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High-Pressure Liquid Chromatographic Method for Determination of Sch 29482 in Human Serum

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A high-pressure liquid chromatographic method was developed for quantitative determination in human serum of a new penem antibiotic known as Sch 29482 (5R,6S,8R-2-ethylthio-6(1-hydroxyethyl)penem-3-carboxylic acid). The method involves serum extraction at an acid pH with ether, followed by separation on a reverse-phase column and UV light detection at 254 nm. This technique produced a good linear relationship between the peak height ratio and the Sch 29482 concentration, which ranged from 0.09 to 35.64 μg/ml. In addition, this procedure proved to be quite specific for Sch 29482, since all beta-lactam antibiotics tested did not interfere with the assay. For high concentrations (>0.5 μg/ml), the mean values obtained from the high-pressure liquid chromatographic method correlated very well (r = 0.997) with those obtained from a microbiological assay. This method is accurate and reproducible, with a sensitivity of about 0.09 μg per ml of serum. It is useful for monitoring serum drug levels in animals and humans and can also be used for drug pharmacokinetic studies in humans.

Sch 29482 (5R,6S,8R-2-ethylthio-6(1-hydroxyethyl)penem-3-carboxylic acid) is a new penem antibiotic, prepared by a synthetic route from 6-aminopenicillanic acid (V. M. Girijavallabhan, A. K. Ganguly, S. W. McCombie, P. Pinto, and R. Rizvi, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 21st, Chicago, Ill., abstr. no. 829, 1981). Sch 29482 has excellent activity against gram-positive organisms and a large number of non-*Pseudomonas* gram-negative organisms (R. S. Hare, G. H. Miller, L. Naples, A. Sabatelli, D. Loebenberg, and J. A. Waitz, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 21st, Chicago, Ill., abstr. no. 831, 1981). It also shows excellent stability against a wide variety of beta-lactamases (K. Dornbush, L. Adamsson, L. Gezelius, and H. O. Hallander, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 21st, Chicago, Ill., abstr. no. 740, 1981).

The concentrations of Sch 29482 in serum have been previously determined by a microbiological assay. However, the method has limited sensitivity and reproducibility. To facilitate absorption and pharmacokinetic studies in animals and humans, we developed a sensitive high-pressure liquid chromatographic (HPLC) method for the measurement of Sch 29482 in serum. This report describes a new HPLC method and its application to the measurement of serum levels of Sch 29482 in humans after oral administration of the antibiotic. The serum levels of Sch 29482 measured by the HPLC method are compared with those measured by the microbiological assay.

MATERIALS AND METHODS

Compounds. Sodium ampicillin, sodium amoxicillin, sodium cloxacillin, sodium carbenicillin, sodium dicloxacillin, sodium methicillin, sodium nafcillin, sodium oxacillin, potassium penicillin G, and potassium penicillin V were purchased from Sigma Chemical Co., St. Louis, Mo. Sch 29482 (sodium salt) and *m*-propionamidophenol (internal standard) were obtained from Schering Corp., Bloomfield, N.J.

Extraction. A 2-ml amount of 0.4% H_3PO_4 and 0.1 ml of *m*-propionamidophenol solution (10 μg of internal standard per ml in acetonitrile) were added to 0.5 ml of serum, and the mixture was extracted twice with 10 ml of diethyl ether (washed with water before use) by high-speed shaking with an Eberbach shaker for 10 min. Each ether portion was dried with 1.0 g of anhydrous Na_2SO_4. The combined ether extract was evaporated to dryness under N_2 at room temperature. The residue was then dissolved in 3 ml of a solution containing 10% acetonitrile and 90% 0.05 M sodium acetate. Afterward, 0.1 ml of the solution was injected into a high-pressure liquid chromatograph through a Waters Intelligent Sample Processor (Waters Associates, Millford, Mass.).

Chromatographic conditions. The HPLC system consisted of a Waters model 6000A pump and a model 440 absorbance detector with a fixed wavelength of 254 nm (Waters Associates). Separation was accomplished on a Partisil 10 ODS-3 column (0.46 by 25 cm; Whatman Inc., Clifton, N.J.). The absorbance detector output was monitored with a 5-mV recorder (model

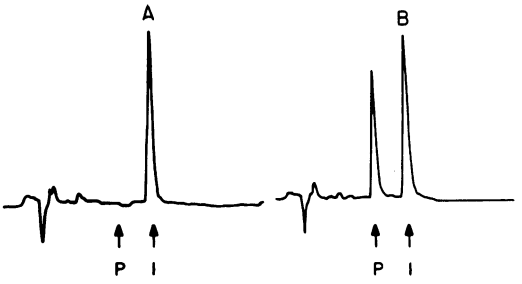

FIG. 1. Typical liquid chromatogram of Sch 29482 (P) and the internal standard (I) from control human serum (A) and control human serum spiked with 5 μg of Sch 29482 per ml (B). The *y* axis represents the detector response, and the *x* axis represents the retention time.

9176; Varian Aerograph, Palo Alto, Calif.), and detector sensitivity was 0.02 optical density units, full scale. The solvent mixture (two parts acetonitrile and eight parts 0.05 M sodium acetate buffer [pH 4.1]) was delivered at 1.0 ml/min at 1,200 lb/in^2. All separations were carried out at ambient temperature.

Calculation. Chromatogram peaks were identified on the basis of retention time. Calculation of the Sch 29482 concentration was based on the ratio of the peak height of Sch 29482 to the peak height of the internal standard. Possible variations in chromatographic response were corrected by frequent injection of the standard (in triplicate) prepared daily by adding 0.1 ml of an aqueous solution, which containined 5 μg of Sch 29482 (sodium salt), and 0.1 ml of *m*-propionamidophenol solution (10 μg/ml in acetonitrile) to 0.5 ml of control human serum and extracted as described previously. Since a good linear relationship was obtained between the peak height ratio and the concentration of Sch 29482 (see Fig. 2), the serum levels of Sch 29482 were calculated from the extracted standard and expressed as Sch 29482 acid equivalent; the conversion factor was 0.893, which also accounts for the moisture content of the drug.

TABLE 1. HPLC retention times of Sch 29482, other beta-lactam antibiotics, the internal standard, and some common drugs

Compound	Retention time (min)
Sch 29482	8
m-Propionamidophenol (internal standard)	10
Sodium ampicillin	<2
Sodium amoxicillin	<2
Sodium cloxacillin	54
Sodium carbenicillin	<2
Sodium dicloxacillin	119
Sodium methicillin	9
Sodium nafcillin	73
Sodium oxacillin	31
Potassium penicillin G	13
Potassium penicillin V	23
Acetaminophen	4.5
Caffeine	5.5
Theophyllin	4
Aspirin	6.5
Chlorpheniramine	55.0
Pseudoephedrine	5

RESULTS AND DISCUSSION

Recently, HPLC methods have been used widely to determine the plasma and serum concentrations of antibiotics such as netilmicin (6), tobramycin (3), erythromycin (9), various peni-

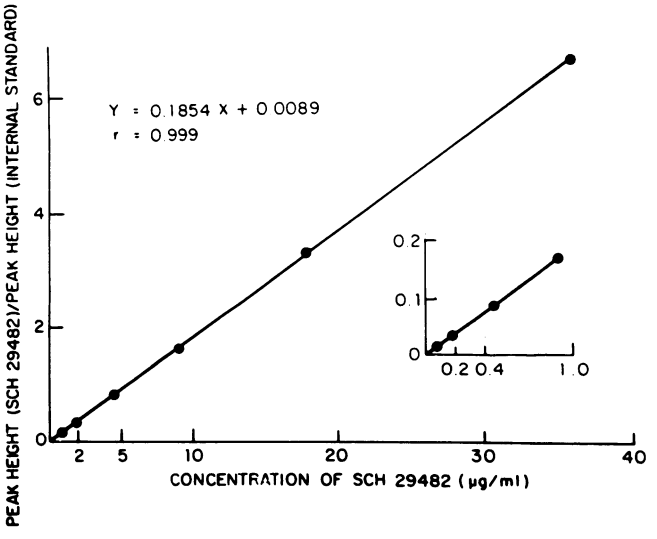

FIG. 2. Standard curve for Sch 29482 extracted from human serum, with *m*-propionamidophenol as the internal standard. The insert indicates low concentration.

TABLE 2. Sensitivity and precision of the HPLC method

Sample no.	Amt of Sch 29482 acid[a] (μg/ml) added to human serum	Amt of Sch 29482 acid (μg/ml) detected[b]
1	0.089	0.089
2	0.089	0.089
3	0.089	0.093
4	0.089	0.092
5	0.089	0.083
6	0.089	0.094

[a] Expressed as Sch 29482 acid equivalent.
[b] Mean, 0.090; coefficient of variation, 4.44%.

cillins (8, 10), and cephalosporins (1, 4, 5, 7). The present paper describes a simple and sensitive HPLC assay for a new penem antibiotic, Sch 29482, in human serum. Figure 1 shows the typical liquid chromatogram of Sch 29482 and the internal standard (*m*-propionamidophenol) extracted from control serum and from the serum spiked with Sch 29482. The retention times for Sch 29482 and the internal standard were 8 and 10 min, respectively. These are clearly different from the retention times for other beta-lactam antibiotics (Table 1). Thus, the HPLC method measures Sch 29482 specifically, and the beta-lactam antibiotics tested do not interfere with the measurement of Sch 29482. We have also shown that Sch 29482 has a retention time different from those of common drugs (Table 1).

The standard curve for Sch 29482 was obtained by plotting the ratio of the peak height of Sch 29482 to the peak height of the internal standard against the concentration of Sch 29482. As shown in Fig. 2, a good linear relationship was obtained between the peak height ratio and the drug concentrations, which ranged from 0.09 to 35.64 μg/ml. Regression analysis of the data revealed a correlation coefficient of 0.999. When the peak height ratio obtained from extracted serum samples was compared with that from corresponding unextracted standards, the recovery of Sch 29482 from serum was found to be about 87%. We have also estimated the sensitivity of the HPLC method to be about 0.09 μg/ml (Table 2).

The within-day reproducibility of the method was demonstrated at two concentrations of Sch 29482 (8.93 and 26.73 μg per ml of serum). Eight serum samples at each concentration were extracted as described above and injected into an HPLC column on the same day. The results (Table 3) show that the HPLC method was accurate and showed good reproducibility. To evaluate the between-day reproducibility of the method, we spiked four serum samples (0.5 ml) with 4.46 μg of Sch 29482 on each of 5 consecutive days. As shown in Table 4, the between-day run (day-to-day) precisions were quite impressive.

TABLE 3. Reproducibility of the HPLC method

Sample no.	Concn of Sch 29482 acid[a] (μg/ml) in human serum	Concn of Sch 29482 acid (μg/ml) detected[b]
1	8.93	8.99
2	8.93	8.71
3	8.93	9.19
4	8.93	8.90
5	8.93	8.70
6	8.93	8.90
7	8.93	9.19
8	8.93	8.91
9	26.73	27.01
10	26.73	27.20
11	26.73	26.63
12	26.73	26.64
13	26.73	27.01
14	26.73	26.64
15	26.73	26.26
16	26.73	26.36

[a] Expressed as Sch 29482 acid equivalent.
[b] For samples 1 through 8, the mean was 8.91, and the coefficient of variation was 2.27%; for samples 9 through 16, the mean was 26.71, and the coefficient of variation was 1.17%.

To investigate the feasibility of using the HPLC method to measure Sch 29482 levels in human serum, we analyzed serum samples from 10 volunteers after oral administration of 125 and 1,000 mg of Sch 29482 (sodium salt). Figure 3 shows the mean serum concentration-time (0 to 10 h) curve obtained with the HPLC method. Sch 29482 levels reached a maximum of about 1 h after drug administration. Thereafter, the drug concentration decreased with time, and the serum half-life ranged from 0.95 to 1.37 h.

The serum samples were also measured for Sch 29482 concentration by a microbiological assay consisting of a cylinder cup and *Sarcina lutea* ATCC 9431 as the test organism. Serum samples were compared with the standard curve by standard assay procedures (2). From 0 to 10 h

TABLE 4. Between-day reproducibility of the HPLC method

Day of assay	Peak height ratio of indicated serum sample				Variation within determinations[a]	
	A	B	C	D	Mean	CV (%)
1	1.64	1.66	1.67	1.67	1.66	0.85
2	1.66	1.62	1.70	1.66	1.66	1.96
3	1.62	1.65	1.63	1.85	1.68	6.46
4	1.57	1.61	1.67	1.61	1.61	2.55
5	1.61	1.64	1.65	1.65	1.63	1.15

[a] For variation between determinations, the mean was 1.65, and the CV was 1.68%.

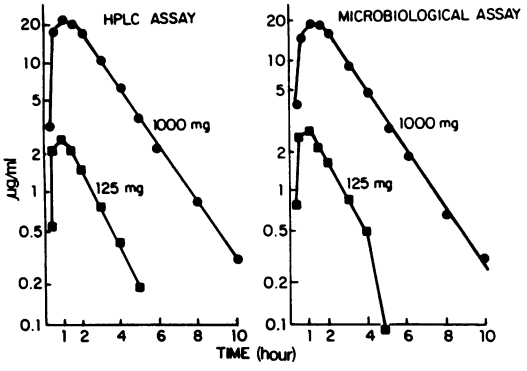

FIG. 3. Mean serum concentrations of Sch 29482 in 10 volunteers after oral administration of the drug.

after administration of a dose of 1,000 mg and from 0 to 4 h after administration of a dose of 125 mg, the microbiological assay and the HPLC method gave similar results (Fig. 3). For these high concentrations, the linear regression analysis for the mean values obtained from the HPLC method and those obtained from the microbiological assay yielded an equation of $y = 1.104x - 0.134$, with a correlation coefficient of 0.997 (Fig. 4).

However, at 5 h after administration of a dose of 125 mg, the serum drug concentration fell below 0.2 μg/ml. The mean value for the microbiological assay was then appreciably lower than that from the HPLC method (Fig. 3). This was due to the sensitivity limit of the microbiological assay, which is approximately eight times lower than that of the HPLC method. Thus, the HPLC method was able to measure the drug levels accurately at later time periods, which was not possible with the microbiological assay. The HPLC method, therefore, is very useful in pharmacokinetic studies of Sch 29482 in animals and humans. The HPLC method is specific for Sch 29482, since all of the beta-lactam antibiotics tested and many common drugs do not interfere with it. The microbiological assay, on the other hand, measures all microbiologically active substances. This would exclude the microbiological assay from use when Sch 29482 is coadministered with other antibiotics. The HPLC method for the determination of Sch 29482 levels described in this report is specific, sensitive, accurate, and reproducible; it is also useful for pharmacokinetic studies in animals and humans.

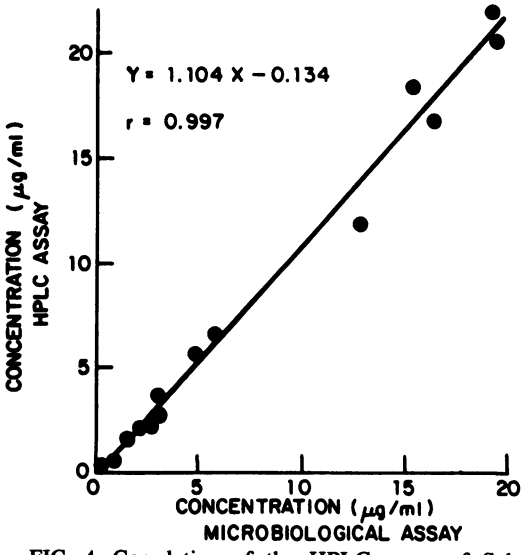

FIG. 4. Correlation of the HPLC assay of Sch 29482 in human serum with the microbiological assay.

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