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Column-switching high-performance liquid chromatographic analysis of BO-2727, a new carbapenem antibiotic, in human plasma and urine by direct injection

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Abstract

A column-switching high-performance liquid chromatographic method has been developed for the simple and sensitive analysis of BO-2727 (I) in human plasma and urine. Plasma samples were diluted with an equal volume of a stabilizer, and the mixture was directly injected onto the HPLC system. The analyte was enriched in a pre-treatment column, while endogenous components were eluted to waste. The analyte was then backflushed onto an analytical column and quantified with ultraviolet detection. Urinary concentrations were determined in a similar way except that the enriched analyte was eluted in the foreflush mode to a cation-exchange column used for chromatographic separation. The standard curves for the drug were linear in the range of 0.05–50 μg/ml in plasma and 0.5–100 μg/ml in urine. The limits of quantification for plasma and urine were found to be 0.05 μg/ml and 0.5 μg/ml, respectively. This method was used to support Phase I clinical pharmacokinetic studies.

1. Introduction

Carbapenems have recently attracted increased attention because of their potent antibacterial activity against a broad spectrum of microorganisms. BO-2727 (I, Fig. 1) is a new carbapenem antibiotic [1]. This compound shows excellent antibacterial activity against infectuous microorganisms, including *P. aeruginosa* which is resistant to many other antibiotics. Furthermore, the drug resists degradation by β-lactamases and hydrolysis by renal dehydropeptidase I. Based on the above characteristic and on the results of safety assessment, Phase I clinical trials have been conducted.

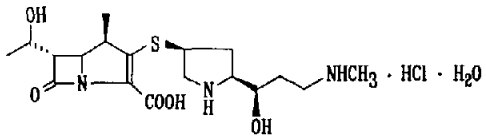

Fig. 1. Chemical structure of BO-2727 (I).

The aim of the present investigation was to develop a high-performance liquid chromatographic (HPLC) method to quantify I in human plasma and urine and support clinical pharmacokinetic studies. HPLC methods found in the literature for the quantification of carbapenems in human plasma and urine are based on direct injection without sample clean-up [2], solid-phase extraction [3], and column-switching [4].

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For the assay of I, rapid sample-processing procedures were required because of its limited stability in biological fluids, as is also found for the other carbapenems. The column-switching technique avoids off-line procedures for sample clean-up. The application of this method allowed plasma and urine samples to be directly injected onto a fully automated HPLC system, thereby resulting in an accurate assay without internal standards. The method was validated at drug concentration ranges of 0.05–50 μg/ml in plasma and 0.5–100 μg/ml in urine. Plasma and urine samples from the Phase I trials were analyzed by the procedure described in this paper.

2. Experimental

2.1. Reagents

Compound I, (1*R*,5*S*,6*S*)-6-[(*R*)-1-hydroxyethyl]-2-[(3*S*,5*S*)-5-{(*R*)-1-hydroxy-3-N-methylaminopropyl}pyrrolidin-3-ylthio]-1-methyl-1-carbapen-2-em-3-carboxylic acid hydrochloride hydrate, was supplied by Banyu Tsukuba Research Institute (Tsukuba, Japan). Acetonitrile, acetone (both HPLC grade) and 2-(N-morpholino)ethanesulfonic acid monohydrate (MES) were purchased from Wako (Osaka, Japan). MES buffer (pH 7, 1 *M*) was used as a stabilizer in this study, unless otherwise stated. Water was purified with a Millipore Milli-Q system (Milford, MA, USA). All other reagents were of analytical reagent grade commercially obtained from Junsei (Tokyo, Japan).

2.2. Instrumentation for plasma assay

A Shimadzu (Kyoto, Japan) LC-10A series HPLC system was used. The system consisted of two pumps (LC-10AD), a column oven (CTO-10A), an autosampler (SIL-10A) with a sample cooler, a degasser (DGU-4A), a six-port two-position valve (FCV-12AH; VA), a seven-port six-position valve (FCV-13AL) used as a three-port two-position valve (VB), and a photodiode-array UV detector (SPD-M10A). The system included a pre-treatment column and an analytical column. The chromatograph, switching valves and autosampler were controlled by a Compaq Prolinea 4/33 analytical workstation (Houston, TX, USA) as a fully automated system. Data were acquired and processed on the workstation.

2.3. Chromatographic conditions for plasma assay

The pre-treatment column (C1) was a Capcell pak C_{18} column (35 mm × 4.6 mm I.D., 5 μm, SG-120) and the analytical column (C2) was a Capcell pak C_{18} (250 mm × 4.6 mm I.D., 5 μm, SG-120) purchased from Shiseido (Tokyo, Japan). The mobile phases were sodium phosphate buffer (50 m*M*, pH 7; MP1), acetone–acetonitrile–water (25:25:50, v/v/v; MP2), and sodium phosphate buffer (50 m*M*, pH 7)–acetonitrile (96:4, v/v; MP3). Mobile phases MP1 and MP3 contained sodium azide (0.02%) as a fungicide. Column C1 was operated at ambient temperature and C2 was maintained at 40°C. The autosampler was set at 2°C. The flow-rate for both columns was 1 ml/min. The detector was operated at a wavelength of 300 nm and a time constant of 0.24 s.

Fig. 2 shows a schematic diagram of the automated system. The system was operated according to the following procedure where

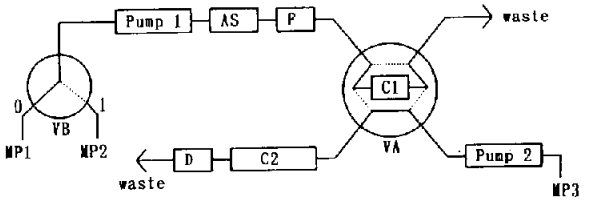

Fig. 2. Automated HPLC system with column-switching used for separation and quantification of I in plasma. C1 and C2 = columns 1 and 2, AS = autosampler, F = filter, D = detector, VA = six-port two-position valve, VB = three-port two-position valve, MP1, MP2 and MP3 = mobile phases 1, 2 and 3. The solid and dotted lines in VA and VB indicate valve positions 0 and 1.

valve positions and switchover times are in parentheses.

Step 1 (VA = 0, VB = 0; 0 min): A plasma sample is injected. Detector data acquisition starts. Step 2 (VA = 0, VB = 0; 0–5 min): The sample is swept in C1 with MP1 from pump 1. The analyte is retained, while proteins and polar substances are eluted to waste. Step 3 (VA = 1, VB = 0; 5–7 min): Mobile phase MP3 from pump 2 passes through C1 in the backflush mode and elutes the analyte onto C2, where the analyte is separated. Step 4 (VA = 0, VB = 1; 7–20 min): Mobile phase MP3 further passes through C2 for chromatographic separation. Concurrently, C1 is washed with MP2 to remove hydrophobic substances retained on the column. Step 5 (VA = 0, VB = 0; 20–30 min): The analysis is continued on C2 with MP3, meanwhile C1 is reconditioned with MP1 and prepared for the next injection.

2.4. Instrumentation and chromatographic conditions for urine assay

Another column-switching system was built with a Shimadzu LC-9A HPLC series, consisting of three pumps (LC-9A), a column oven (CTO-6A), a UV detector (SPD-6A), a six-port two-position valve (FCV-2AH), and an AS-4000 autosampler (Hitachi; Tokyo, Japan) with a cooler and two GT-102 degassers (Tokyo Kasei; Tokyo, Japan). The system was controlled by a Chromatopac C-R4AX (Shimadzu) data processor, which also acquired and processed the data. This system was similar to that used for the plasma samples, but here two independent pumps were used to deliver MP1 and MP2 instead of one pump and one valve (VB). In addition, the enriched analyte in C1 was fore-flushed to an analytical column. For analysis, a cation exchange column, Kaseisorb LC SCX-60-5 (150 mm × 4.6 mm I.D., 5 μm), purchased from Tokyo Kasei was employed, and the mobile phase was phosphate buffer (0.1 *M*, pH 7)–acetonitrile (80:20, v/v). The SCX column was maintained at 35°C. The other conditions were the same as those described for the plasma assay.

2.5. Standard solutions

Compound I was dissolved in MES buffer at a concentration of 1 mg/ml as free base. This solution was divided into *ca.* 1-ml portions, which were frozen at −80°C and used as stock standard solution. A portion of the solution was further diluted with MES buffer to produce standard solutions with concentrations of 0.05, 0.1, 0.5, 5 and 50 μg/ml for the plasma assay, and 0.5, 1, 5, 50 and 100 μg/ml for the urine assay. Each solution was divided into *ca.* 300-μl portions, which were stored at −80°C until use. The solutions were found to be stable for at least three months without measurable decomposition.

2.6. Standard curves

The standard curve for I was constructed by addition of standard solution (125 μl) to drug-free plasma (125 μl) and urine (125 μl). The mixtures were filtered through an Ultrafree (C3GV, 0.22 μm, Millipore) by centrifugation at 500 *g* for 5 min at 4°C. The filtrate (50 μl) was injected onto the HPLC system. Plots of peak area against drug concentration were used to calculate the linear regression equation.

2.7. Preparation of plasma and urine samples

Plasma was separated from heparinized blood samples by centrifugation at 1500 *g* for 15 min at 4°C. Immediately after collection of plasma and urine samples, they were diluted with an equal volume of MES buffer and the mixtures were stored at −80°C until analysis. After thawing, the mixtures (200 μl) were treated in the same manner as described for standard curves. The filtrates (50 μl) were then subjected to HPLC analysis.

3. Results and discussion

In initial studies, I was found to be reasonably stable in saline and distilled water at room temperature, but the drug in biological fluids was

stable for only one or two hours at room temperature. For the quantitative determination of such unstable compounds by HPLC, stabilization of the compounds in biological fluids and quick sample-processing procedures are required.

MES buffer (1 *M*, pH 7) was found to act as a stabilizer. In a 1:1 mixture of the buffer and human plasma, the drug stability was enhanced such that hardly any loss of the compound occurred at 2°C for 16 h, or at room temperature for 6 h. Under similar conditions, the drug in urine was even more stable. No additional studies on drug stability were performed, since the drug was stable long enough to place samples in an autosampler set at 2°C for overnight analysis. However, since the drug stability was still limited, it was advisable to avoid sample clean-up procedures using liquid–liquid extraction, solid-phase extraction and deproteination prior to chromatography.

For quick sample processing, a method based on column-switching HPLC was employed. This technique has been increasingly used for on-line sample clean-up and/or enrichment of analytes [5], and is widely adopted for the analysis of antibiotics in biological matrices [4–11].

Attempts to use internal surface reversed-phase precolumns were unsuccessful because I was insufficiently retained on these columns. Short C_{18} columns were found to retain the analyte for longest periods, and variations in room temperature had little influence on the retention time of I. Generally, direct injection of crude biological samples onto columns consisting of conventional silica-based supports causes clogging or a rapid loss of column efficiency mainly due to denatured protein [12]. Denaturation of proteins is partly caused by active hydrogens located on the surface of the silica gel. This prompted us to use a column packed with silicone-coated silica where direct contact between the hydrogens and the proteins is prevented. Thus, a Capcell pak column was selected for on-line sample clean-up.

Preliminary studies showed that this column maintained its efficiency even after more than 200 plasma samples had been directly injected under various conditions. However, direct injection, when repeated at short intervals, appeared to cause more serious damage to the column. In the assay of clinical samples, the pre-treatment column was replaced by a new one every 120–130 samples including those of the standard curves and quality control.

To determine urinary concentrations of I, a cation-exchange column was employed. This column could separate I from a number of abundant organic acids contained in urine because the drug is an amphoteric compound. In this assay, the analyte was foreflushed to elute from the precolumn, resulting in better chromatographic separation.

The present method was validated to support Phase I clinical pharmacokinetic studies. Fig. 3 shows chromatograms of drug-free plasma, plasma spiked with I, and a plasma sample from the Phase I study. Also chromatograms of urine samples are presented in Fig. 3. The selectivity of the assay was checked by measurement of drug-free plasma, urine and pre-dose samples from the clinical study. No endogeneous interferences were observed.

The on-line recovery of I from plasma and urine was determined by comparison of the peak areas obtained after injection of the fluids spiked with known concentrations of the drug to that

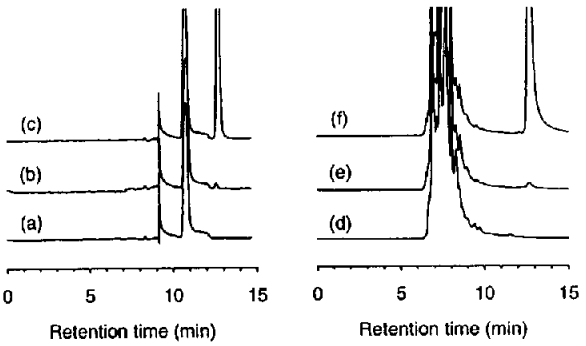

Fig. 3. Typical chromatograms of human plasma and urine samples. (a) Drug-free plasma, (b) spiked plasma (0.1 μg/ml), and (c) plasma collected from a volunteer 4 h after infusion at a dose of 1000 mg of I. (d) Drug-free urine, (e) spiked urine (1 μg/ml), and (f) urine (4-fold dilution with a 1:1 mixture of drug-free urine and MES buffer) collected from a volunteer 4–6 h after infusion at a dose of 1000 mg of I.

produced by the same concentrations of the drug dissolved in MES buffer (0.5 *M*, pH 7). The samples were prepared by the method described for standard curves. Replicate assays ($n = 5$) indicated that the recovery from plasma was 97.0–100.4% at a concentration range of 0.05–50 μg/ml, and 96.3–100.3% at 0.5–100 μg/ml for urine (Table 1).

Standard curves for I in human plasma and urine were linear. A typical standard curve for the former is given by the equation, $y = 32673.6x - 222.6$ ($r = 0.9994$), and for the latter $y = 4704.3x - 101.7$ ($r = 0.9997$), where y indicates the peak area, x represents the drug concentration in μg/ml, and r is the correlation coefficient. The discrepancy in the slope of these equations is chiefly attributable to differences in the data processing system.

The within-day precision of the method for plasma samples was examined by replicate analyses ($n = 5$) of human plasma spiked with known amounts of the drug at concentrations of 0.05, 0.1, 0.5, 5 and 50 μg/ml. The precision, expressed by coefficient of variation (C.V.), is given in Table 1. The C.V.s were <5% for all tested concentrations, showing that the method provided good reproducibility without an internal standard. The between-day precision was determined by assaying spiked plasma samples (0.05–50 μg/ml) on three different days (Table 2). The C.V.s did not exceed 5% for all concentrations, demonstrating the good stability and repeatability of this assay system. Thus, the limit of quantification (LOQ) was defined as 0.05 μg/ml, at a signal-to-noise (S/N) ratio of 9:1.

For the urine assay, within-day and between-day precisions were determined by a procedure virtually identical to that used for the plasma samples except that urine was spiked at concentrations of 0.5, 1, 5, 50 and 100 μg/ml. The results are summarized in Tables 1 and 2. The LOQ was 0.5 μg/ml at an S/N ratio of 50:1, which was sufficient for the determination of urinary concentrations.

In the assay of clinical samples, when the concentrations of the drug in both fluids were higher than the range of the standard curves, the samples were appropriately diluted with a 1:1 mixture of the corresponding matrices and MES buffer to perform reassays. The results of the assay and pharmacokinetic studies will be published in detail elsewhere [13]. Thus, the method presented here is capable of characterizing the pharmacokinetic parameters in the Phase I

Table 1
Within-day precision, accuracy and recovery for the assay of I in human plasma and urine

Spiked concentration (μg/ml)	Measured concentration (mean ± S.D., μg/ml)	Coefficient of variation (%)	Accuracy[a] (%)	Recovery (%)
Plasma				
0.0500	0.0518 ± 0.00239	4.6	104	97.0
0.100	0.100 ± 0.00362	3.6	100	99.2
0.500	0.494 ± 0.00583	1.2	99	100.2
5.00	4.87 ± 0.0317	0.7	97	99.8
50.0	50.2 ± 1.95	3.9	100	100.4
Urine				
0.500	0.558 ± 0.0246	4.4	112	96.3
1.00	1.03 ± 0.0371	3.6	103	97.1
5.00	4.29 ± 0.0616	1.4	86	96.7
50.0	49.5 ± 0.361	0.7	99	100.3
100	101 ± 1.74	1.7	101	99.5

[a] Accuracy = (measured plasma concentration/spiked plasma or urine concentration) · 100.

Table 2
Between-day precision for the assay of I in human plasma and urine

Spiked concentration (μg/ml)	Measured concentration (μg/ml)				Coefficient of variation (%)
	day 1	day 2	day 3	mean ± S.D.	
Plasma					
0.0500	0.0510	0.0523	0.0477	0.0503 ± 0.00237	4.7
0.100	0.100	0.0967	0.0967	0.0978 ± 0.00191	2.0
0.500	0.470	0.453	0.463	0.462 ± 0.00854	1.8
5.00	4.63	4.51	4.79	4.64 ± 0.140	3.0
50.0	50.4	50.4	50.3	50.4 ± 0.0577	0.1
Urine					
0.500	0.430	0.497	0.493	0.473 ± 0.0376	7.9
1.00	0.853	0.893	0.910	0.885 ± 0.0293	3.3
5.00	4.56	4.25	4.33	4.38 ± 0.161	3.7
50.0	48.5	48.2	50.2	48.9 ± 1.07	2.2
100	99.8	101	100	100 ± 0.643	0.6

study. However, for the assay of metabolites of I or for therapeutic drug monitoring in co-medication, alternative methods will have to be developed.

4. References

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