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# Determination of a new oral cephalosporin, S-1090, in human plasma and urine by direct injection high-performance liquid chromatography with ultraviolet detection and column switching

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**Abstract**

Direct injection high-performance liquid chromatographic (HPLC) methods with column switching and UV detection were developed for the rapid and accurate determination of S-1090 in human plasma and urine. An internal-surface reversed-phase pre-column and a $C_{18}$ analytical column were used for the plasma assay. Two pre-columns packed with cyano and phenyl materials and a $C_{18}$ analytical column were used for the urine assay. The calibration curves for plasma and urine assays were linear in the ranges 0.09–9 μg/ml and 0.5–100 μg/ml of S-1090, respectively. The relative standard deviations for plasma and urine assays were less than 6% with low relative errors. The established HPLC methods were demonstrated to be useful for clinical pharmacokinetic studies after oral administration of S-1090. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Cephalosporins; S-1090

## 1. Introduction

Compound S-1090, (−)-(6*R*,7*R*)-7-[(*Z*)-2-(2-aminothiazol-4-yl)-2-hydroxyiminoacetamido]-8-oxo-3-(1*H*-1,2,3-triazol-4-yl)thiomethylthio-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid monohydrochloride monohydrate (Fig. 1), is a new oral cephalosporin developed by our laboratories [1,2]. It was found to be highly active against a variety of gram-positive and gram-negative bacteria, and to have a high extent of absorption in various laboratory animals [2,3].

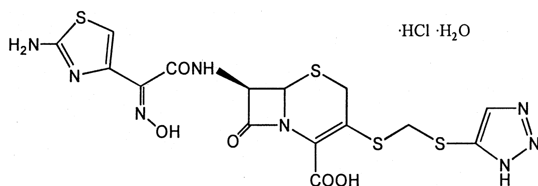


Fig. 1. Chemical structure of S-1090.

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The recent development of high-performance liquid chromatography (HPLC) methods for the assay of cephalosporins in biological fluids was well reviewed [4]. Most of the methods used off-line sample pretreatment such as protein precipitation and solid-phase extraction. Now, we have developed direct injection HPLC methods with a column switching technique and UV detection for the rapid and accurate determination of S-1090 in human plasma and urine. A direct sample injection system involving a column switching technique is substantially advantageous in terms of time- and labor-

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saving features [5,6]. Column switching systems using pre-column and analytical column in series were adopted in the present study: an internal-surface reversed-phase pre-column and a $C_{18}$ analytical column for plasma assay, two pre-columns packed with cyano and phenyl materials, and a $C_{18}$ analytical column for urine assay.

In this paper, we describe the HPLC methods established for the human plasma and urine assays of S-1090 and demonstrate their usefulness for clinical pharmacokinetic studies after oral administration of S-1090.

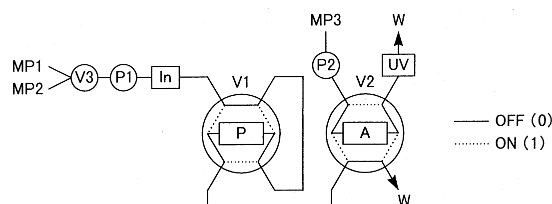


Fig. 2. Schematic diagram of the column switching HPLC system for the assay of S-1090 in human plasma. MP1, MP2 and MP3, mobile phases I, II and III; P1 and P2, pumps 1 and 2; In, injector; P, pre-column; A, analytical column; V1 and V2, six-port switching valves 1 and 2 for column switching; V3, six-port switching valve 3 for mobile phase switching; UV, UV detector; W, waste.

## 2. Experimental

### 2.1. Materials

Compound S-1090 was synthesized in our laboratories. Sodium 1-heptanesulfonate (1-hydrate) was of special grade for ion-pair HPLC (Eastman Kodak, New York, USA). Methanol and water were of HPLC grade. All other reagents were of analytical reagent grade.

### 2.2. HPLC system

The assay of S-1090 in human plasma was performed with a liquid chromatograph composed of two solvent delivery pumps, LC-6A and LC-6AD, and an SIL-6B autosampler, which were all controlled automatically by an SCL-6B controller, and also an SPD-6AV ultraviolet detector set at 290 nm, and a C-R4A integrator (Shimadzu, Kyoto, Japan). The analytical separation was done on an L-1180 pre-column (10 μm, 30 mm×4.6 mm I.D., Chemicals Inspection and Testing Institute, Tokyo, Japan) and a YMC-Pack ODS-AM AM-302 analytical column (5 μm, 150 mm×4.6 mm I.D., YMC, Kyoto, Japan), using a column switching system. As shown in Fig. 2, the column switching system was connected through a PT-8000 self-controllable valve actuator (Tosoh, Tokyo, Japan) equipped with two six-port switching valves, V1 and V2, for column switching and four three-port switching valves, one (V3) of which was used for mobile phase switching. Mobile phase I for adsorption of S-1090 on the pre-column was of 0.05 *M* phosphate buffer ($KH_2PO_4$–$H_3PO_4$, pH 2.5). Mobile phase II for desorption of S-1090 from the pre-column was composed of 0.05 *M* phosphate buffer (pH 2.5)–methanol (75:25). Mobile phase III for analysis on the analytical column was a 0.05 *M* phosphate buffer (pH 2.5)–methanol (80:20) mixture containing 1 m*M* sodium 1-heptanesulfonate. According to the time program shown in Table 1, mobile phases I and II were switched using switching valve V3 and their flow-rates were changed, while the flow-rate of mobile phase III was kept at 1.2 ml/min. All of the mobile phases were filtered with a Type FR-70 membrane filter (0.7 μm, Fuji Photo Film, Tokyo, Japan) and degassed under reduced pressure before use. The chromatography was carried out at the well-controlled room temperature of 25±2°C. Before and after analysis of each run, checks were done of the retention time (ca. 10 min) of S-1090 on the pre-column and that (ca. 27 min) on the analytical column in the column switching system. Each column switching valve in position 0 (off) or 1 (on) was initially set for the sample injection at the position (V1=0, V2=0) shown in Fig. 2. The time program of the column switching system is also shown in Table 1.

The assay of S-1090 in human urine was performed in a similar manner to the plasma assay described above. Different points are described below. Three pumps, an LC-6A and two LC-6AD were used for urine assay. The analytical separation was accomplished on a Nucleosil CN pre-column I (5 μm, 50 mm×4.6 mm I.D., Chemco Scientific, Kyoto, Japan), a Spherisorb Ph pre-column II (5 μm, 50 mm×4.6 mm I.D., Chemco Scientific), and a

Table 1
Time program of the column switching HPLC system for the assay of S-1090 in human plasma

| Time (min) | V1 | V2 | Mobile phase | Flow-rate (ml/min) | Function |
|---|---|---|---|---|---|
| 0 | 0 | 0 | I | 1.5 | Start (1st), adsorption |
| 4 | 0 | 0 | II | 1.5 | Remove early-eluting peaks |
| 7 | 0 | 0 | II | 1.0 | Remove early-eluting peaks |
| 8.5 | 0 | 1 | II | 1.0 | Desorption from pre-column |
| 12.7 | 1 | 0 | II | 3.0 | Analysis, back-flush (pre-column) |
| 16.0 | 1 | 0 | I | 3.0 | Analysis, back-flush (pre-column) |
| 16.7 | 1 | 0 | I | 1.5 | Analysis, back-flush(pre-column) |
| 18.0 | 0 | 0 | I | 1.5 | Analysis, equilibration |
| 21.2 | 0 | 0 | I | 1.0 | Analysis, equilibration |
| 22.0 | 0 | 0 | I | 1.5 | Analysis (end 31 min), start (2nd) |

Nucleosil $C_{18}$ analytical column (5 μm, 150 mm× 4.6 mm I.D., Chemco Scientific), using a column switching system. As shown in Fig. 3, the column switching system was connected through the two PT-8000 self-controllable valve actuators equipped with three six-port switching valves, V1, V2 and V3, for column switching. Mobile phase I for the two pre-columns was a 0.05 *M* phosphate buffer ($KH_2PO_4$–$H_3PO_4$, pH 2.5)–methanol (97:3) mixture containing 1 m*M* sodium 1-heptanesulfonate.

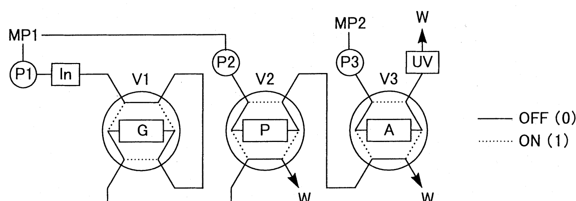


Fig. 3. Schematic diagram of the column switching HPLC system for the assay of S-1090 in human urine. MP1 and MP2, mobile phases I and II; P1, P2 and P3, pumps 1, 2 and 3; In, injector; G, pre-column I; P, pre-column II; A, analytical column; V1, V2 and V3, six-port switching valves 1, 2 and 3 for column switching; UV, UV detector; W, waste.

Mobile phase II for analysis on the analytical column was a 0.05 *M* phosphate buffer (pH 2.5)–methanol (76:24) mixture containing 3 m*M* sodium 1-heptanesulfonate. The flow-rate of mobile phase I for pre-column I was changed according to the time program shown in Table 2, while the flow-rates of mobile phase I for pre-column II and mobile phase II were kept at 1.0 ml/min. Before and after analysis of each run, we checked the retention time (ca. 5 min) of S-1090 on pre-column I, that on pre-column II (ca. 13 min), and that on the analytical column (ca. 30 min) in the column switching system. Each column switching valve was set for the sample injection at the position (V1=0, V2=0, V3=0) shown in Fig. 3. The time program of the column switching system is shown in Table 2.

### 2.3. Analytical procedure

Plasma and urine samples were stored at −70°C until analysis within two months.

A 150–200 μl portion of human plasma and a 300–400 μl portion of human urine were filtered with a Ultrafree C3-HV membrane filter (0.45 μm,

Table 2
Time program of the column switching HPLC system for the assay of S-1090 in human urine

| Time (min) | V1 | V2 | V3 | Flow-rate (ml/min) | Function |
|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0.5 | Start (1st), separation on pre-column I |
| 4 | 0 | 1 | 0 | 0.5 | Transfer from pre-column I |
| 7.7 | 1 | 0 | 0 | 1.0 | Separation on pre-column II |
| 10 | 1 | 0 | 1 | 1.0 | Transfer from pre-column II |
| 15.4 | 0 | 0 | 1 | 0.5 | Transfer from pre-column II |
| 16 | 0 | 0 | 0 | 0.5 | Analysis, equilibration |
| 23 | 0 | 0 | 0 | 0.5 | Analysis (end 33 min), start (2nd) |

Nihon Millipore, Tokyo, Japan) by centrifugation at 1940 *g* for 5 min with a Kaiten-kun portable centrifuge (Funakoshi, Tokyo, Japan). After each 100 μl portion of the plasma filtrate and 200 μl portion of the urine filtrate was transferred into a 2-ml VT-032P silicon-coated tube (Terumo, Osaka, Japan), 10 μl and 20 μl each of a 0.05 *M* phosphate buffer (pH 2.5)–methanol (75:25) mixture was added for human plasma and urine assays, respectively. After the mixtures were well mixed with an NS-8 vortex mixer (Pasolina, Tokyo, Japan), 200 μl of 0.5 *M* phosphate buffer (pH 2.2) and 800 μl of 0.5 *M* phosphate buffer (pH 2.5) were added for human plasma and urine assays, respectively. The resulting mixtures were vortex-mixed, then a 150 μl portion and a 50 μl portion were injected onto the HPLC system for human plasma and urine assays, respectively.

### *2.4. Standard curve*

For each run of the plasma and urine assays, accurately weighed 6.8 mg and 23 mg portions of S-1090 were dissolved in 5 ml of methanol and diluted to 20 ml with 0.05 *M* phosphate buffer (pH 2.5). Appropriate dilutions were done with 0.05 *M* phosphate buffer (pH 2.5)–methanol (75:25) to obtain standard solutions containing five points with duplicates for each point in the range of 0.9–90 μg/ml of S-1090 for the plasma assay, and six points with duplicates for each point in the range of 5–1000 μg/ml of S-1090 for the urine assay. A 150–200 μl portion of human blank plasma and a 300–400 μl portion of human blank urine were filtered by centrifugation as described in the previous section. Plasma and urine standard solutions were prepared in the range of 0.09–9 μg/ml and 0.5–100 μg/ml of S-1090 by spiking a 100 μl portion of the plasma filtrate with 10 μl each of the standard solutions for plasma assay and by spiking a 200 μl portion of the urine filtrate with 20 μl each of the standard solutions for urine assay. Plasma and urine standard curves were constructed separately in the two ranges of 0.09–0.9 and 0.9–9 μg/ml and 0.5–10 and 10–100 μg/ml by plotting the concentration of S-1090 in human plasma and urine versus the peak area and by determining the best-fit line from the weighted linear regression analysis by the method of Aarons et al. [7].

### *2.5. Validation study*

#### *2.5.1. Selectivity*

Chromatograms of blank plasma and urine from several sources before administration of S-1090 were examined.

#### *2.5.2. Linearity*

Plasma and urine standard curves were constructed using five and six points with duplicates for each point in the ranges of 0.09–9 μg/ml and 0.5–100 μg/ml, respectively.

The slopes of log–log plots of the concentration versus the peak area were calculated to evaluate linearity.

#### *2.5.3. Precision and accuracy*

The precision and accuracy of the plasma and urine assays were examined by testing six replicate blank plasma and urine samples each spiked with 0.09, 0.3, 0.9 or 3 μg/ml of S-1090 and with 0.5, 2, 15 or 70 μg/ml of S-1090 in the same run and by testing each sample over six different runs.

#### *2.5.4. Recovery*

Over three different runs, plasma and urine standard curves were constructed using five points with duplicates for each point in the range of 0.09–9 μg/ml and six points with duplicates for each point in the range of 0.5–100 μg/ml, respectively. Over the three different runs, the corresponding standard curves were constructed from aqueous methanol solutions prepared by spiking 100 μl of water with 10 μl of a 0.05 *M* phosphate buffer (pH 2.5)–methanol (75:25) mixture containing 0.9–90 μg/ml of S-1090 for plasma assay, and by spiking 200 μl of water with 20 μl of a 0.05 *M* phosphate buffer (pH 2.5)–methanol (75:25) mixture containing 5–1000 μg/ml of S-1090 for urine assay, respectively.

The absolute analytical recoveries were determined from the slopes of the weighted linear regression analyses constructed by plotting the spiked concentrations in plasma or urine versus the concentrations calculated from the weighted linear stan-

dard curves which were obtained from the aqueous methanol solutions.

### 2.5.5. Quality control

Quality control samples in duplicates at the concentrations of 0.3, 0.9 and 3 μg/ml of S-1090 in plasma, and 2, 15 and 75 μg/ml of S-1090 in urine were stored at −70°C and included in each run in plasma and urine assays.

### 2.5.6. Stability

Stabilities at the concentrations of 0.5 and 3 μg/ml of S-1090 in plasma and of 5 and 30 μg/ml of S-1090 in urine were tested over two months at −20°C and −70°C. Blank plasma and urine samples, each spiked with 0.4 or 2 μg/ml of S-1090 and with 7 or 45 μg/ml of S-1090, were stored at −70°C, and the effect of freeze–thaw cycles was examined.

## 2.6. Microbiological method

Assays of S-1090 in human plasma and urine were also carried out by a microbiological method using *Providencia stuartii* IFO 12930 as a test organism and Mueller–Hinton agar as the test medium as described previously [8].

## 3. Results and discussion

### 3.1. HPLC system

In order to assay many clinical plasma and urine samples of S-1090, we adopted direct injection HPLC systems with a column switching technique [5,6], which were very useful and practical without the disadvantages of the classical labor-intensive and time-consuming preparation process. Samples were injected directly onto the HPLC systems after filtration and acidification. On these systems, the next sample can be injected about 10 min before the end of the analysis of the current sample. Therefore, analysis time was shortened by 30% and sample throughout could be increased to 150%. Acidic mobile phases were effective for obtaining quantitative recoveries of S-1090 without loss due to adsorption on HPLC columns. Addition of internal standard was not required for the established HPLC methods which gave highly reproducible results with quantitative recoveries.

An internal-surface reversed-phase column, L-1180 [9], which consists of porous silica support coated with a hydrophilic 3-glycerylpropylsilyl phase on the surface inside the pores and with a hydrophobic octadecyldimethylsilyl phase on the surface outside the pores, was used as a pre-column to discard plasma components and to concentrate S-1090 prior to eluting it to a conventional octadecyl column. The pre-column was replaced after injection of about 500 samples.

A less retentive cyano pre-column was used to discard many highly polar urine components. Another pre-column packed with phenyl material allowed sufficient cleaning of the cyano pre-column by back-flushing during pre-separation of S-1090 with stronger retention. Finally, the S-1090 fraction transferred from the phenyl pre-column was finely separated on an octadecyl analytical column.

Attempts to separate S-1090 by reversed-phase chromatography on cyano, phenyl and octadecyl columns with mobile phases consisting of methanol and aqueous buffer resulted in weak retentions and interferences from endogenous components in plasma and urine.

The aminothiazole group of S-1090 having a p$K_a$ of 3.2 would be mostly protonated under the mobile phase pH condition, pH 2.5.

Addition of the ion-pair reagent, sodium 1-heptanesulfonate, caused stronger retentions of S-1090 on the columns and led to satisfactory separations on the octadecyl analytical columns.

### 3.2. Assay validation

Typical chromatograms under the plasma and urine assay conditions are shown in Fig. 4. These assay methods were selective as demonstrated by the lack of interfering peaks in the blank plasma and urine. We also confirmed no carry-over effects by checking the chromatograms of blank plasma and urine after injection of plasma and urine samples in the assay range.

Linearities of plasma and urine standard curves were evaluated over nine analytical runs in the ranges 0.09–9 μg/ml and 0.5–100 μg/ml, respectively. The slopes of log–log plots for the con-

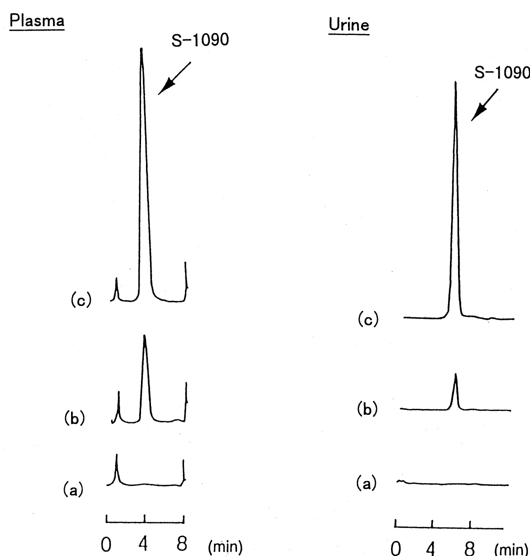


Fig. 4. HPLC chromatograms of S-1090 in human plasma and urine. (a) Blank plasma and urine. (b) Blank plasma and urine spiked with 0.9 μg/ml of S-1090 and 10 μg/ml of S-1090, respectively. (c) Sample after administration of 200 mg of S-1090 (found concentration: 4.3 μg/ml in plasma collected 4 h after administration; 61 μg/ml in urine collected 4–6 h after administration). The actual retention times of compound S-1090 in human plasma and urine assays are 22 min and 23 min longer than the time shown in the figure, respectively.

centration versus the peak area of plasma and urine standard curves ranged from 0.99 to 1.02 (mean±SD, 1.00±0.01) and from 0.99 to 1.03 (mean±SD, 1.00±0.01), respectively. The plasma and urine standard curves were evaluated as being linear because the slopes were close to one.

Assay precision and accuracy were determined by analyzing six replicate blank plasma and urine samples each spiked with 0.09, 0.3, 0.9 or 3 μg/ml of S-1090 and with 0.5, 2, 15 or 70 μg/ml of S-1090, respectively, on the same run and by analyzing each sample over six runs. All of the relative standard deviations for within-run and between-run assays were less than 6% with relative errors of −6 to −1% at 0.3, 0.9 and 3 μg/ml levels of S-1090 in plasma, and were less than 4% with relative errors of −2 to +6% at 2, 15 and 70 μg/ml levels of S-1090 in urine.

The limits of quantitation for plasma and urine assays were 0.09 and 0.5 μg/ml, respectively, where the relative standard deviations for within-run and

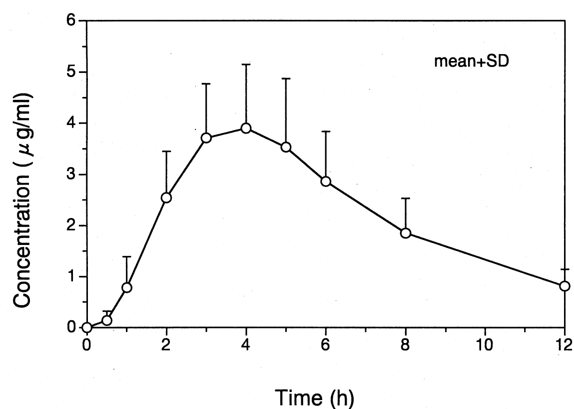


Fig. 5. Mean concentration of S-1090 in human plasma after single oral administration of S-1090 (200 mg, $n=12$).

between-run assays were less than 6% with acceptable low relative errors.

Absolute analytical recoveries were calculated for the three different runs from the slope of the weighted linear regression analysis between the spiked concentration and the concentration calculated from the weighted linear standard curves which were constructed using the aqueous methanol solutions of S-1090. The absolute analytical recoveries of S-1090 in the ranges of 0.09–0.9 and 0.9–9 μg/ml in plasma were 96.5–102.3% and 95.8–100.3%, respectively, and those in the ranges of 0.5–10 and 10–100 μg/ml in urine were 100.2–101.5% and 100.0–100.7%, respectively.

Quality control samples were subjected to six analytical runs of a batch of samples. Both plasma and urine assays over the six runs were demonstrated

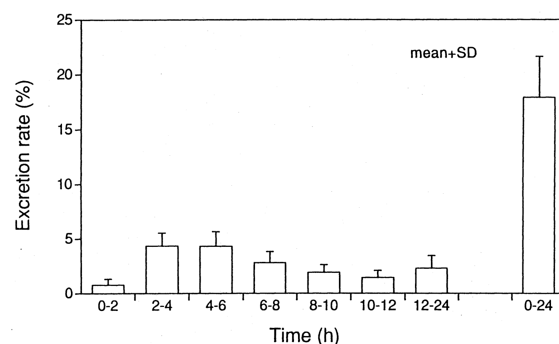


Fig. 6. Mean excretion rate of S-1090 after single oral administration of S-1090 (200 mg, $n=12$).

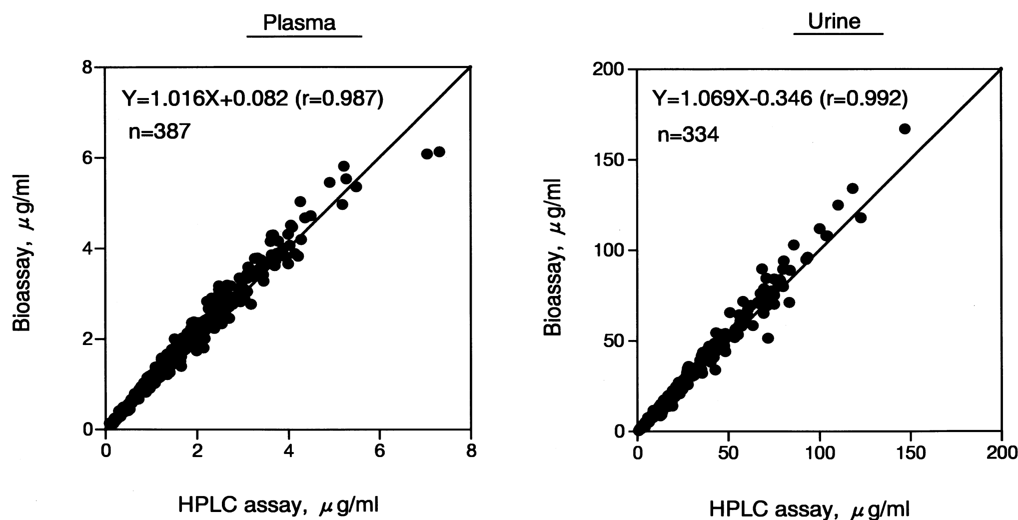


Fig. 7. Relationship between HPLC assay and bioassay.

to be acceptable, because all of the quality control samples gave relative errors less than 15%.

Plasma samples were stable for at least two months at −70°C, but 10–15% decrease in the S-1090 contents had occurred after one month at −20°C. Urine samples were stable for at least two months at −20°C and −70°C. Plasma and urine samples were stable over five freeze–thaw cycles.

### *3.3. Application to clinical samples*

The established HPLC methods were applied to various clinical pharmacokinetic studies after oral administration of S-1090.

Figs. 5 and 6 show the representative plasma concentration–time and urinary excretion rate–time profiles of S-1090 after oral administration of 200 mg of S-1090 to 12 healthy human volunteers.

Comparison with the microbiological method was tested in a single-dose (50–200 mg) safety and tolerance study of compound S-1090 in normal volunteers [8].

As shown in Fig. 7, plots of the concentration by HPLC method ($x$) vs. the concentration by bioassay method ($y$) in plasma and urine gave good linear regression curves, $y=1.016x+0.082$ ($r=0.987$, $n=387$), and $y=1.069x-0.346$ ($r=0.992$, $n=334$), respectively, with slopes near one and intercepts near zero.

## 4. Conclusions

The HPLC methods described in this report are extremely simple, rapid, accurate and precise. By direct injection into the column switching HPLC systems, the plasma and urine samples can be assayed without any pre-separation procedures. The methods should be useful for various clinical studies of compound S-1090.

## References

[1] M. Kume, T. Kubota, Y. Kimura, H. Nakashimizu, K. Motokawa, M. Nakano, J. Antibiot. 46 (1993) 177.
[2] M. Kume, T. Kubota, Y. Kimura, H. Nakashimizu, K. Motokawa, Chem. Pharm. Bull. 41 (1993) 758.
[3] M. Tsuji, Y. Ishii, A. Ohno, S. Miyazaki, K. Yamaguchi, Antimicrob. Agents Chemother. 39 (1995) 2544.
[4] F. Péhourcq, C. Jarry, J. Chromatogr. A 812 (1998) 159.
[5] D.J. Anderson, Anal. Chem. 65 (1993) 434R.
[6] P. Campíns-Falcó, R. Herráez-Hernández, A. Sevillano-Cabeza, J. Chromatogr. 619 (1993) 177.
[7] L. Aarons, S. Toon, M. Rowland, J. Pharmacol. Methods 17 (1987) 337–346.
[8] M. Nakashima, T. Oguma, Y. Kimura, S. Sasaki, Y. Sendo, H. Imoto, J. Infect. Chemother. 2 (1996) 271.
[9] J. Fujii, N. Inotsume, M. Nakano, I. Matsukane, A. Higashi, I. Matsuda, Y. Sudo, Y. Takahata, J. Chromatogr. 530 (1990) 469.