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High-Pressure Liquid Chromatographic Analysis of BMY-28142 in Plasma and Urine

R. H. BARBHAIYA,* S. T. FORGUE, W. C. SHYU, E. A. PAPP, AND K. A. PITTMAN

Department of Metabolism and Pharmacokinetics, Pharmaceutical Research and Development Division, Bristol-Myers Company, Syracuse, New York 13221

Received 21 July 1986/Accepted 20 October 1986

A high-pressure liquid chromatographic assay was developed for the quantitative analysis of a new cephalosporin, BMY-28142, in plasma and urine. The plasma method involved protein precipitation with acetonitrile and trichloroacetic acid followed by extraction of the acetonitrile into dichloromethane. After centrifugation, the organic phase was discarded, the aqueous solution was injected into a reverse-phase column, and peaks were detected at 280 nm. The urine method involved dilution of a urine sample with sodium acetate buffer (pH 4.25) and direct injection into the high-pressure liquid chromatography system. The assay validation data indicate that the assays for BMY-28142 in plasma and urine were specific, accurate, and reproducible. The analytical methods were applied to the determination of protein binding in human serum and to a pharmacokinetic study in rats. The results of the protein-binding study indicated that BMY-28142 was 16.3% bound to human serum proteins. In the pharmacokinetic study in rats, the maximum level in plasma of 38.7 μg/ml was achieved at 2.33 h after administration of a subcutaneous dose of 100 mg/kg. The levels in the plasma then declined with an elimination half-life of about 0.56 h. The mean values for the steady-state volume of distribution and total body clearance were 0.46 liters/kg and 11.9 ml/min per kg, respectively. The 0- to 24-h excretion of intact BMY-28142 in urine accounted for 88.6% of the dose.

BMY-28142 is a recently developed aminothiazole methoximino cephalosporin which bears a quaternized 1-methylpyrrolidine group at position 3 of methylene (Fig. 1). It is very active against *Staphylococcus aureus* and *Pseudomonas aeruginosa* and more potent than other new broad-spectrum β-lactam antibiotics against some members of the *Enterobacteriaceae* family (3, 6, 11). BMY-28142 is also very effective against many β-lactamase-producing strains. The excellent spectrum of BMY-28142, its virtually complete resistance to the action of common β-lactamases, and its low affinity for these enzymes are characteristics that make it a promising drug candidate.

The concentrations of BMY-28142 in plasma were previously determined by a microbiological assay (6). Although bioassays have been traditionally used for the determination of antibiotics, they have limited sensitivity and reproducibility. The additional disadvantages of bioassays are their lack of specificity and the length of time required for sample analysis. High-pressure liquid chromatography (HPLC) provides a sensitive and specific alternative, with the additional advantages of high precision and rapid turnaround time. We developed a sensitive HPLC assay for the measurement of BMY-28142 in plasma and urine to facilitate pharmacokinetic studies in animals and humans. This paper describes the HPLC method and its application for protein-binding studies and pharmacokinetic studies in rats.

MATERIALS AND METHODS

Reagents. BMY-28142 and cefadroxil were prepared at Bristol-Myers Co., Syracuse, N.Y. Ceftazidime was obtained from Glaxo Pharmaceuticals, Ltd., Greenford, England. Glacial acetic acid, phosphoric acid, and dichloromethane were purchased from J. T. Baker Chemical Co. Phillipsburg, N.J. HPLC-grade methanol, acetonitrile, and tetrahydrofuran and trichloroacetic acid were purchased from Fisher Scientific Co., Pittsburgh, Pa.; 1-octanesulfonic acid (Pic B-8) was from Waters Associates, Inc., Milford, Mass., and sodium dodecyl sulfate (specially pure; lot 44244) was from BDH, Poole, England.

Preparation of standards and quality control samples. An accurately weighed 13.2-mg portion of BMY-28142 · H_2SO_4, equivalent to 10.9 mg of BMY-28142 dipolar ion, was dissolved in 10.0 ml of 0.005 M octanesulfonic acid (Pic B-8; Waters Associates). Plasma standards containing 0.1 to 54.8 μg of BMY-28142 per ml were prepared by dilution of this stock solution with control human plasma.

For the preparation of BMY-28142 urine standards, drug-free human urine was diluted threefold with 200 mM sodium acetate buffer (pH 4.25). Then 36.0 mg of BMY-28142 · H_2SO_4, equivalent to 29.9 mg of BMY-28142 dipolar ion, was dissolved in 29.9 ml of buffer-diluted human urine. All urine standards, containing 2 to 1,000 μg of BMY-28142 per ml, were prepared by dilution of this stock solution with buffered human urine.

Spiked quality control samples were prepared in the biological matrices for determination of the accuracy and precision of the plasma and urine assays. These quality control samples were prepared and analyzed in a blinded manner.

Sample processing. A cefadroxil solution (0.1 ml; 100 μg/ml) (internal standard) was added to 0.5 ml of plasma. The plasma proteins were precipitated by the addition of 0.3 ml of 5% trichloroacetic acid solution and 0.5 ml of acetonitrile. After addition of 1.5 ml of dichloromethane, the mixture was vortexed for about 10 s, and the organic phase containing the acetonitrile and dichloromethane was separated from the aqueous solution by centrifugation (5°C and 500 to 600 × *g* for 10 min). Approximately 200 μl of supernatant (aqueous solution) was pipetted into a microvial, and 25 μl was injected into the HPLC system through a Waters Intelligent Sample Processor.

* Corresponding author.

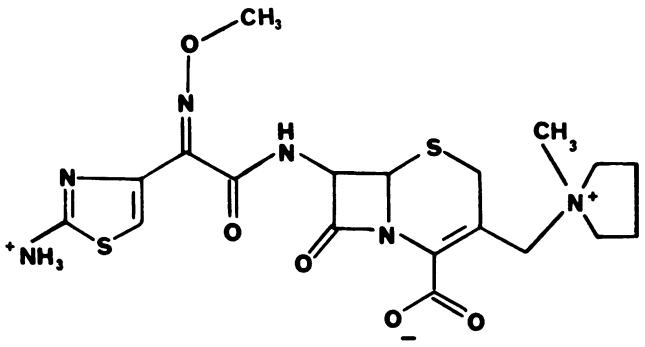

FIG. 1. Structure of BMY-28142, 7-[α-(2-aminothiazol-4-yl)-α-(Z)-methoximinoacetamido]-3-[(1-methyl-1-pyrrolidino)-methyl]-3-cephem-4-carboxylate.

The urine samples were diluted threefold with 200 mM sodium acetate buffer (pH 4.25). A ceftazidime solution (0.1 ml; 1,500 μg/ml) (internal standard) was added and vortexed for 30 s. A 100-μl sample of this solution was transferred to a microvial, and 10 μl was injected into the HPLC system.

Chromatographic conditions. The HPLC system consisted of a Waters Model 590 solvent delivery system and a Model 441 absorbance detector with a fixed wavelength of 280 nm. The chromatographic separation for the urine and plasma assays was accomplished on reverse-phase C18 Partisil 5 ODS-3RAC (0.94 by 10 cm; Whatman Inc., Clifton, N.J.) and Nova-Pak (0.4 by 15 cm; Waters Associates) columns, respectively. A precolumn (0.4 by 2.3 cm), packed with C_{18} packing material (Coracil; 37 to 50 μm; Waters Associates), was fitted just before the inlet junction of each analytical column. The eluting solvent mixture for the plasma assay was acetonitrile–0.005 M 1-octanesulfonic acid (12:88), and that for the urine assay was methanol–0.01 M sodium dodecyl sulfate–5% (wt/vol) trichloracetic acid–0.85 M phosphoric acid–tetrahydrofuran (49.7:40.4:3.9:0.7:5.3). The mobile phase for the urine assay was prepared by mixing 1,500 ml of 0.01 M sodium dodecyl sulfate solution (adjusted to pH 3.0 with glacial acetic acid), 1,846 ml of methanol, 144 ml of 5% (wt/vol) trichloroacetic acid, 28 ml of 0.85 M phosphoric acid, and 197 ml of tetrahydrofuran. The eluting solvent was delivered at a flow rate of 1.0 ml/min for the plasma assay and 2.8 ml/min for the urine assay.

Data processing. The analog output of the UV detector was digitized by an analog-to-digital converter and recorded by the Hewlett-Packard Model 3357 Laboratory Automation System (Hewlett-Packard Co., Avondale, Pa.). Each chromatogram was analyzed by the system software, using an internal-standard method to obtain the maximum signals for the drug and internal standard. The ratio of the peak height of BMY-28142 to that of cefadroxil (plasma assay) or ceftazidime (urine assay) was calculated for each chromatogram. The least-squares regression of peak height ratio on concentration for each analytical standard, weighted by the inverse of the nominal concentration of each standard, was determined. The concentrations of BMY-28142 in the samples were estimated by inverse prediction from the regression line.

Protein binding. Binding of BMY-28142 to human serum proteins was determined by using an ultrafiltration technique (8, 10). Briefly, fresh human serum containing 40, 200, or 400 μg of BMY-28142 per ml was incubated at 37°C for 0, 15, and 30 min. At the end of each incubation time, a 1.0-ml sample was transferred to a micropartition system and was centrifuged at 37°C for 5 min at 1,000 × *g* to obtain about 100 μl of ultrafiltrate. The concentrations of BMY-28142 in the serum sample and ultrafiltrate were determined by using the assay procedures described above.

Rat study. The pharmacokinetics and excretion of BMY-28142 in urine were investigated, in support of safety evaluation of the compound, in six Sprague-Dawley rats after administration of a subcutaneous dose of 100 mg/kg. On the day before being dosed, the rats were weighed, and a catheter was implanted in one jugular vein by a procedure described previously (1). On the day of administration of BMY-28142, each rat was weighed again and inspected to ensure patency of the jugular vein. Approximately 200-μl blood samples were withdrawn at 0.05, 0.25, 0.50, 1.0, 2.0, 3.0, 4.0, 6.0, 8, and 10 h after administration of the drug.

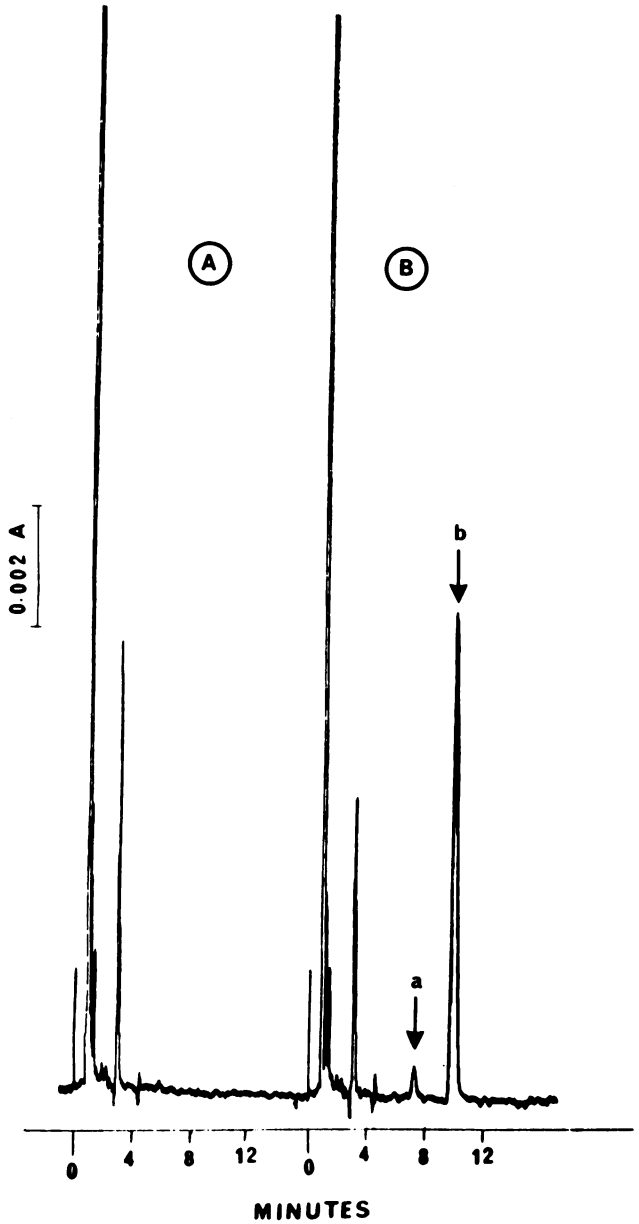

FIG. 2. Typical chromatograms for plasma containing no drugs (A) and plasma containing 0.5 μg of BMY-28142 per ml (B, peak a) and 16.6 μg of cefadroxil per ml (B, peak b).

Model-independent techniques were used to describe the pharmacokinetics of BMY-28142 (2, 4, 9). The area under the plasma concentration-time curve was obtained by using the linear trapezoidal rule up to the final measured plasma concentration and, from that point, by extrapolation to infinity. The elimination rate constant was calculated from the slope of the postdistributive terminal portion of the plasma concentration-time curve for the determination of half-life. The mean absorption time (MAT) and absorption rate constant (k_a) were calculated by using the following equations: (i) $MAT = MRT_{sc} - MRT_{iv}$, where MRT_{sc} and MRT_{iv} are the mean residence times after subcutaneous and intravenous administrations, respectively; and (ii) k_a = 1/MAT. The steady-state volume of distribution and total body clearance were calculated by using the standard methods (4). For the calculation of the steady-state volume of distribution and total body clearance, the fraction of the BMY-28142 dose absorbed was assumed to be 1 (S. T. Forgue, W. C. Shyu, C. R. Gleason, K. A. Pittman, and R. H. Barbhaiya, submitted for publication).

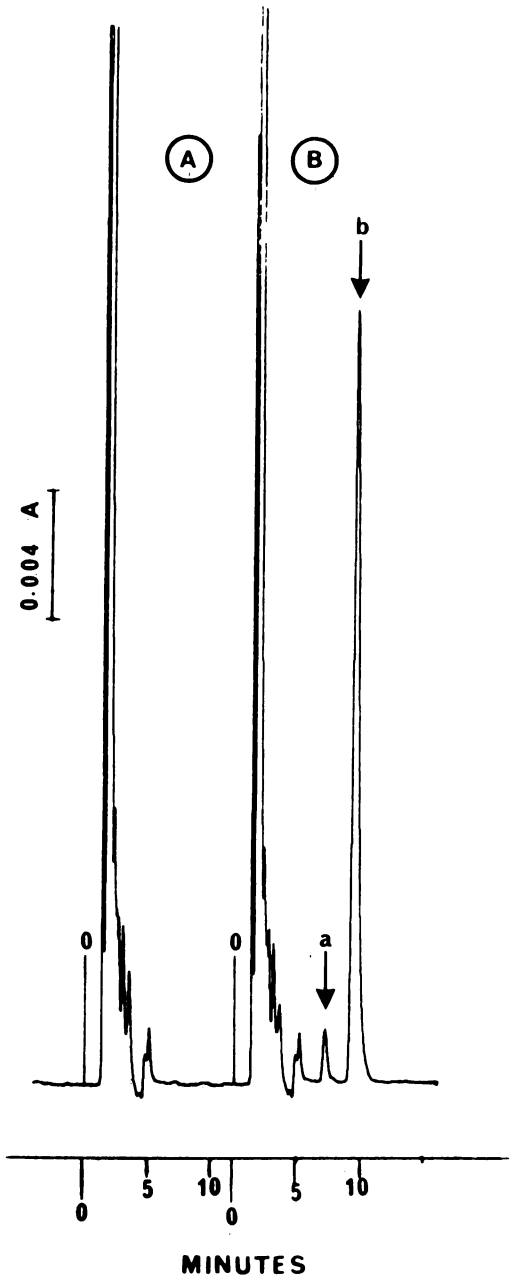

FIG. 3. Typical chromatograms for urine containing no drugs (A) and urine containing 5.0 μg of BMY-28142 per ml (B, peak a) and 165 μg of ceftazidime per ml (B, peak b).

TABLE 1. Accuracy and precision of assays for BMY-28142 in plasma and urine

Assay (n)	BMY-28142 concn (μg/ml)		SD	% CV[a]	% Deviation from nominal
	Nominal	Mean			
Plasma (11)	0.90	0.83	0.04	4.81	−7.7
	22.0	23.6	0.50	0.50	+7.3
Urine (10)	38.2	38.6	1.19	3.10	+1.0
	761.9	763.4	23.7	3.10	+0.2

[a] CV, Coefficient of variation.

RESULTS AND DISCUSSION

Plasma and urine assays. Typical chromatograms obtained for human plasma samples without drugs and for samples containing BMY-28142 and cefadroxil are shown in Fig. 2. BMY-28142 was completely separated from the internal standard, and there was no interference at the retention time of the drug or internal standard from any endogenous substance in the plasma samples obtained from humans or rats. The retention times for BMY-28142 and cefadroxil were approximately 7.0 and 10 min, respectively, and the response (concentration-versus-peak-height ratio) was linear in the range of 0.10 to 54.8 μg/ml. Standard curves obtained from repeated determination of plasma standards were $y = 0.188x + 0.0127$ ($r = 0.999$). When the slope of the standard curve obtained for extracted plasma samples was compared with that for the corresponding unextracted standards, the recovery of BMY-28142 from rat and human plasma was found to be about 97%. Typical chromatograms obtained for urine samples without drugs for samples containing BMY-28142 and ceftazidime are shown in Fig. 3. Base-line separation was obtained between BMY-28142 and ceftazidime, and there was no interference at the retention time of BMY-28142 or ceftazidime from any endogenous substance in the urine samples obtained from humans or rats. The retention times for BMY-28142 and ceftazidime were approximately 7.5 and 10 min, respectively. The urine assay was linear in the range of 2.0 to 1,000 μg/ml. Standard curves obtained from repeated determination of urine standards were $y = 0.0122x - 0.0017$ ($r = 0.999$). The within-day accuracy and precision data for the analysis of BMY-28142 in plasma and urine are shown in Table 1. Excellent data for accuracy and consistently less than 4.81% coefficients of variation suggest that the assays for BMY-28142 in plasma and urine were accurate and precise. The between-day coefficient of variation values at concentrations of BMY-28142 in plasma of 0.9 ($n = 15$) and 22.0 ($n = 15$) μg/ml were 6.7 and 8.4, respectively. The between-day coefficient of variation values for concentrations of BMY-28142 in urine of 38.1 ($n = 15$) and 762 ($n = 15$) μg/ml were 5.8 and 6.3, respectively.

BMY-28142 was stable in human plasma for up to 51 days at −20°C and 138 days at −70°C. The stability of BMY-28142 in diluted buffered urine was established for up to 86 days at

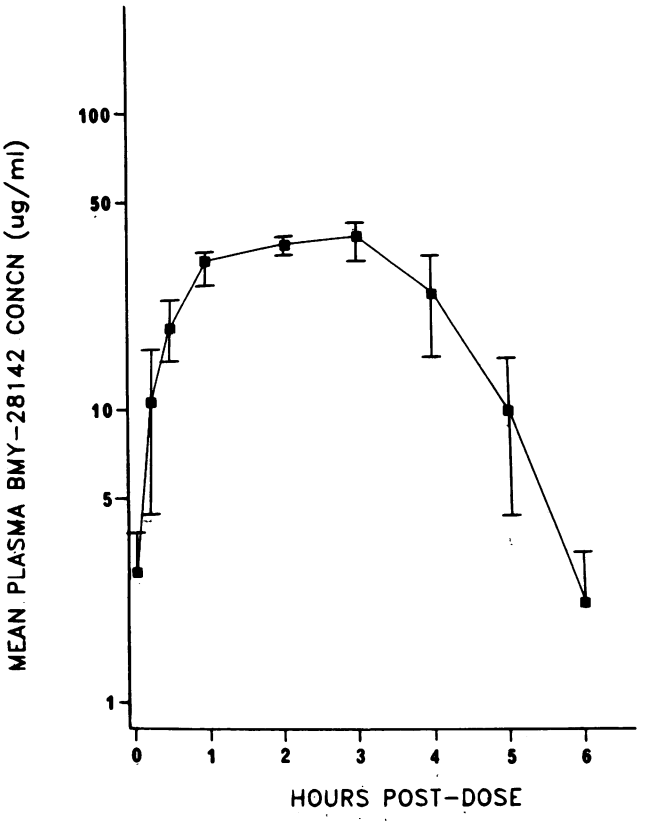

FIG. 4. Mean concentration of BMY-28142 in plasma specimens from six rats after a subcutaneous dose of 100 mg/kg.

−20 and −70°C. With the advent of the automatic sample injector, a large number of samples may be processed at a time, and as a result, the total time of analytical runs has become long. It has therefore become necessary to determine the stability of the drug and the internal standard in the processed samples (injection solvent). For the plasma assay, the stability of BMY-28142 and cefadroxil in the injection solvent was assessed by repeatedly injecting the same processed sample for 40 h. Both BMY-28142 and cefadroxil were stable for up to 40 h in the injection solvent. For the urine assay, the stability of BMY-28142 and ceftazidime was established for up to 22 h.

Protein binding. The newly developed HPLC assay was applied to the determination of protein binding of BMY-28142 in human serum. The mean percentage of protein binding of BMY-28142 in human serum, incubated at 37°C, was low, ranging from 14.5 to 18.9% within a concentration range of 40 to 400 μg/ml. The overall mean value for BMY-28142 protein binding was 16.4%, similar to that reported for ceftazidime (5, 12). Kessler et al. (6) recently found a similar value for protein binding of BMY-28142 in serum by using an agar diffusion assay procedure. No concentration or time dependency was observed for the binding of BMY-28142 to human serum proteins.

Rat study. The assay described above was also used to elucidate the pharmacokinetics of BMY-28142 in rats. The mean concentrations of BMY-28142 in plasma after a subcutaneous dose of 100 mg/kg are presented in Fig. 4. A mean maximum level in plasma of 38.7 μg/ml was achieved at 2.33 h after drug administration. The estimates for the area under the plasma concentration-time curve were very close to those obtained after an intravenous administration in a separate study (Forgue et al., submitted). Therefore, it is assumed that BMY-28142 is 100% available after a subcutaneous dose of 100 mg/kg. The compartmental model-independent pharmacokinetic parameters for BMY-28142 are presented in Table 2. The estimates for the terminal half-life (0.56 h), total body clearance (11.9 ml/min per kg), and steady-state volume of distribution (0.46 liters/kg) were very similar to those obtained after an intravenous administration (Forgue et al., submitted). As a result of prolonged absorption, as indicated by an MAT of 2.07 h, the MRT (2.59 h) after subcutaneous administration was considerably longer than that observed after an intravenous administration (0.69 h). The 0- to 24-h excretion of intact BMY-28142 in urine accounted for 88.6% of the dose. The value for the recovery of BMY-28142 in urine is typical of that for most of the expanded-spectrum cephalosporins. However, the elimination half-life of BMY-28142 in rats was significiantly longer than that reported for other cephalosporins of the same class (7).

TABLE 2. Pharmacokinetic parameters for BMY-28142 after administration of subcutaneous dose of 100 μg/kg in rats

Parameter[a]	Value (mean ± SD)
C_{max} (μg/ml)	38.7 ± 5.17
T_{max} (h)	2.33 ± 1.03
MRT (h)	2.59 ± 0.36
MAT (h)	1.90 ± 0.37
k_a (h^{-1})	0.54 ± 0.13
k_{el} (h^{-1})	1.22 ± 0.32
$t_{1/2}$ (h)	0.56 ± 0.0
AUC (μg · h/ml)	142 ± 17.5
V_{ss} (liters/kg)	0.46 ± 0.04
CL_p (ml/min per kg)	11.9 ± 1.47
Recovery in urine (% of dose)	88.6 ± 5.00

[a] C_{max}, Maximum concentration; T_{max}, time (after administration) maximum concentration achieved; MRT, mean residence time; MAT, mean absorption time; k_a, absorption rate constant; k_{el}, elimination rate constant; $t_{1/2}$, half-life; AUC, area under concentration-time curve; V_{ss}, volume of distribution at steady state; CL_p, total body clearance.

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