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Note

Determination by high-performance liquid chromatography of pipemidic acid in human serum and urine

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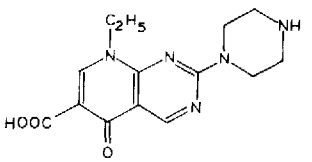
Pipemidic acid is an antibacterial agent [1—3] used in the treatment of urinary tract infections. It is normally given as a 400-mg dose every 12 h. Our experience is, that at this dose, over this period of time, serum levels of 0—4 μg/ml and urine levels of 0—800 μg/ml may be expected (see Tables I and II).



Pipemidic acid: 8-ethyl-5,8-dihydro-5-oxo-2-(1-piperazinyl)-pyrido[2,3-*d*]pyrimidine-6-carboxylic acid

Previously pipemidic acid has been determined in these two body fluids either by microbiological [4] or fluorescence methods [5].

Two recent papers have used high-performance liquid chromatography (HPLC) to determine the drug in biological fluids obtained from animal studies. The first [6] utilises a complex extraction procedure prior to normal-phase

TABLE I

SERUM LEVELS (μg/ml) OBTAINED FROM A TYPICAL PATIENT AFTER A SINGLE 400-mg DOSE OF PIPEMIDIC ACID

	Time (h)								
	0	1	2	3	4	5	6	7	8
Pipemidic acid (μg/ml)	<0.1	<0.1	2.76	3.64	3.60	3.19	2.95	1.90	1.13

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TABLE II

URINE LEVELS (mg) OBTAINED FROM A TYPICAL PATIENT AFTER A SINGLE 400-mg DOSE OF PIPEMIDIC ACID

	Time period (h)						Total	Percent dose
	0–2	2–4	4–6	6–8	8–12	12–24		
Pipemidic acid excreted (mg)	28.7	69.3	40.3	45.5	48.8	27.4	260.0	65.0

chromatography, whilst the second [7] uses reversed-phase chromatography followed by post-column derivatisation and fluorescence detection.

Our procedure offers a simple fast alternative for quantitative determination of the drug in both serum and urine and may be easily automated to cope with large sample numbers.

EXPERIMENTAL

Chromatography system

Reversed-phase chromatography was carried out on a Waters Assoc. (Milford, MA, U.S.A.) chromatography system consisting of a Waters pre-packed column (stainless-steel 30 cm × 3.9 mm I.D.) packed with μBondapak C_{18}, 10 μm, a Waters 6000A solvent delivery pump, a Waters 440 fixed-wavelength detector with 280 nm phosphor and a Waters Wisp 710A automatic sample injector. The analytical column was protected by a guard column, stainless-steel 5 cm × 5 mm I.D., packed with Co:Pell ODS C_{18} pellicular packing material, 30 μm (Whatman, Clifton, NJ, U.S.A.). A 10-mV recorder was used and peak integration was carried out using a Hewlett-Packard 3351 laboratory data system.

Mobile phase

The mobile phase for the urine analysis, mobile phase A, consisted of 46.8 g of sodium dihydrogen orthophosphate dihydrate, $NaH_2PO_4 \cdot 2H_2O$, (Analar) dissolved in a mixture of 275 ml of HPLC grade methanol and 725 ml of water.

For the serum analysis the mobile phase was altered to separate the pipemidic acid peak from background peaks due to the serum. Mobile phase B consisted of 46.8 g of $NaH_2PO_4 \cdot 2H_2O$ dissolved in a mixture of 75 ml methanol (HPLC grade), 75 ml of acetonitrile (HPLC grade) and 850 ml of water.

Both mobile phases were filtered through Whatman GF/F glass microfibre filters (Whatman, Springfield Mill, Maidstone, Great Britain) under vacuum to degas them before use.

Sample preparation and chromatography

Urine. A 1-ml volume of the urine sample under test was diluted to 50 ml with mobile phase A and filtered through a Millipore GS, 0.2-μm, filter (Millipore, London, Great Britain). A 50-μl sample of the resulting filtrate

was injected into the chromatograph. The flow-rate of mobile phase A was 1.5 ml/min. Chromatography was at ambient temperature and detection was at 280 nm with a detector sensitivity of 0.05 absorbance units full scale (a.u.f.s.).

The retention time of pipemidic acid under these conditions is approximately 7 min (see Fig. 1). Peak areas in the sample chromatograms were quantitated by the external standard technique using a standard solution of pipemidic acid trihydrate reference standard (Dainippon Pharmaceuticals, Osaka, Japan) dissolved in mobile phase A.

Serum. A 1-ml volume of the serum under test was diluted by addition of 3 ml of dilute acetic acid (60 ml of glacial acetic acid in 1 l of water). The resulting solution was filtered through a Millipore GS, 0.2-μm filter and 200 μl of the fitrate were injected. The flow-rate of mobile phase B was 1.8 ml/min. Chromatography was at ambient temperature with detection at 280 nm, 0.02 a.u.f.s.

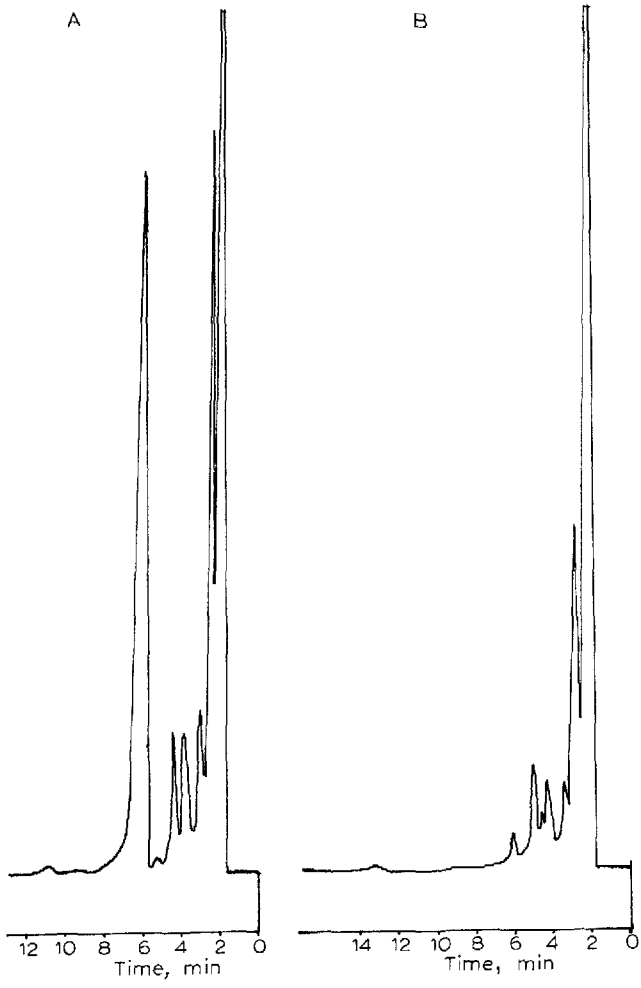

Fig. 1. Chromatograms of blank urine (B) and urine containing 324 μg/ml (A) of pipemidic acid obtained from a typical patient. Conditions as stated in the text (mobile phase A, 0.05 a.u.f.s.).

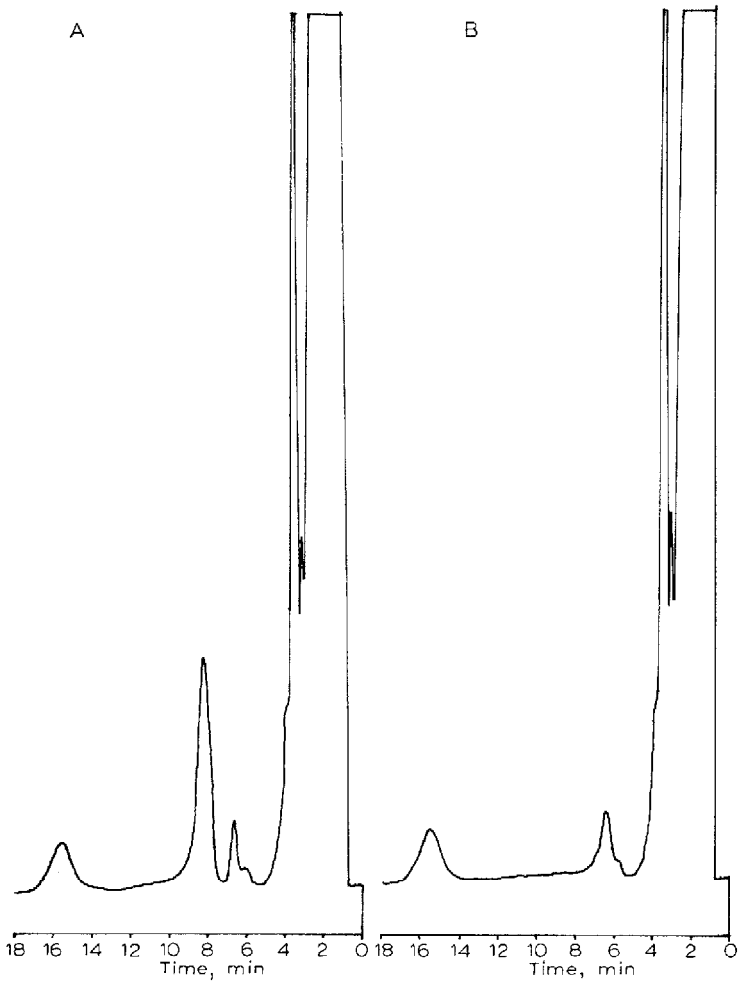

Fig. 2. Chromatograms of blank serum (B) and serum containing 1.40 μg/ml (A) of pipemidic acid obtained from a typical patient. Conditions as stated in the text (mobile phase B, 0.02 a.u.f.s.).

Quantitation was again carried out by comparison of peak areas with those from an external standard. The retention time of pipemidic acid under these conditions was approximately 9 min (see Fig. 2).

Recovery experiments were performed by spiking 1-ml aliquots of unmedicated human serum and urine with various amounts of pipemidic acid trihydrate reference standard dissolved in dilute acetic acid. These samples were then analysed as outlined above and a factor for the recovery of pipemidic acid from the two body fluids was obtained (see Tables III and IV).

The precision of the method was determined by analysis of six aliquots from one sample each of urine and serum containing pipemidic acid (see Table V).

TABLE III

RECOVERY OF PIPEMIDIC ACID FROM URINE

Pipemidic acid added, (μg/ml, x)	Pipemidic acid found, (μg/ml, y)	Recovery (%)
25.3	25.4	100.4
63.1	66.1	104.8
108	108	100.0
216	198	91.7
324	326	100.6
432	438	101.4
540	535	99.1
	Mean	99.7%

Slope = 0.9989; linear correlation coefficient = 0.9992; y intercept = —1.4.

TABLE IV

RECOVERY OF PIPEMIDIC ACID FROM SERUM

Pipemidic acid added, (μg/ml, x)	Pipemidic acid found, (μg/ml, y)	Recovery (%)
0.568	0.50	88.0
1.136	1.17	103.0
2.272	2.11	92.9
3.408	3.18	93.3
4.544	4.20	92.4
5.680	5.55	97.7
	Mean	94.6%

Slope = 0.959; linear correlation coefficient = 0.9986; y intercept = —0.028.

TABLE V

PRECISION OF THE ASSAYS OF PIPEMIDIC ACID IN BODY FLUIDS

Body fluid	Pipemidic acid found (μg/ml)	Mean ± relative standard deviation (%)
Urine	353 351 361 346 329 338	346 ± 3.29 (n = 6)
Serum	1.94 1.92 1.83 2.00 1.80	1.90 ± 3.99 (n = 6)

RESULTS AND DISCUSSION

Choice of chromatography systems

Pipemidic acid is amphoteric in nature and from consideration of its pK values [8] the best approach to developing a suitable mobile phase appeared to be an anionic ion-pairing system combined with a reversed-phase column.

Some of the mobile phases tried were: (1) methanol—water—acetic acid (45:55:0.1); (2) methanol—water—acetic acid (60:40:0.8) containing 0.005 *M* sodium lauryl sulphate; (3) methanol—water (30:70) containing sodium dihydrogen orthophosphate at different strengths.

The major problem with systems 1 and 2 was that the pipemidic acid peak was affected by very bad tailing. It was found that addition of sodium dihydrogen orthophosphate to the mobile phase produced sharper peaks and that increasing its concentration eventually eliminated tailing. The optimum concentration was found to be 0.3 *M*. Such a high salt concentration in the mobile phase can have a detrimental effect on the μBondapak C_{18} column packing over a sustained period of time, as it slowly dissolves away the silica support phase eventually resulting in a void space in the column which affects chromatographic efficiency. This may be overcome by use of a pre-column packed with 30-μm silica inserted between the pump and injector of the chromatograph. In this way the mobile phase becomes saturated with silica before entering the main guard column—analytical column system, and any void generated in the silica pre-column should not affect the chromatography.

Sample preparation

A 1-cm layer of a solution of pipemidic acid (1%, w/v) in aqueous acetic acid (1%, v/v) has been shown to have an absorbance (*A* 1 cm, 1%) of circa 1900 at a wavelength of 275 nm [9]. This strong absorbance meant that the drug could easily be estimated in urine by dilution followed directly by chromatography without any prior extraction step. It was decided to try a similar procedure for the serum samples. Due to the large number of analyses involved, avoidance of prior extraction would cut down the time required for each analysis. Using a direct dilution method on serum samples did have some disadvantages. Firstly, there were often a considerable number of background peaks in the chromatogram from the serum (see Fig. 2) and the mobile phase had to be carefully adjusted so that the pipemidic acid peak was well separated from them. Secondly, the continual injection of diluted serum onto the reversed-phase C_{18} chromatography column resulted in irreversible adsorption of non-polar compounds contained in the serum which eventually affected the chromatographic efficiency. For this reason the use of the guard column is very important and it was repacked with fresh Co:Pell ODS after every 50 sample injections to protect the main analytical column from contamination.

Sensitivity, linearity and precision

Using these methods the lower limit of detection for pipemidic acid in urine is estimated to be ca. 5 μg/ml, and in serum ca. 0.1 μg/ml. However, the peaks in the chromatogram become too small for accurate quantitation at urine levels below ca. 15 μg/ml and at serum levels below ca. 0.3 μg/ml. For this reason linearity has only been demonstrated over the range for which accurate determination of peak areas is possible. Below these levels, which are outside the range of interest for this particular investigation, the drug concentrations may only be approximated.

The data in Table III show the recovery of pipemidic acid from urine to be essentially quantitative with a linear detector response in the range 25—540 μg/ml. The precision of the method is satisfactory with a relative standard deviation of 3.29% (Table V).

The recovery of pipemidic acid from serum was slightly lower (Table IV), and detector response varied linearly with pipemidic acid concentration in the range 0.50—5.55 μg/ml. The precision of the assay for serum samples was also satisfactory, relative standard deviation = 3.99% (Table V).

The sensitivity, linearity and precision of the methods were found to be adequate for the determination of pipemidic acid in both serum and urine.

Applications

These methods have been used successfully to assay 200 serum samples and 130 urine samples during a bioavailability comparison of several pipemidic acid formulations.

The simple sample preparation steps and the use of an auto-injector on overnight runs, enabled one analyst to complete the analysis of 40 samples of serum or urine in less than the equivalent of one working day; this included the preparation of samples and standards, carrying out chromatography and calculation of results.

CONCLUSION

Pipemidic acid in body fluids may be determined rapidly and accurately using the reversed-phase HPLC methods described.

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