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Note

Simple method for the determination of pipemidic acid in biological fluids by high-performance liquid chromatography

KATSUHARU FUKUHARA and YASUHIKO MATSUKI*

Hatano Research Institute, Food and Drug Safety Center, Ochiai, Hadano 257 (Japan)

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Pipemidic acid, 8-ethyl-5,8-dihydro-5-oxo-2-(1-piperazinyl)pyrido[2,3-*d*]pyrimidine-6-carboxylic acid, is known to be a synthetic antibacterial agent [1, 2]. It has been established that pipemidic acid acetate, formate and oxopipemidic acids are major urinary metabolites in humans, exhibiting less potent pharmacological activity than the parent drug [3]. Various methods for the determination of pipemidic acid have been developed, including high-performance liquid chromatography (HPLC) with UV detection [4, 5], fluorometry [6] and a thin-layer cup method using *Escherichia coli* Kp [6]. These methods, however, are not necessarily suitable for the assay of pipemidic acid in biological specimens with respect to sensitivity and reliability. An urgent need to clarify the pharmacokinetics of pipemidic acid orally administered to human subjects prompted us to develop a simple and sensitive method.

This paper describes a simple method for the determination of pipemidic acid in blood and urine by HPLC with fluorometric detection.

EXPERIMENTAL

Reagents and materials

Dorcol® (Dainippon Pharmaceutical, Japan), pipemidic acid and norfloxacin, which was used as an internal standard (I.S.), were kindly donated by Yohshindo (Toyama, Japan). MILLEX-SR (1 μm) was purchased from Millipore, and other chemicals employed were of analytical-reagent grade.

High-performance liquid chromatography

The apparatus used was a Shimadzu Model LC-3A solvent delivery system (Shimadzu, Kyoto, Japan) equipped with a Hitachi Model 650-10LC fluores-

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cence spectrophotometer (Hitachi, Tokyo, Japan) and a Shimadzu Model SPD-2A spectrophotometer. The test samples were applied to the chromatograph by a Rheodyne Model 7125 sample loop injector (Rheodyne, Cotati, CA, U.S.A.). A Shim-Pack CLC-ODS (Shimadzu, 5 μm) column (15 cm$\times$6 mm I.D.) was used at ambient temperature. The eluate was monitored by fluorescence detection (excitation wavelength 277 nm, emission wavelength 440 nm). Acetic acid–acetonitrile–water (2:2:25) was employed as a mobile phase at a flow-rate of 0.8 ml/min.

Standard solution

Standard solutions of pipemidic acid and norfloxacin were prepared at a concentration of 100 μg/ml in methanol and stored at 4°C. After evaporation of the solvent containing pipemidic acid (0.42–6.8 ng), the residue was dissolved in mobile phase and a 20-μl aliquot of the resulting solution was injected into the chromatograph.

Assay procedure

To a plasma specimen (1 ml) was added ethanol (3 ml) containing a known amount of I.S. (ca. 5 μg), and the resulting solution was shaken for 10 min. The mixture was centrifuged at 1600 *g* for 5 min, and the precipitate was treated twice with ethanol (5 ml) as described above. The supernatant was combined and evaporated under reduced pressure below 40°C, and the residue was dissolved in the mobile phase (5 ml). After filtration through a MILLEX-SR filter, a 20-μl aliquot was injected onto the column. The assay of pipemidic acid in urine was carried out in a similar manner.

Recovery test for pipemidic acid added to human plasma and urine

The test samples were prepared by dissolving pipemidic acid (1 or 0.2 μg) and I.S. (5 μg) in plasma (1 ml), and pipemidic acid (1 μg) and I.S. (5 μg) in urine (1 ml), respectively. Pipemidic acid was determined according to the procedure described above.

Determination of pipemidic acid in blood and urine

Sixteen healthy male volunteers were orally given a tablet of Dorcol containing pipemidic acid (250 mg per tablet). Blood samples were taken at 0, 20, 40 min, 1, 1.5, 2, 3, 5, 8 and 12 h, and the plasma was obtained by centrifugation at 1600 *g* for 15 min. The urine was collected for 24 h into a beaker. The determination of pipemidic acid was carried out according to the procedure described above.

RESULTS AND DISCUSSION

Initially, the fluorescent nature of pipemidic acid was examined for establishing suitable detection conditions. As shown in Fig. 1, pipemidic acid exhibited two maxima at 277 and 330 nm on the excitation spectrum and a maximum at 440 nm on the emission spectrum.

Separation of pipemidic acid was investigated on ODS and Nucleosil$_5$ C_{18} col-

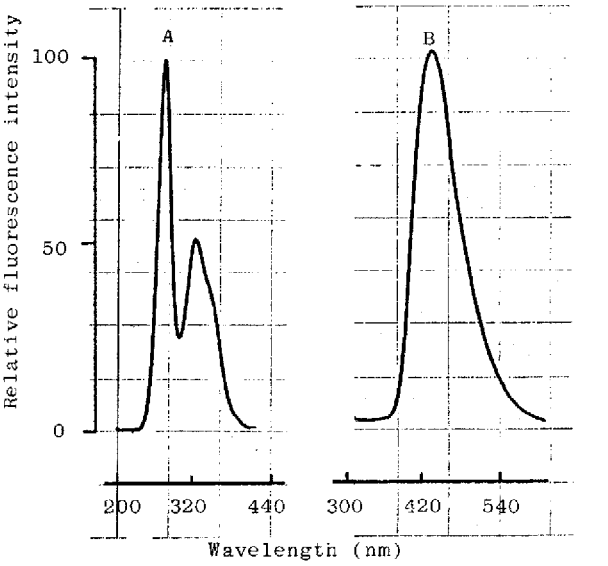

Fig. 1. (A) Excitation and (B) emission spectra of pipemidic acid in acetic acid–acetonitrile–water (2:2:25).

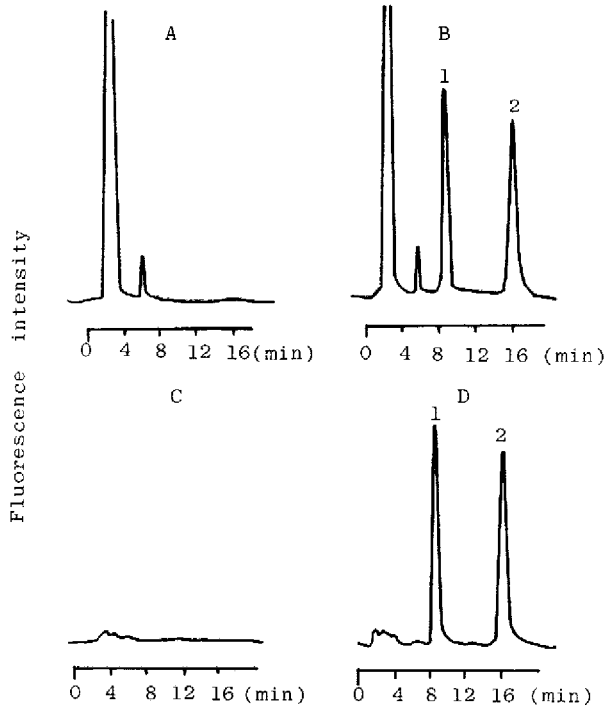

Fig. 2. Chromatograms of pipemidic acid and I.S. (A) Plasma blank; (B) plasma sample containing pipemidic acid (4.2 ng) and I.S. (20 ng); (C) urine blank; (D) urine sample containing pipemidic acid (4.0 ng) and I.S. (20 ng). Conditions: column, Shim-Pack CLC-ODS; mobile phase, acetic acid–acetonitrile–water (2:2:25); flow-rate, 0.8 ml/min; fluorescence detection, excitation wavelength 277 nm, emission wavelength 440 nm. Peaks: 1 = pipemidic acid; 2 = internal standard.

TABLE I

RECOVERY TEST FOR PIPEMIDIC ACID ADDED TO HUMAN PLASMA AND URINE

Sample	Concentration (μg/ml)		Recovery (mean ± S.D., $n=6$) (%)
	Added	Found	
Plasma	1.00	0.977	97.7 ± 1.36
	0.20	0.197	98.5 ± 2.54
Urine	1.00	0.994	99.4 ± 1.22

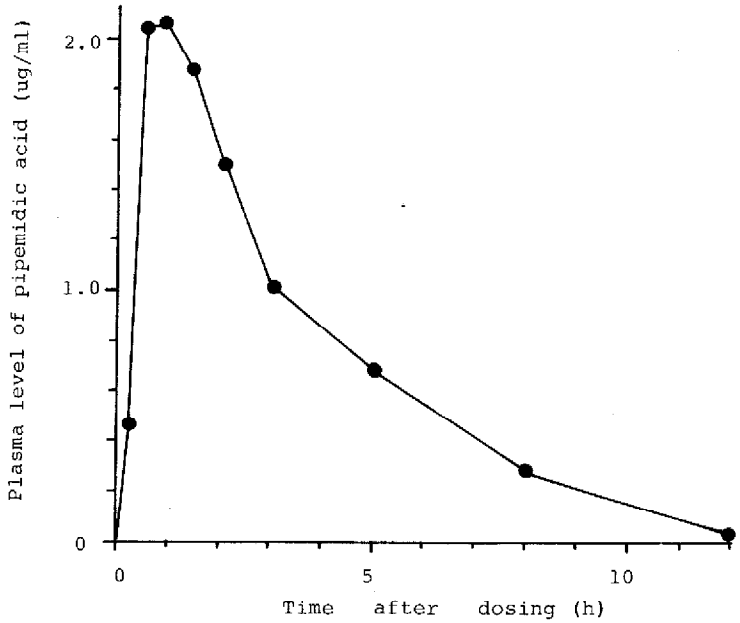

Fig. 3. Change in plasma level of pipemidic acid in sixteen human subjects orally administered the drug (250 mg).

TABLE II

URINARY EXCRETION OF PIPEMIDIC ACID FOR 24 h AFTER ORAL ADMINISTRATION OF THE DRUG (250 mg PER TABLET) TO HUMAN SUBJECTS

Subject	Amount excreted (mg)	Percentage of dose
A	81.36	32.5
B	97.65	39.1
C	77.06	30.8
D	97.97	39.2

umns with several kinds of solvent system. Consequently, when chromatographed on a CLC ODS column with acetic acid–acetonitrile–water (2:2:25), pipemidic acid gave a single peak of the correct theoretical shape with a satisfac-

tory response on the chromatogram. A typical chromatogram of pipemidic acid and I.S. obtained by fluorometric detection is illustrated in Fig. 2.

A calibration graph was constructed by plotting the ratio of the peak height of pipemidic acid to that of I.S. against the amount of the former, and satisfactory linearity was observed in the range 0.5–30 ng of pipemidic acid. The regression equation of the calibration graph is expressed as $y=2.339x-0.0015$, where x is the amount of pipemidic acid and y is the peak-height ratio of these two. The detection limit of pipemidic acid was estimated to be 0.5 ng.

Pipemidic acid in plasma and urine was efficiently separated by extracting with ethanol. In order to check the validity of the proposed method, a known amount of pipemidic acid was added to human plasma and urine, and their recovery rates were determined by the proposed method. As listed in Table I, the recovery rate of pipemidic acid in plasma was 97.7–98.5% and that in urine was 99.4%.

The blood levels of pipemidic acid were determined with sixteen healthy male volunteers orally administered Dorcol containing pipemidic acid (250 mg per tablet). A blood level profile is illustrated in Fig. 3. The blood level of pipemidic acid reached a peak concentration of 2.19 μg/ml at 1 h after administration and then gradually decreased until 12 h. When calculated from the trapezoidal rule, the mean area under the concentration–time curve (AUC) for pipemidic acid was estimated to be 8.36 μg h/ml. The urinary excretion rate of pipemidic acid was 30.8–39.2% (Table II). The limit of determination of pipemidic acid in blood was estimated to be 0.1 μg/ml.

The proposed method for the determination of pipemidic acid in biological fluids has proved to be satisfactory with respect to sensitivity and reliability. It is hoped that the availability of a simple method for the quantitation of pipemidic acid will serve to clarify the bioequivalence and bioavailability of this drug.

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