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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF CLAVULANATE IN HUMAN PLASMA AND URINE BY FLUORIMETRIC DETECTION

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SUMMARY

A high-performance liquid chromatographic method with fluorimetric detection has been developed for the determination of clavulanate in human urine and plasma. Clavulanate in plasma samples was ultrafiltered using YMT membrane and reacted with benzaldehyde in phosphate buffer solution (pH 3.8) at 100°C for 20 min. Clavulanate in urine samples was filtered with a polyacrylate membrane after ten-fold dilution, and reacted under the same conditions as those for plasma samples. The fluorescent product thus formed from clavulanate was separated from ordinary components of plasma and urine on a reversed-phase C_{18} column followed by fluorimetric detection (λ_{ex} = 386 nm, λ_{em} = 460 nm). The within- and between-run precisions were of the order of 4.02% (n = 10) and 6.23% (n = 5) for plasma samples at a level of 0.67 μg/ml. The detection limit was as low as 10 ng/ml in plasma samples with a 50-μl injection. Coexisting ticarcillin, amoxicillin or 1-amino-4-hydroxybutan-2-one (which is a metabolite of clavulanate in rat and dog) did not interfere in the clavulanate assay.

INTRODUCTION

Clavulanate is a potent, irreversible β-lactamase inhibitor [1]. The in vitro [2—7] and in vivo [8- 15] experiments revealed that a combined use of a

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conventional β-lactam antibiotic with clavulanate is effective against various β-lactamase-producing Gram-positive and Gram-negative bacteria. A clinical trial for coadministration with ticarcillin is now being investigated in Japan.

The assays of clavulanate in pharmaceutical preparations and in body fluids have been achieved mainly by microbiological methods [16]. A few papers have described high-performance liquid chromatographic (HPLC) methods for the determination of clavulanate in urine and serum [17—21]. However, since clavulanate has no ultraviolet (UV) absorption maximum above 210 nm [2], the UV detection at an accessible wavelength limits the assay of clavulanate concentration as high as 5 μg/ml in human urine [17]. Therefore, in order to enhance the detectability, pre-column and post-column derivatization procedures were investigated for the determination of lower levels of clavulanate: Foulstone and Reading [18] presented the assay method based on the pre-column reaction with imidazole followed by detection at 312 nm, while we reported the HPLC assay using post-column alkaline degradation followed by detection at 270 nm [19, 20].

This paper deals with a fluorimetric HPLC method using pre-column reaction with benzaldehyde in phosphate buffer (pH 3.8) for the determination of clavulanate in urine and plasma, and application of the method to the assay of clavulanate after intravenous infusion of a mixture of ticarcillin and clavulanate.

EXPERIMENTAL

Reagents and materials

Potassium clavulanate and amoxicillin trihydrate were kindly donated from Beecham Yakuhin (Tokyo, Japan). Benzaldehyde was purchased from Nakarai Chemicals (Kyoto, Japan) and used without further purification. Other chemicals used were of analytical-reagent grade. Deionized, glass-distilled water and glass-distilled methanol were used for the preparations of buffer solutions and HPLC eluents.

A 2% benzaldehyde solution was prepared by diluting 2 ml of benzaldehyde to 100 ml with methanol. A 1 *M* phosphate buffer solution was prepared by dissolving 15.601 g of $NaH_2PO_4 \cdot 2H_2O$ in 80 ml of water, adding 1 ml of 1 *M* phosphoric acid and diluting to 100 ml with water.

HPLC equipment and conditions

A liquid chromatograph (LC-5A, Shimadzu, Kyoto, Japan) equipped with a spectrofluorimeter (RF-540, Shimadzu) with a 12-μl flow-through cell was used. For clavulanate assay, a reversed-phase C_{18} column (15 cm × 4.6 mm I.D.) packed with Develosil ODS-5 (5 μm) (Nomura Chemicals, Seto, Japan) was used. A pre-column (3 cm × 4.6 mm I.D.) packed with the same packing materials was used to guard the main column. The eluent used was water—methanol (1:2) for both plasma and urine samples. The flow-rate was maintained at 0.8 ml/min. Fluorescence detection was performed with excitation at 386 nm and emission at 460 nm. All chromatographic operations were carried out at ambient temperature. For the separation of clavulanate and amoxicillin fluorophores, the same HPLC equipment and conditions as those for the

clavulanate assay were used, except that the eluent used was water—methanol (1:1).

Pre-column reaction procedure of clavulanate in plasma and urine

Plasma samples. A 150–200 μl aliquot of the plasma sample was ultrafiltered using an MPS-1 micropartition system (Amicon, Tokyo, Japan) with an Amicon YMT membrane at 1500 *g* for 10 min at ambient temperature. To a 50-μl aliquot of the ultrafiltrate, 150 μl of 1 *M* phosphate buffer (pH 3.8) solution and 20 μl of 2% benzaldehyde solution were added. Then, the reaction solution was kept at 100°C for 20 min and was immediately cooled to room temperature. A 20–50 μl portion of the solution was exactly injected into an HPLC column.

Urine samples. The urine samples were diluted ten-fold with water and filtered with a 0.45-μm acrylate copolymer membrane (Gelman Science, Tokyo, Japan). To a 100-μl aliquot of the ultrafiltrate, 300 μl of 1 *M* phosphate buffer (pH 3.8) solution and 40 μl of 2% benzaldehyde solution were added. The solution was treated according to the same procedures as those for plasma samples described above.

Assay of clavulanate in serum and urine

Ticarcillin (3 g) and clavulanate (200 mg) were dosed intravenously at a constant rate for 0.75–1 h to four healthy male volunteers. A blood sample was collected 0, 0.5, 1, 1.5, 2, 4, 6 and 8 h after initiation of infusion. The blood sample was centrifuged at 1500 *g* for 10 min to separate the plasma layer. A urine sample was collected 0, 0.5, 1.5, 3, 5, 7 and 9 h after initiation of infusion. The plasma and urine samples were added to a small amount of solid carbon dioxide and stored at −80°C until assay. These samples were treated according to the procedures described above.

The standard solutions were prepared by dissolving known amounts of clavulanate in control plasma or urine, and treated in the same manner as described above. A calibration graph of peak height versus concentration was constructed.

RESULTS AND DISCUSSION

Pre-column reaction conditions

In previous papers [22, 23], we reported that clavulanate was degraded in 0.1 *M* Na_2HPO_4 solution (pH 9.2) to four pyrazine derivatives which have UV absorption maxima at 280 nm and exhibit fluorescence with an excitation maximum at 280 nm and an emission maximum at 340 nm. However, the UV absorption and fluorescence intensity of these degradation products were not strong enough to allow the quantitation of clavulanate in body fluids. On the other hand, when clavulanate was degraded in weakly acidic solutions in the presence of benzaldehyde, it yielded an intensely fluorescent product. The excitation and emission spectra of this product are shown in Fig. 1. Therefore, we applied this acidic degradation reaction to the assay of clavulanate in plasma and urine. The fluorescent product was separated from the ordinary components of plasma and urine on a C_{18} column using methanol—water as an

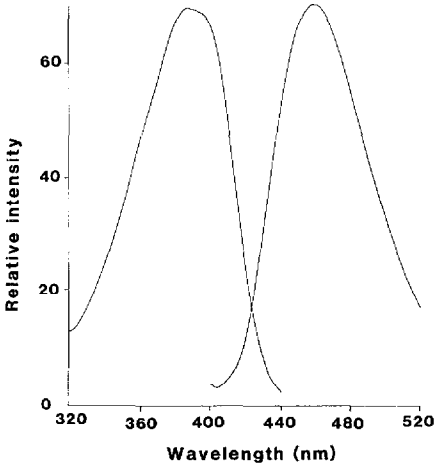

Fig. 1. Excitation and emission spectra of the fluorescent product of clavulanate. To a 1-ml aliquot of a clavulanate solution (30 μg/ml), 3 ml of 1 *M* phosphate buffer solution (pH 3.8) and 400 μl of 2% benzaldehyde solution were added. The solution was allowed to stand at 100°C for 20 min, and was immediately cooled to room temperature. The reaction solution was diluted five-fold with water—methanol (1:2), and the fluorescent spectra were measured.

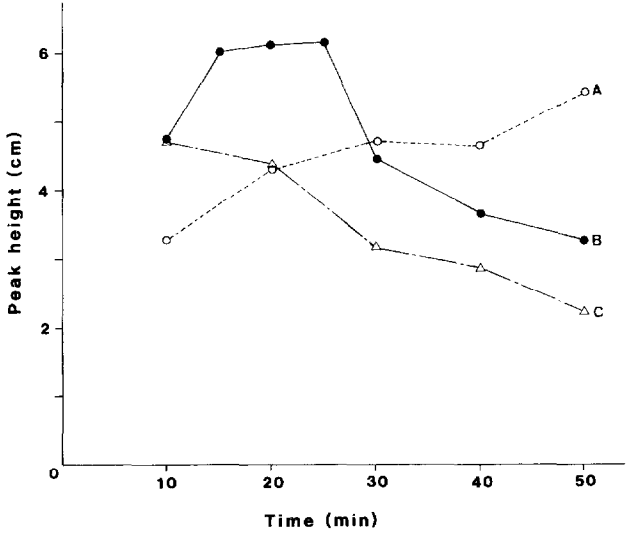

Fig. 2. Effect of pH on the formation of the clavulanate fluorophore in a plasma sample. Clavulanate (1.67 μg/ml) in plasma samples was reacted with 2% benzaldehyde in 1 *M* phosphate buffer solution adjusted to (A) pH 3.3, (B) pH 3.8 and (C) pH 4.5. A 20-μl portion of each sample solution was loaded onto a column, and the peak height was plotted against reaction time.

effluent, and detected at excitation wavelength 386 nm and emission wavelength 460 nm.

The factors affecting the pre-column reaction such as concentration and pH of phosphate buffer solution, benzaldehyde concentration and reaction temperature were examined. The change in the phosphate buffer concentration

between 0.5 and 2 *M* showed almost no effect on the fluorescence intensity. As found in Fig. 2, when the 1 *M* phosphate buffer solution at pH 3.8 was used, the maximum and constant fluorescence intensities were obtained at reaction times between 15 and 25 min. The effect of benzaldehyde concentration upon fluorescence is shown in Fig. 3, which indicates that the fluorescence intensity obtained by the reaction with benzaldehyde at a 2% concentration was stronger than that at a concentration of 1 or 3%. At a temperature lower than 100°C, the reaction rate and the maximum fluorescence intensity were decreased. Thus, the reaction conditions were selected as described under Experimental for the assay of clavulanate in plasma samples. Similar results were also obtained for the reaction of clavulanate in urine samples and so the same reaction conditions were adopted.

The fluorescent product was stable for at least 1 h at room temperature.

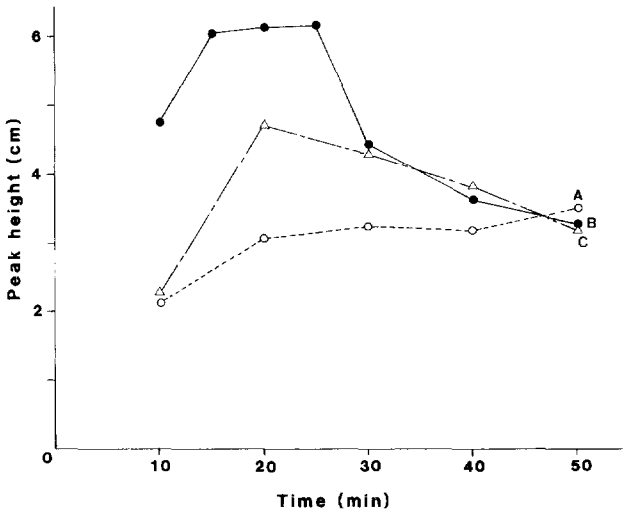

Fig. 3. Effect of concentration of benzaldehyde on the formation of the clavulanate fluorophore in a plasma sample. Clavulanate (1.67 μg/ml) in plasma samples was reacted with (A) 1%, (B) 2% and (C) 3% benzaldehyde in 1 *M* phosphate buffer solution (pH 3.8). A 20-μl portion of each sample solution was loaded onto a column, and the peak height was plotted against reaction time.

HPLC separation and interference

Figs. 4 and 5 show the separation of clavulanate fluorophore from the background components of plasma and urine after administration of a mixture of ticarcillin and clavulanate. It was eluted within 5 min without the interference of the background components. The presence of ticarcillin or 1-amino-4-hydroxybutan-2-one (a metabolite of clavulanate in rat and dog [24]) did not interfere in the assay of clavulanate, whereas amoxicillin, which can also be coadministered with clavulanate, yielded the fluorescent product having the same retention time as that of clavulanate fluorophore. Therefore, for the plasma or urine samples collected following combined dose of clavulanate and amoxicillin, the mobile phase composition should be changed to water–methanol (1:1), which can separate the fluorophore of clavulanate from those of amoxicillin and amoxicilloic acid (a metabolite of amoxicillin) (Fig. 6).

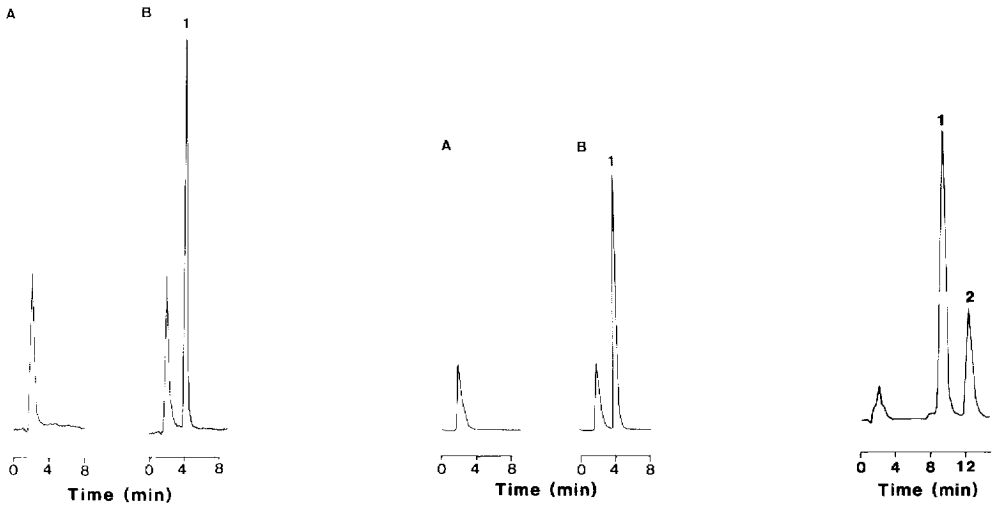

Fig. 4. Chromatogram of clavulanate fluorophore in human plasma. The eluent was monitored with excitation at 386 nm and emission at 460 nm. Pre-dose plasma sample (A) and 2-h post-dose plasma sample (B) were treated according to the procedures described under Experimental. A 20-μl portion of each sample solution was loaded onto a column. Peak 1 is clavulanate fluorophore. Clavulanate concentration was estimated to be 3.25 μg/ml.

Fig. 5. Chromatogram of clavulanate fluorophore in human urine. The eluent was monitored with excitation at 386 nm and emission at 460 nm. Pre-dose urine sample (A) and 1.5–3 h post-dose urine sample (B) were treated according to the procedures described under Experimental. A 20-μl portion of each sample solution was loaded onto a column. Peak 1 is clavulanate fluorophore. Clavulanate concentration was estimated to be 35.2 μg/ml.

Fig. 6. Separation of clavulanate and amoxicillin fluorophores. To a 100-μl aliquot of clavulanate (6.7 μg/ml) and amoxicillin (8.3 μg/ml) solutions, 300 μl of 1 *M* phosphate buffer solution (pH 3.8) and 40 μl of 2% benzaldehyde solution were added. The solution was allowed to stand at 100° C for 20 min, and was immediately cooled to room temperature. A 20-μl portion of the sample solution was loaded onto a column. Peaks 1 and 2 are clavulanate and amoxicillin fluorophores, respectively.

The structural investigations of these reaction products will be discussed elsewhere in the near future.

Recovery and precision

Table I shows the total recovery of clavulanate from plasma and urine, and the coefficients of variation. The within- and between-run precisions were of the order of 4.02% (n = 10) and 6.23% (n = 5) for plasma samples at a level of 0.67 μg/ml. For urine samples at a level of 16.8 μg/ml, the within- and between-run precisions were of the order of 3.15% (n = 10) and 5.23% (n = 5).

Linearity and sensitivity

The calibration graphs over the range 0.05—20 μg/ml for plasma samples and 5—400 μg/ml for the urine samples were linear and passed through the origin, with the correlation coefficient above 0.99. The detection limit was 10 ng/ml in plasma samples and 100 ng/ml in urine samples with a 50-μl injection (signal-to-noise ratio = 3).

TABLE I

RECOVERY OF CLAVULANATE FROM PLASMA AND URINE

Sample	Added (μg/ml)	Recovery (%)	Coefficient of variation (n = 5) (%)
Urine	33.5	76.9	4.03
Plasma	1.34	79.7	2.64

Comparison with previously reported HPLC method

Ticarcillin and clavulanate were intravenously administered to healthy male volunteers, and plasma and urine samples were collected at predetermined time intervals. The plasma and urine levels of clavulanate were assayed by the proposed and previously reported [20] HPLC methods. For the correlation of the clavulanate assay in plasma samples, the equation for the regression line was $HPLC_{proposed}$ = 1.082 (HPLC) + 0.077, with a correlation coefficient of 0.97. For the correlation of the clavulanate assay in urine samples, the equation for the regression line was $HPLC_{proposed}$ = 0.931 (HPLC) + 0.777, with a correlation coefficient of 0.97. In a previous paper [20], we found that the HPLC assay could agree very well with bioassay. Therefore, we conclude that there are good agreements between the proposed HPLC method and bioassay.

Application

Fig. 7 shows the mean plasma concentration—time curve of clavulanate following intravenous infusion of ticarcillin and clavulanate to four healthy male volunteers. The urinary excretion data are listed in Table II.

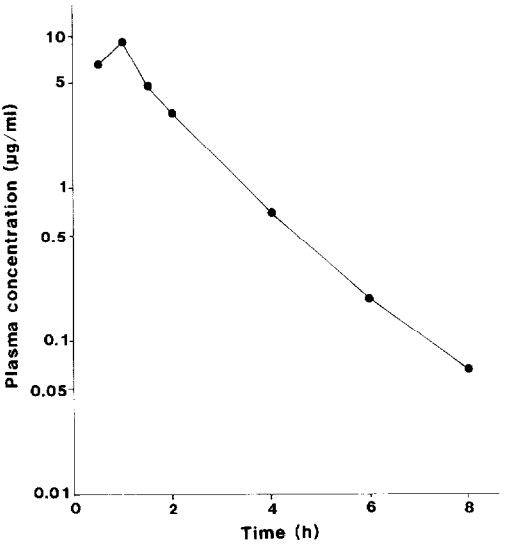

Fig. 7. Mean plasma concentrations of clavulanate after intravenous infusion to four healthy male subjects.

TABLE II

URINARY EXCRETION OF CLAVULANATE AFTER INTRAVENOUS INFUSION OF TICARCILLIN AND CLAVULANATE

Data are expressed as percentages of the dose.

Subject	0—9 h Excretion (%)
1	56.7
2	42.0
3	39.5
4	38.8
Mean	44.3
S.D.	8.41

CONCLUSION

The proposed HPLC method for the determination of clavulanate in plasma and urine is specific to intact clavulanate in the presence of ticarcillin, amoxicillin or 1-amino-4-hydroxybutan-2-one, and permits the detection of clavulanate concentrations down to 10 ng/ml in plasma with a 50-μl injection. The assay method developed here is suitable for the estimation of clavulanate after a combined dose with ticarcillin in clinical samples.

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