4 is a chromatogram obtained from a urine extract from a subject taking the ticarcillin—clavulanate mixture; the 30% methanol solvent was used.

Linearity. The summed area of the ticarcillin peak has been found to be linear over the range 0—600 mg/l for serum and 0—6000 mg/l for urine, this is equivalent to 0—30 μg on-column weight.

Sensitivity. The sensitivity for each ticarcillin isomer is 1 mg/l, i.e. a summed sensitivity of 2 mg/l equivalent to an on-column weight of 200 ng.

Recovery. By comparison with the summed areas from directly injected standards a recovery relative to the internal standard of 98.1 ± 8.3% (n = 20) was found at 80 mg/l.

Precision. The within-batch precision at 5 mg/l was 7.8% (n = 21) and at 80 mg/l was 6.0% (n = 60). The between-batch precision was 12.4% (n = 58) and 8.4% (n = 62), respectively.

Accuracy. The co-eluting endogenous peak had an area equivalent to 3.5

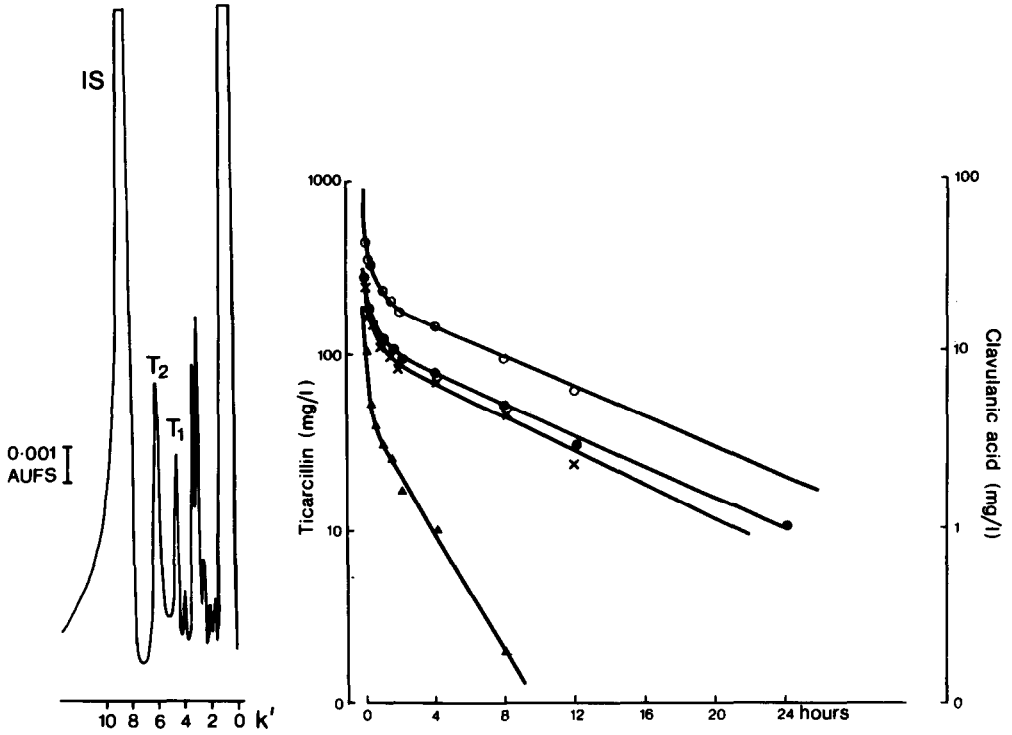

Fig. 4. Chromatogram following assay of ticarcillin in urine. Eluent: methanol—0.05 *M* phosphoric acid (30:70); chart speed 2 mm/min. Peaks: IS = internal standard, thienylbutyric acid; T_1 = ticarcillin isomer 1; T_2 = ticarcillin isomer 2.

Fig. 5. Plasma concentration—time curve for clavulanate and ticarcillin following a 3-g ticarcillin—200-mg clavulanate oral dose in a patient with renal failure. Creatinine clearance 20 ml/min. ○, Total ticarcillin; X, ticarcillin isomer 1; ●, ticarcillin isomer 2; ▲, clavulanate.

mg/l ticarcillin. No medication yet encountered has given rise to spurious results.

Pharmacokinetics. The plasma drug concentration—time curves for total ticarcillin, ticarcillin isomer 1, ticarcillin isomer 2 and clavulanate from a subject with renal failure (creatinine clearance 20 ml/min), given 3 g ticarcillin—200 mg clavulanate intravenously are shown in Fig. 5. The half-lives of elimination were: total ticarcillin, 7.0 h; ticarcillin isomer 1, 6.5 h; ticarcillin isomer 2, 6.8 h; and clavulanate 1.8 h; the ticarcillin half-lives are comparable within the error of the assay and are excreted in a constant proportion.

DISCUSSION

Clavulanate

The sample preparation procedure used here was closely related to that of Foulstone and Reading [10], but had not been validated by them for a routine procedure. The Syva assemblies used here are very similar to the Amicon MPS-1 system and acceptable results should be obtainable using this. In the sample preparation the buffer was used primarily to stabilise the clavulanate, as

glycerol [10] did not interfere in the chromatography. The pre-column derivatisation step is preferred although it should be possible to develop a satisfactory post-column reaction procedure if desired. The proportions of reagent to sample were satisfactory and were chosen for assay simplicity.

The reasons for the chosen solvent composition are as follows: the potassium phosphate was used to maintain an acid pH at which the clavulanate derivative was relatively stable; pentane sulphonic acid was required to ion-pair the reaction product 1-(8-hydroxy-6-oxo-4-azaoct-2-enoyl)imidazole [10] to improve its retention. No products were found for ticarcillin or its pencillinoic acid when incubated with imidazole reagent.

When well capped reversed-phase material was used some tailing of the peak shape resulted; however, if end-capping was incomplete it was found that peak tailing was excessive; in either case excellent peak shape could be obtained by the addition of a cation which covered the residual silanol groups and also acted as a counter-ion; ethanolamine was found to be satisfactory and adjustment of its concentration could be used to control the retention of the clavulanate—imidazole reaction product, an increase causing a decrease in the k' value and a decrease an increased k' value. The use of longer-chain alkyl cationic surfactants resulted in excessive desorption with a decrease in retention to a k' of < 1. An internal standard was unnecessary as there was little sample preparation for urine samples and a simple preparation for serum with reproducible recovery.

The product stability was such that adjustment for degradation loss within-run was necessary although reproducible within the run; this was well identified with the repeat sampling of the target standard.

The precision of the assay was better than that previously noted with a microbiological assay [8].

Ultrafiltration of serum samples is essential for the maintenance of column selectivity as direct injection of serum, although feasible [10] led to rapid loss of retention; this would be acceptable for small sample numbers only, especially if imidazole, that has not been specially purified, was used owing to the interfering peaks that would be found.

Ticarcillin

Of particular note in the assay described for ticarcillin was the ability to separate and quantitate the two ticarcillin isomers, the pharmacokinetics of which can be studied for the first time. The interference by the small serum peak can be avoided by increasing the retention to allow resolution, by subtraction of the peak area found in a pre-dose sample or by running a penicillinase blank; of the options the second was the most rapid and was routinely adopted.

As would be expected, manual measurement and summation of total ticarcillin was less precise than integrated summation of area, with correction for background especially if faster elution times are used. However, measurement of peak height for each isomer and summation compared well with blank-corrected integrated area; using peak height in this way a negligible contribution from the endogenous peak was noted.

Despite investigating a number of penicillins and their penicillinoic acids,

none had a k' value appropriate for a useful internal standard. The internal standard used was suitable in terms of its chromatographic retention; however, early problems of variable relative recovery proved attributable to variable recovery of thienylbutyric acid, a problem resolved by using a mechanical shaker. There are a number of other areas in the ticarcillin assay which can lead to imprecision; in the extraction step it was found to be essential to add the acid after the diethyl ether and to mix immediately; delay resulted in significant and variable losses of ticarcillin which is acid-labile. Losses, owing to residual acidity, can occur, if the extract is then evaporated to dryness; extracts were therefore concentrated by evaporation into phosphate buffer.

The precision attainable with this assay is superior to that reported for a microbiological assay [8].

CONCLUSION

Since both clavulanic acid and ticarcillin are labile, correct storage and handling of samples are essential for accurate results; the use of HPLC provides an ideal approach for the fast, reliable, precise and accurate assay of both analytes in serum and urine.

The ticarcillin assay is the first to show the existence of the two isomers. The assays developed here are suitable for the estimation of both clavulanic acid and the ticarcillin isomers in clinical studies.

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