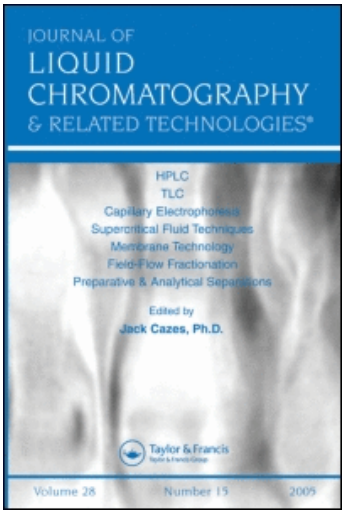

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Improved High Performance Liquid Chromatographic Assay of Clavulanic Acid And Sulbactam by Postcolumn Alkaline Degradation

J. Haginaka[a]; J. Wakai[a]; H. Yasuda[a]; T. Uno[a]; T. Nakagawa[b]
[a] Faculty of Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya, Japan [b] Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

To cite this Article Haginaka, J. , Wakai, J. , Yasuda, H. , Uno, T. and Nakagawa, T.(1985) 'Improved High Performance Liquid Chromatographic Assay of Clavulanic Acid And Sulbactam by Postcolumn Alkaline Degradation', Journal of Liquid Chromatography & Related Technologies, 8: 13, 2521 — 2534
To link to this Article: DOI: 10.1080/01483918508076585
URL: http://dx.doi.org/10.1080/01483918508076585

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(13), 2521-2534 (1985)

IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF CLAVULANIC ACID AND SULBACTAM BY POSTCOLUMN ALKALINE DEGRADATION

J. Haginaka[1]*, J. Wakai[1], H. Yasuda[1], T. Uno[1], and T. Nakagawa[2]

[1]*Faculty of Pharmaceutical Sciences*
Mukogawa Women's University
4-16 Edagawa-cho
Nishinomiya 663, Japan
[2]*Faculty of Pharmaceutical Sciences*
Kyoto University
Sakyo-ku
Kyoto 606, Japan

ABSTRACT

Improved high-performance liquid chromatographic methods have been developed for the determination of clavulanic acid and sulbactam in plasma and urine. The plasma samples containing clavulanic acid or sulbactam were ultrafiltered using Amicon YMT membranes, and the urine samples were filtered with a 0.45-μm acrylate-copolymer membrane after ten-fold dilution with distilled water. Thereby, they were easily separated from the normal components of plasma and urine on a C_{18} reversed-phase column using an eluent containing methanol as an organic modifier. The effluent was led to postcolumn alkaline degradation in 0.5-0.75 M sodium hydroxide solution, which had the same methanol content as that of the HPLC eluent. Subsequently, the degradation products were detected at 272 nm for

*To whom correspondence should be addressed.

 0148-3919/85/0813-2521$3.50/0

clavulanic acid and at 278 nm for sulbactam. These methods, which are accurate and precise, permit determination of 25 ng/mL of clavulanic acid and sulbactam in plasma. At a clavulanic acid or sulbactam concentration of 0.5 μg/mL in plasma, within- and between-run precisions were of the order of 1.89-5.65% and 1.33-3.21%, respectively.

INTRODUCTION

The increase in the prevalence of β-lactamase-producing strains of both Gram-positive and Gram-negative bacteria has narrowed the antibacterial spectrum of β-lactam antibiotics. One way to resolve this problem is the coadministration of a β-lactamase inhibitor with a conventional β-lactam antibiotic. Clavulanic acid and sulbactam, respectively, are the potent β-lactamase inhibitors isolated from ATCC 27064 by Beecham groups (1) and semi-synthesized by Pfizer groups (2).

In previous papers (3,4), we reported that clavulanic acid and sulbactam are rapidly degraded in alkaline methanolic solutions to methyl 8-hydroxy-6-oxo-4-aza-2-octenoate and 5-carboxy-6-methyl-6-sulfino-4-aza-2-heptenoate, which show ultraviolet absorption maxima at 267 and 276 nm, respectively, and we developed accurate, sensitive high-performance liquid chromatographic (HPLC) methods for the determination of clavulanic acid and sulbactam in plasma and urine using the above reaction as postcolumn derivatization. Those methods, however, needed relatively long run times (10-20 min), and the detection limits were 0.1 μg/mL in plasma. This paper deals with the improved HPLC assays of clavulanic acid and sulbactam in plasma and urine. In order to attain faster and more sensitive assays, we improved the HPLC methods with respect to the following points. 1) The separation is performed using a high-efficiency small particle column (5-7 μm). 2) Plasma and urine samples, respectively, are ultrafiltered using YMT membranes and filtered with 0.45-μm microfilters after 10-fold dilution with distilled water. 3) The postcolumn reagent used is 0.5-0.75 M sodium hydroxide solution, which has the same methanol content as

that of an HPLC eluent. The improved methods allow the assays to be performed twice or three times as fast and four times as sensitive as the previous methods (3,4).

MATERIALS AND METHODS

Reagents

Potassium clavulanate and sodium sulbactam were donated from Beecham Yakuhin (Tokyo, Japan) and Pfizer-Taito (Tokyo), respectively. Tetrabutylammonium bromide (TBAB) and other chemicals of reagent grade were purchased from Nakarai Chemicals (Kyoto, Japan). Deionized, glass-distilled water and glass-distilled methanol were used for the preparations of HPLC eluents.

Phosphate buffer, 0.005 M. Prepared by dissolving 1.791 g of Na_2HPO_4 $12H_2O$ and 0.780 g of NaH_2PO_4 2 H_2O in water and diluting to 1 L.

HPLC Equipment and Operating Conditions

Two pumps were used, one for delivering an HPLC eluent (Trirotar-V, Japan Spectroscopic, Tokyo) and the other for delivering a postcolumn reagent (NP-DX-2, Nihon Seimitsu Kagaku, Tokyo). A portion of a sample solution was loaded onto a column via a Model VL-614 loop injector (Japan Spectroscopic). The detector used was a UVIDEC-100-V spectrophotometer (Japan Spectroscopic) equipped with an 8-μL flow-through cell. Develosil ODS-5 (5 μm) (Nomura Chemicals, Seto, Japan) packed in a 150 x 4.6 mm i.d. stainless steel tubing was used as a stationary phase except for the assay of clavulanic acid in plasma, for which Zorbax ODS-7 (7 μm)(Du pont Instrument, System, Delaware, USA) was used. The precolumn (30 x 4.6 mm i.d.) packed with Develosil ODS-10 (10 μm) was used and frequently repacked to guard the main column. The eluents used were as follows; eluents A and B, 5 mM TBAB plus 5 mM phosphate buffer:methanol (2.7/1 and 3.5/1, v/v) for the assay of clavulanic acid in plasma; eluent C, 5 mM TBAB plus 1 mM phosphate buffer:

methanol (2.5/1, v/v) for clavulanic acid in urine, eluent D, 5 mM TBAB plus 0.5 mM phosphate buffer:methanol (1.5/1, v/v) for sulbactam in plasma; eluent E, 5 mM TBAB plus 0.5 mM phosphate buffer: methanol (2/1, v/v) for sulbactam in urine. The flow rates of the eluent in all cases were maintained at 0.8 mL/min. The postcolumn reagent was 0.5 M sodium hydroxide solution for clavulanic acid and 0.75 M for sulbactam (which had the same methanol content as that of the HPLC eluent), and delivered at a flow rate of 0.2 mL/min. A 0.5 mm i.d. x 1 m Teflon tubing was used as a reactor. Detection was performed at 272 nm for clavulanic acid and 278 nm for sulbactam. All separations and postcolumn reactions were carried out at ambient temperature.

Sample Preparations

For plasma samples, a clavulanic acid or sulbactam standard was dissolved in human control plasma. The plasma samples were ultrafiltered using an Amicon MPS-1 micropartition system with Amicon YMT membrane at 1500 x g for 10 min. A 20-μL portion of the ultrafiltrate was accurately loaded onto an HPLC column. For urine samples, a clavulanic acid or sulbactam standard was dissolved in human control urine. The urine samples were diluted 10-fold with distilled water, and filtered with a 0.45-μm acrylate-copolymer membrane (Showa Denko, Tokyo). A 20-μL portion of the filtrate was accurately loaded onto the HPLC column.

Comparison with Reported HPLC Method or Bioassay

Clavulanic acid spiked at various concentrations in plasma or urine samples was assayed by the modification of the method reported by Foulstone and Reading (5). Sulbactam spiked at various concentrations in plasma or urine samples was assayed by an agar diffusion method with *Escherichia coli* C/R ms 149 as the test organism. Although sulbactam itself dose not inhibit growth of the test organism at the concentration employed, it inactivates β-lactamase, thus rendering the organism susceptible to inhibition by ampicillin in the medium. The samples mentioned above, respec-

tively, were also assayed by the improved HPLC methods, and the assay values were compared (Figures 3 and 4).

Assay of Clavulanic Acid in Plasma

Clavulanic acid (200 mg) and ticarcillin (3 g) were administered to a healthy male volunteer by a 1-h intravenous infusion. A blood sample was collected at 0.5, 1, 1.5, 2, 4, 6, and 8 h after initiation of infusion. The plasma layer was obtained, and stored at -80°C until assay. The plasma levels of clavulanic acid were assayed according to the procedures described above.

RESULTS

HPLC Separation and Detection

In this study, a high-efficiency small particle C_{18} column (5-7 μm) was used instead of a 10-μm diameter packing material used in the previous studies (3,4), and the flow rate of an HPLC eluent containing methanol as an organic modifier was maintained at 0.8 mL/min. In order to obtain the optimum postcolumn reaction conditions, the flow injection technique was adopted. A carrier solution (eluent A) was delivered at a flow rate of 0.8 mL/min, and then the factors affecting the postcolumn alkaline degradation such as concentration and flow rate of sodium hydroxide solution, and reaction coil length were examined. The maximum and constant response was obtained with sodium hydroxide solution at 0.5 M (or more) for clavulanic acid and at 0.75 M (or more) for sulbactam, when the reagent was delivered at a flow rate of 0.2 mL/min to a 1-m reaction coil (0.5 mm i.d.). The residence time was about 12 s, when the 1-m reaction coil was used. The actual detection wavelength was determined by varying the wavelength of HPLC detector. The optimal detection wavelengths were 272 nm for clavulanic acid and 278 nm for sulbactam.

Under the precolumn reaction conditions thus established, the separation and detection of these β-lactamase inhibitors were per-

formed. Figure 1 shows the chromatograms of clavulanic acid and sulbactam in plasma treated according to the pretreatment procedures described in the experimental section. Figure 2 shows the chromatograms of clavulanic acid and sulbactam in 10-fold diluted urine. They were well separated from the ordinary components of plasma and urine, and eluted within 6 min for plasma samples, and 9 min for urine samples. The assay of a low concentration (< 0.1 μg/mL) of clavulanic acid in plasma, which was interfered with the ordinary trace components, was performed by decreasing methanol content of eluent A (eluent B).

The calibration graphs for clavulanic acid and sulbactam were linear in the concentrations ranging from 0.05 to 10 μg/mL for plasma samples and from 1.0 to 50 μg/mL for neat urine samples, and passed through the origin. The lower limits of accurate determination of clavulanic acid and sulbactam were as low as 25 ng/mL for plasma samples and 0.5 μg/mL for neat urine samples.

Recovery, Precision and Interference

Table 1 shows recovery of clavulanic acid and sulbactam from spiked plasma, and coefficients of variation. The recovery of clavulanic acid and sulbactam from urine was almost 100%.

Tables 2 and 3 show within- and between-run precisions of clavulanic acid and sulbactam assays in plasma and urine.

Coexisting ampicillin, ticarcillin or cefoperazone did not interfere with the clavulanic acid or sulbactam assay.

Comparison with HPLC or Microbiological Method

Plasma samples spiked at various clavulanic acid concentrations were assayed by using both the improved and previously reported (5) HPLC methods (Figure 3). The equation for regression line was $HPLC_{improved}$ = 1.007 x (HPLC) + 0.140, with a correlation coefficient of 0.994. Figure 4 shows the correlation of the sulbactam assay in plasma samples by using the improved HPLC method and bioassay. The equation for regression line was $HPLC_{improved}$ = 0.960 x (bioassay) - 0.016, with a correlation coefficient of

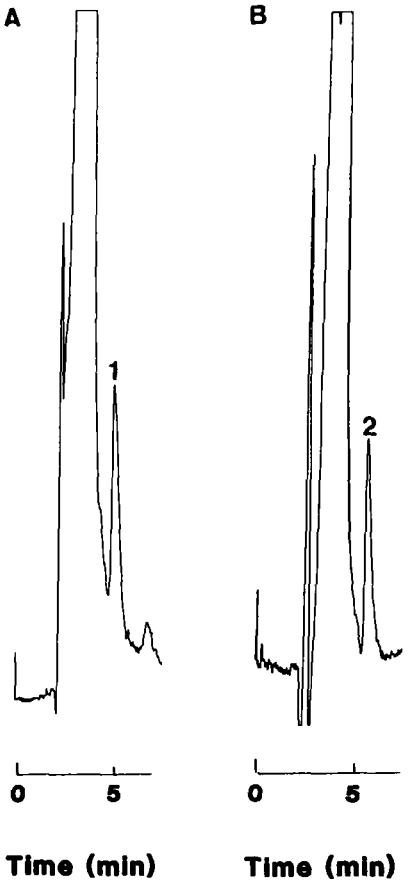

Figure 1. Chromatograms of clavulanic acid (A) and sulbactam (B) in control plasma. Control plasma spiked at a clavulanic acid or sulbactam concentration of 0.5 µg/mL was treated according to the procedures described in the text. Eluent: A, eluent A; B, eluent C. Sample volume: 20 µL. Sensitivity: 0.008 aufs. Keys: 1, clavulanic acid; 2, sulbactam.

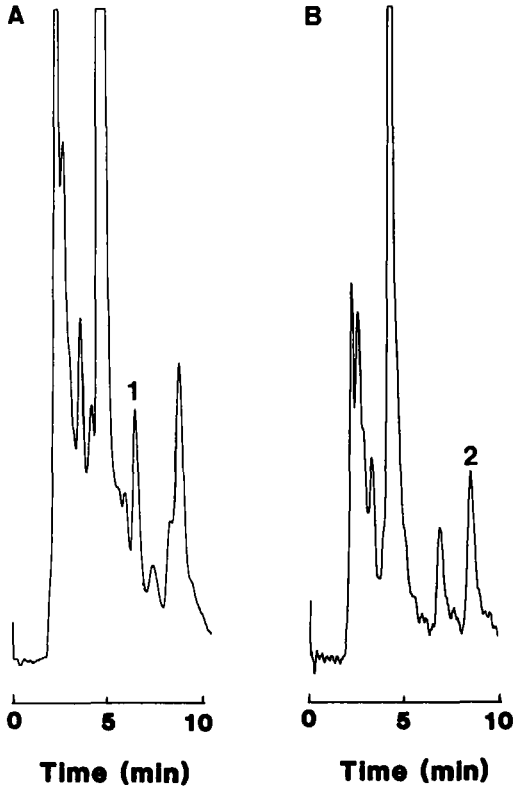

Figure 2. Chromatograms of clavulanic acid (A) and sulbactam (B) in control urine. Control urine spiked at a clavulanic acid or sulbactam concentration of 5.0 μg/mL was treated according to the procedures described in the text. Eluent: A, eluent D; B, eluent E. Sample volume: 20 μL. Sensitivity: 0.008 aufs. Keys are the same as those of the legend to Figure 1.

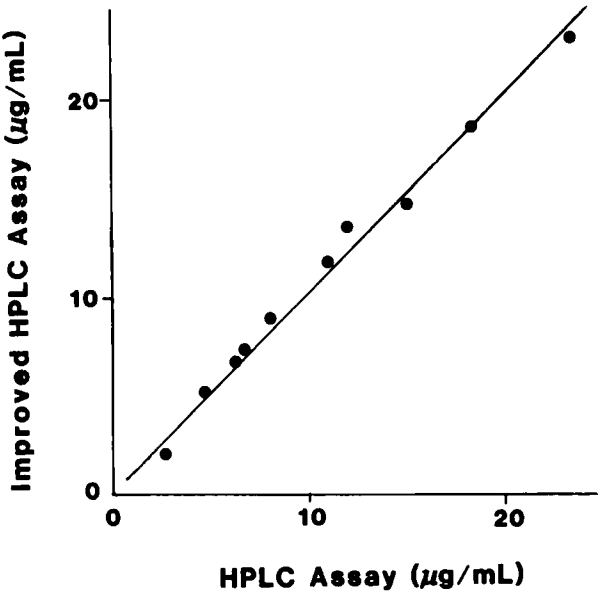

Figure 3. Comparison of the improved and previously reported HPLC methods for the assay of clavulanic acid.

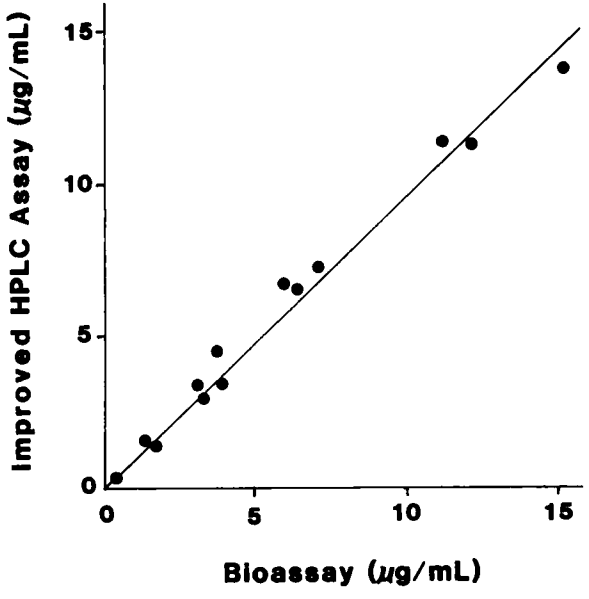

Figure 4. Comparison of the improved HPLC and microbiological methods for the assay of sulbactam.

0.992. Results similar to those described above were obtained with urine samples spiked with clavulanic acid or sulbactam.

Assay of Clavulanic Acid in Plasma

The plasma levels of clavulanic acid after a 1-h intravenous infusion to a healthy male volunteer were determined by the improved HPLC method. Figure 5 shows a semi-logarithmic plot of the time course data.

DISCUSSION

The advantages of the improved HPLC methods are that run times are as short as 6 min for the assays of clavulanic acid and sulbactam in plasma samples and the limits of accurate determination are as low as 25 ng/mL in plasma samples. These were due to high-efficiency column, ultrafiltration technique, and postcolumn reagent containing methanol. Clavulanic acid and sulbactam were well separated from the ordinary components of plasma and urine in a short time by using a high-efficiency small particle column. It has been shown (5-7) that the ultrafiltration technique using a membrane filter, which is commonly used for the drug-protein binding studies, is useful for the pretreatment procedures of plasma samples for HPLC. When plasma proteins were deproteinized with organic solvents (acetonitrile, methanol or acetone), interfering peaks occurred on the chromatograms. The main advantages of the ultrafiltration technique are that there are almost no interfering peaks and the samples need not be diluted. The addition of methanol to the reagent solution resulted in the decrease in the noise level.

Foulstone and Reading (5) reported that their HPLC method had a good correlation with bioassay. Although we were not able to perform bioassay with clavulanic acid, our improved HPLC assay agreed very well with their HPLC assay. Therefore, we concluded that there are good agreements between the improved HPLC method and bioassay. We also found a good correlation between the improved

TABLE 1

Recovery of Clavulanic Acid and Sulbactam from Plasma

	Clavulanic acid		Sulbactam	
Concentration, μg/mL	0.5	5.0	0.5	5.0
Recovery, %	87.3	100	89.1	95.1
CV[a], %	4.46	2.29	3.57	0.89

[a]Coefficient of variation of five analyses.

TABLE 2

Accuracy and Precision of Clavulanic Acid and Sulbactam Assay in Plasma[a]

Assay	Clavulanic acid		Sulbactam	
1[b]	0.483	5.65%	0.498	2.11%
2	0.498	3.28%	0.494	1.89%
3	0.467	3.22%	0.507	3.46%
between-run[c]	0.483	3.21%	0.500	1.33%

[a]Control plasma samples spiked with clavulanic acid or sulbactam at a concentration of 0.5 μg/mL were treated according to the procedures described in the text. [b]Mean ± CV of five replicates. [c]Mean ± CV of three replicates.

TABLE 3

Accuracy and Precision of Clavulanic Acid and Sulbactam Assay in Urine[a]

Assay	Clavulanic acid		Sulbactam	
1[b]	5.01	0.87%	5.02	1.96%
2	5.04	2.61%	5.13	1.75%
3	4.96	3.67%	4.90	1.64%
between-run[c]	5.01	0.83%	5.02	2.29%

[a]Control urine samples spiked with clavulanic acid or sulbactam at a concentration of 5.0 μg/mL were treated according to the procedures described in the text. [b]Mean ± CV of five replicates. [c]Mean ± CV of three replicates.

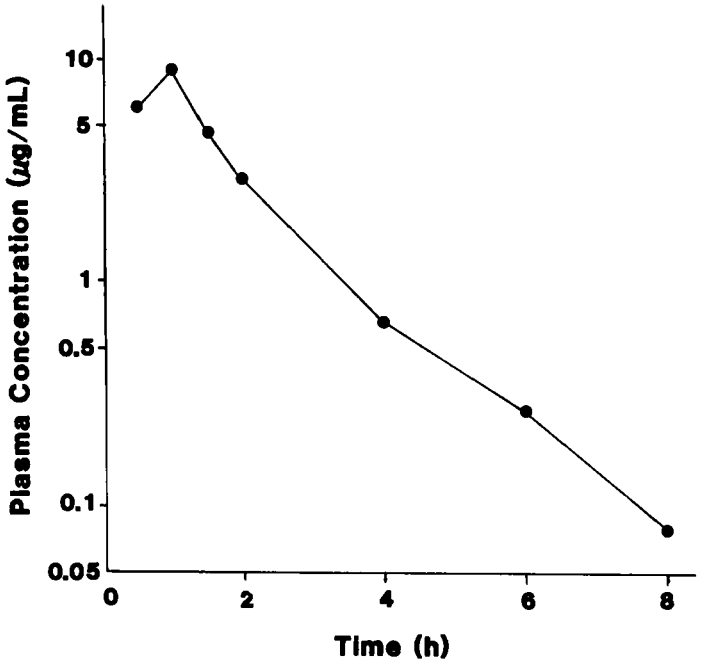

Figure 5. Semi-logarithmic plot of plasma concentration of clavulanic acid after intravenous infusion to a healthy male volunteer.

HPLC assay of sulbactam and bioassay.

The disposition kinetics of clavulanic acid were estimated by moment analysis (8,9). The area under plasma concentration-time curve was 15.6 (μg mL^{-1} h); the mean residence time after intravenous bolus injection, 1.25 (h); the steady-state volume of distribution, 16.0 (L); the total body clearance, 12.8 (L h^{-1}). The pharmacokinetic behavior obtained is consistent with that reported previously (10).

In conclusion, the improved HPLC methods, which are accurate, precise and sensitive, are useful for the determination of clavulanic acid and sulbactam in plasma and urine after therapeutic dose.

ACKNOWLEDGMENTS

The authors thank Pharmaceuticals Research Center, Kanebo Ltd. (Osaka, Japan) for performing the bioassay of sulbactam.

REFERENCES AND NOTES

1. Brown, A. G., Butterworth, D., Cole, M., Hamscomb, G., Hood, J. D., Reading, C., and Rolinson, G. N., Naturally-occurring β-Lactamase Inhibitors with Antibacterial Activity, J. Antibiot., 29, 668, 1976.

2. English, A. R., Retsema, J. A., Girard, A. E., Lynch, J. E., and Barth, W. E., CP-45,899, a Beta-Lactamase Inhibitor That Extends the Antibacterial Spectrum of Beta-Lactams: Initial Bacteriological Characterization, Antimicrob. Agents Chemother., 14, 414, 1978.

3. Haginaka, J., Yasuda, H., Uno, T., and Nakagawa, T., Alkaline Degradation of Clavulanic Acid and High Performance Liquid Chromatographic Determination by Post-column Alkaline Degradation, Chem. Pharm. Bull., 31, 4436, 1983.

4. Haginaka, J., Yasuda, H., Uno, T., and Nakagawa, T., Sulbactam: Alkaline Degradation and Determination by High-performance Liquid Chromatography, Chem. Pharm. Bull., 32, 2752, 1984.

5. Foulstone, M. and Reading, C., Assay of Amoxicillin and Clavulanic Acid, the Components of Augmentin, in Biological Fluids with High-Performance Liquid Chromatography, Antimicrob. Agents Chemother., 22, 753, 1982.

6. Haginaka, J. and Wakai, J., High-Performance Liquid Chromatographic Assay of Carbenicillin, Ticarcillin and Sulbenicillin Using Precolumn Reaction with 1,2,4-Triazole and Mercury(II) Chloride, Analyst, in press.

7. Haginaka, J. and Wakai, J., High-Performance Liquid Chromatographic Assay of Ampicillin, Amoxicillin and Ciclacillin Using Precolumn Reaction with 1,2,4-Triazole and Mercury(II) Chloride, Analyst, under submission.

8. Chan, K. K. H. and Gibaldi, M., Estimation of Statistical Moments and Steady-State Volume of Distribution for a Drug Given by Intravenous Infusion, J. Pharmacokin. Biopharm., 10, 551, 1982.

9. Yamaoka, K., Nakagawa, T., and Uno, T., Moment Analysis for Disposition Kinetics of Several Cephalosporins in Rats, J., Pharm. Phamacol., 35, 19, 1983.

10. Data from Beecham Research Laboratories (Brentford, Middlesex, UK).