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Note

Estimation of pipemidic acid and N-nitrosopipemidic acid by high-performance liquid chromatography

CESARE A. ZIO and GABRIELE MASSARANI
Italiana Schoum S.p.A., Via Kuliscioff 6, 20100 Milan (Italy)
and
PIERGIORGIO PIETTA*
Istituto Chimica Organica ed Analitica, Via Celoria 2, 20133 Milan (Italy)
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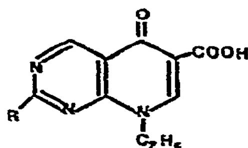
Pipemidic acid (I) (8-ethyl-5-oxo-2-(1-piperazinyl)-5,8-dihydro[2,3-*d*]-pyrido-6-pyrimidinecarboxylic acid)[1] has become an important chemotherapeutic agent with a broad-spectrum activity[2–6]. The antibacterial activity of I proved to be higher than that of piromidic acid (II) and nalidixic acid (III)[7–10].



I

II

III

Recently, the pharmacokinetic[11–16] and pharmacological properties[17–23] of I in humans and animals have been extensively studied, and the toxicity of I found to be very low. However, the presence of monosubstituted piperazine in I makes possible the reaction with sodium nitrite, "*in vitro*" and "*in vivo*", to yield N-nitrosopiperazine derivatives. The toxicity of N-nitroso derivatives is well known[24,25]; therefore it is very important to investigate the formation of such derivatives.

For these reasons, the reaction between pipemidic acid and sodium nitrite was studied. In the course of this study, the cleavage of I to give N-nitrosopiperazine and

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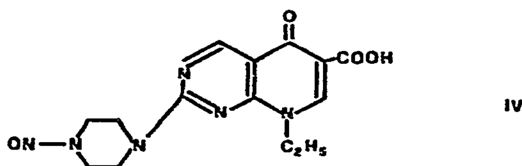
N,N-dinitrosopiperazine was excluded[26], N-nitrosopipemidic acid (IV) being the only product. Its identity was confirmed by infrared (IR), nuclear magnetic resonance (NMR) and mass spectrometry.



IV

A selective and sensitive assay was needed to evaluate IV in the presence of large amounts of I. Thin-layer chromatography proved to be unsuitable, due to the low mobility of I, except in *n*-butanol–acetic acid–water (4:1:1 v/v) or similar systems, where IV is easily decomposed. The high molecular weights of I and IV and the variety of polar functional groups precluded the use of gas–liquid chromatography. On the other hand, high-performance liquid chromatography (HPLC) was very effective in the simultaneous analysis of I and IV.

EXPERIMENTAL

Equipment

Spectra were recorded on Perkin-Elmer Model 297, Model R-25B, Pye Unicam Model SP 1700 and Hitachi Perkin-Elmer Model RMU-6D spectrophotometers. A liquid chromatograph (Perkin-Elmer Model 601) equipped with a Model 7105 injector (Rheodyne), a 0.26 × 25 cm polar bonded-phase column (Amino Sil-X-1, Perkin-Elmer) and a Model LC-55 variable-wavelength detector operating at 275 nm (Perkin-Elmer) was used

Materials

Powdered samples of pipemidic acid (B.T.B., Milan, Italy) were used in the analytical procedure and in the preparation of standard curves The solvents were redistilled in all-glass apparatus; other chemicals were of the highest purity commercially available

Compound IV was synthesized in our laboratory as follows. To a solution of I (5 g) in acetic acid (1000 ml), sodium nitrite (4 g) was added portionwise within 20 min with stirring After 30 min the solvent was decanted off, the precipitate filtered off and thoroughly washed with water The product was dried under vacuum over phosphorus pentoxide at room temperature Yield: 4.2 g (77%); m.p. 302°C (with decomposition). It is soluble in alkalies and acids, in dimethyl sulphoxide, dimethylformamide and carbon tetrachloride, but insoluble in methanol, ethanol, diethyl ether and water. Calc. for $C_{14}H_{16}N_6O_4$; C, 50.60; H, 4.85; N, 25.29%. Found: C, 50.92; H, 4.74; N, 25.0%. IR (in Nujol): 1530, 1430, 1120, 1020 and 980 cm^{-1}. NMR: $\delta = 1.4$–1.7 (t, 3; CH_3CH_2, $J = 8$ Hz); $\delta = 4.4$–4.7 (m, 10; piperazine ring); $\delta = 9.1, 9.4$ (s, 1; C_4, C_7). UV: log $\varepsilon = 4.6733$ at $\lambda_{max.} = 276$ nm, also $\lambda_{max.} = 325$ nm in 50% v/v N,N-dimethylformamide. Mass spectrum: parent at *m/e* 332; fragments at *m/e* 302 (loss of NO) and *m/e* 287 (loss of COOH).

Solutions

Stock solutions (0.2% w/v) of I and IV were prepared by dissolving each powder in N,N-dimethylformamide. Appropriate dilutions yielded solutions of 10–200 μg/ml of I and 3.5–310 μg/ml of IV in N,N-dimethylformamide–chloroform (1:1 v/v). The solutions were used within 24 h.

Urine samples were doped with I and IV to yield solutions of 10–100 μg/ml of I and 1–10 μg/ml of IV. A 5-ml specimen was acidified with 6 *N* hydrochloric acid to pH 2.5, saturated with sodium chloride and extracted with three 5-ml portions of ethyl acetate. The combined extracts were then treated with 4 *N* NaOH to pH 10 and extracted with three 5-ml portions of ethyl acetate. The specimen was finally neutralized to pH 5.6 and extracted with three 5-ml portions of carbon tetrachloride. The pooled extracts were dried over anhydrous sodium sulphate, filtered and evaporated to dryness *in vacuo*. The residue was dissolved in N,N-dimethylformamide–chloroform (1:1 v/v) in preparation for assay by HPLC.

Chromatographic conditions

The mobile phase was N,N-dimethylformamide–chloroform–acetic acid (50:50:1, v/v/v), the components of which were separately filtered through membrane filters (Type FH 0.5 μm, Millipore) before mixing. The flow-rate of the mobile phase was 0.5 ml/min. The column effluent was monitored by UV absorption at 275 nm.

Chromatograms were recorded on a potentiometric recorder (Model PM 8010/02, Philips) and peak areas were used for quantitation. Replicate 2-μl injections of standard solutions were made using a 10-μl Hamilton syringe. The column was maintained at 60°C for all separations.

RESULTS AND DISCUSSION

Compound I was easily converted into IV by reaction with sodium nitrite in acetic acid. IV showed the spectral properties of N-nitroso derivatives (see *Materials*).

As shown in Fig. 1A and 1B, compounds I and IV were eluted as sharp, symmetrical and well-defined peaks with respect to the baseline. They were completely separated and eluted in less than 20 min (Fig. 1C), with retention times of 172 (IV) and 416 sec (I) under these chromatographic conditions.

The calibration curves of I and IV, obtained by four or more injections of seven standard solutions of each substance, showed in the investigated range a linear relationship between concentration and peak area (mm^2) with a slope of 3.89 mm^2/ml/μg, intercept of 0.156 mm^2 and r^2 of 0.9974 for I, and a slope of 5.40 mm^2/ml/μg, intercept of 4.422 mm^2 and r^2 of 0.9984 for IV. The highest relative standard deviation of the areas (within each group) at any concentration was 2.68% (at 25.16 μg/ml, five injections) for I and 2.37% (at 38.4 μg/ml, six injections) for IV.

The detection limit, on the basis of the amount of the injected compound that caused an absorption of twice the standard deviation of the baseline noise, was found to be 8.10 (I) and 1.25 ng (IV). The analytical sensitivity of the photometric method, expressed as the amount of drug giving a detector response of 0.0044 a.u. (absorbance units), was calculated to be about 42.6 (I) and 8.6 ng (IV). The recoveries in the specified concentration ranges were 99.6 ± 2.1% (mean ± S.D.) with a range of 98.1–100.8% for I, and 99.8 ± 1.8% with a range of 98.1–100.4% for IV. There were

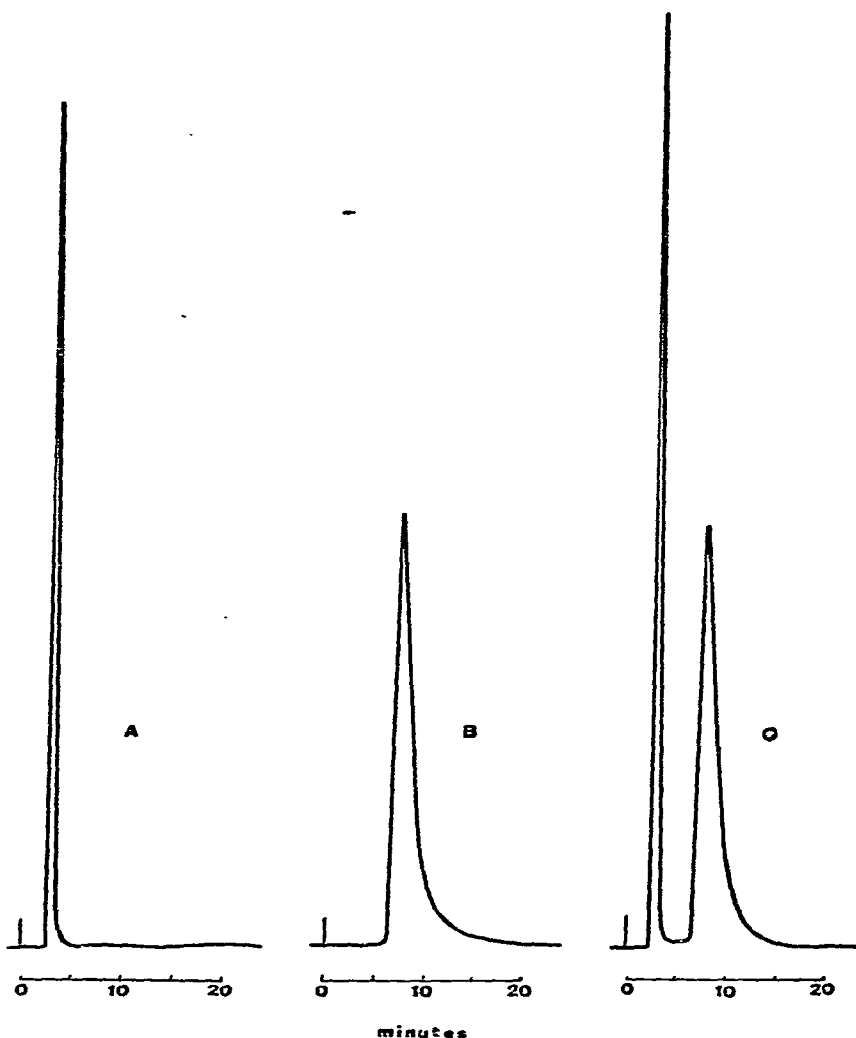


Fig. 1. High-performance liquid chromatogram of N-nitrosopipemidic acid (A), pipemidic acid (B) and their mixture (C).

no significant differences between the areas obtained by injecting 2-μl samples of a standard solution containing known amounts of I and IV and the areas obtained by injecting 8-μl samples of the same solution diluted by a factor of four.

No interference from known impurities (pipemidic acid methyl ester) was noted. The described HPLC procedure is precise, accurate and specific for I and IV. It has been applied to the analytical control and assay of I either as bulk drug or as a component in pharmaceutical preparations (capsules), and the results indicated the absence of IV. The importance of the last result is marked by the recent findings[27] that pure drugs containing secondary and/or tertiary amino groups can react with nitrogen oxides in the air to give N-nitroso derivatives. Therefore these compounds may be present as impurities, despite the use of a nitrite-free synthetic process.

This method was also applied to the analysis of samples of urine doped with I and IV. In this case a preliminary extraction at acidic and basic pH values was necessary. The recoveries were 96.8 ± 2.5% for I and 97.8 ± 1.8% for IV.

In conclusion, the present method is specific and sensitive enough to detect very small amounts of IV, resulting from drug–nitrite interaction, even in the presence of a large quantity of I.

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