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A critical study on degradation of azathioprine in aqueous solutions

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The aspects of degradation kinetics of azathioprine in aqueous solutions at 73°C were recently discussed by Mitra and Narurkar (1986). In our laboratory a similar investigation at comparable temperature (80°C) was carried out earlier (Kumar, 1983; Gupta et al., 1983) with an aim to establish kinetic parameters pertinent to stability of this compound in aqueous media.

Mitra and Narurkar (1986) report that 6-mercaptopurine is formed as primary degradation product of azathioprine over the pH range 1–13. It is stated that a peak with HPLC retention time corresponding to pure 6-mercaptopurine was observed in all kinetic runs.

At the time when kinetic studies were carried out in our laboratory, we observed that samples withdrawn during kinetic runs above pH 7 were colorless but those belonging to acidic pH region below pH 7 were light yellow to yellow in colour. To get an insight into the products formed at various pH and to understand pathways of their formation, we alkalinized the reaction samples and subjected them to spectral studies. For the same purpose investigations involving thin-layer chromatographic separation and identification of products were made. It was shown that in situations where reaction samples were colorless, 6-mercaptopurine along with the associated degradant, 1-methyl-4-nitro-5-hydroxyimidazole, were produced as major degradation products of azathioprine. Under acidic conditions the products were altogether different and they were identified as hypoxanthine and 1-methyl-4-nitro-5-thioimidazole, the latter being responsible for the appearance of the yellow colour in the reaction solutions. 6-Mercaptopurine was shown to degrade further under the reaction conditions in a sequential manner through purine-6-sulphinate to hypoxanthine. There were several cases when no evidence was presented for the presence of 6-mercaptopurine in the reaction samples. The imidazole products were also indicated to be unstable under the condition in which they were formed. The total reaction scheme and other details are contained in our recent publication (Gupta et al., 1987).

It is evident that the observations made by us as above and those of Mitra and Narurkar (1986) are not in conformity with each other. Further studies were, therefore, taken up in our laboratory to examine critically what exactly is the degrada-

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tion pattern of azathioprine in aqueous solutions. The present paper is concerned with high-performance liquid chromatographic examination of the degradation of azathioprine.

Azathioprine and 6-mercaptopurine were generous gifts from The Wellcome Foundation Ltd., London. 1-Methyl-4-nitro-5-thioimidazole was obtained as a gift from Burroughs Wellcome Co., Research Triangle Park, NC, U.S.A. Hypoxanthine was purchased from BDH Chemicals Ltd., Poole, England. Purine-6-sulphinate was prepared from 6-mercaptopurine (Doerr et al., 1961). Buffer substances and all other chemicals were analytical grade. Fresh triple distilled water from an all-glass still was used in all studies.

The hydrolytic reactions were performed for 2.5×10^{-4} M drug and at several pH values between 1.11 and 11.20. Hydrochloric acid, acetate, phosphate, borate and sodium hydroxide were used as buffers. An ionic strength of 0.2 was maintained for each buffer by adding calculated amounts of sodium chloride. The solutions were sealed into 5-ml glass ampoules and thermostated at $80 \pm 0.5\,^{\circ}$C. Samples were withdrawn at appropriate intervals and stored at $-20\,^{\circ}$C to stop further reaction.

The HPLC separations were carried out by following the procedure described by Fell et al. (1979). This method was preferred over other HPLC procedures (reviewed by Wilson and Benezra, 1981) as it allows separation and chromatographic identification of all azathioprine degradation products. The studies were done on a Beckman 340 series LC equipped with Kipp&Zonen BD 41 recorder and Altex model C-RIA recorder/integrator. The conditions were: column,

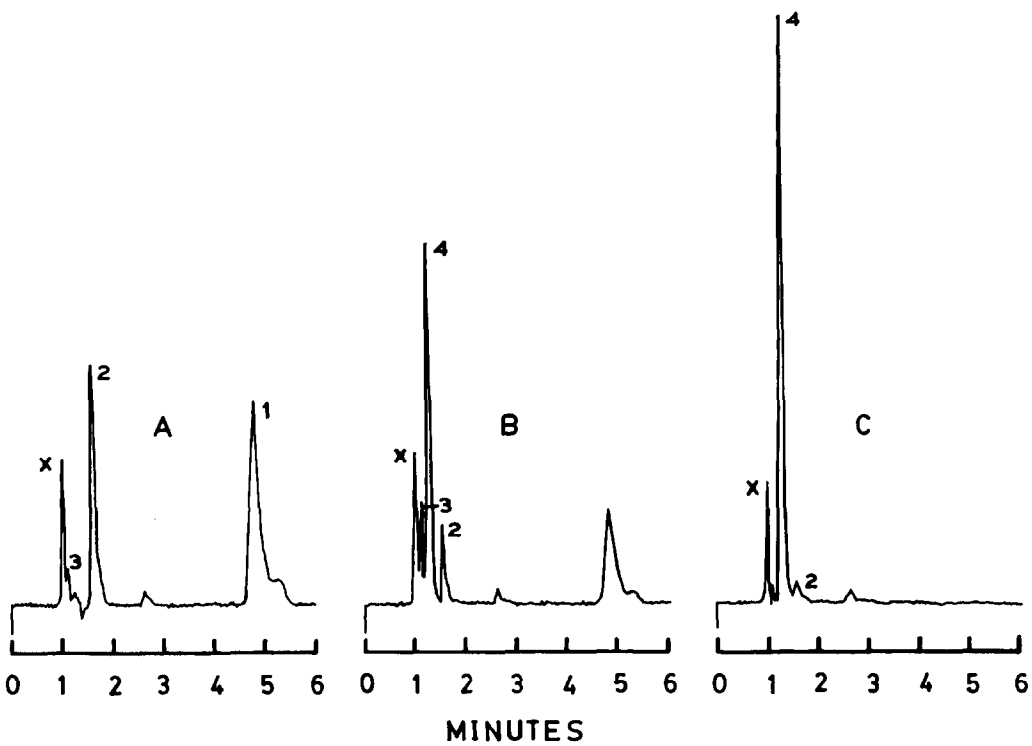


Fig. 1. High-performance liquid chromatograms of reaction samples at 80 °C and $\mu = 0.2$ representing A, 33% degradation of azathioprine at pH 10.55; B, 69% degradation of azathioprine at pH 10.55 and C, total degradation of azathioprine at pH 10.55. (1) azathioprine; (2) 6-mercaptopurine; (3) purine-6-sulphinate; (4) hypoxanthine; (×) not established.

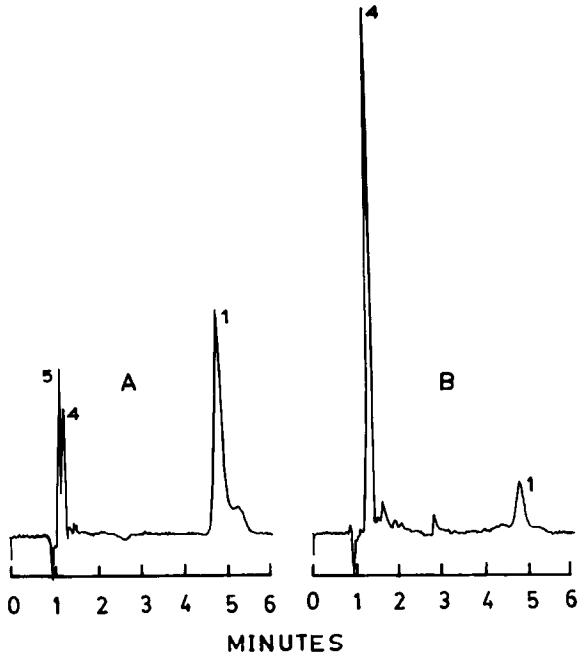


Fig. 2. High-performance liquid chromatograms of reaction samples at 80 °C and $\mu = 0.2$ representing A, 24% degradation of azathioprine at pH 1.1; B, 83% degradation of azathioprine at pH 1.1. (4) hypoxanthine; (5) 1-methyl-4-nitro-5-thioimidazole.

Altex Ultrasphere ODS (4.6 × 150 mm, 5 μm); mobile phase, methanol –25 mM potassium dihydrogen phosphate–glacial acetic acid (20 : 79.5 : 0.5) (pH adjusted to 4.50 with ammonium carbonate); flow rate, 1.5 ml/min; detection, 240 nm. The products were identified by comparison of HPLC retention times with authentic samples. The retention times (min) for various products were: azathioprine, 4.84; 6-mercaptopurine, 1.62; hypoxanthine, 1.27; 1-methyl-4-nitro-5-thioimidazole, 1.17; purine-6-sulphinate, 1.12.

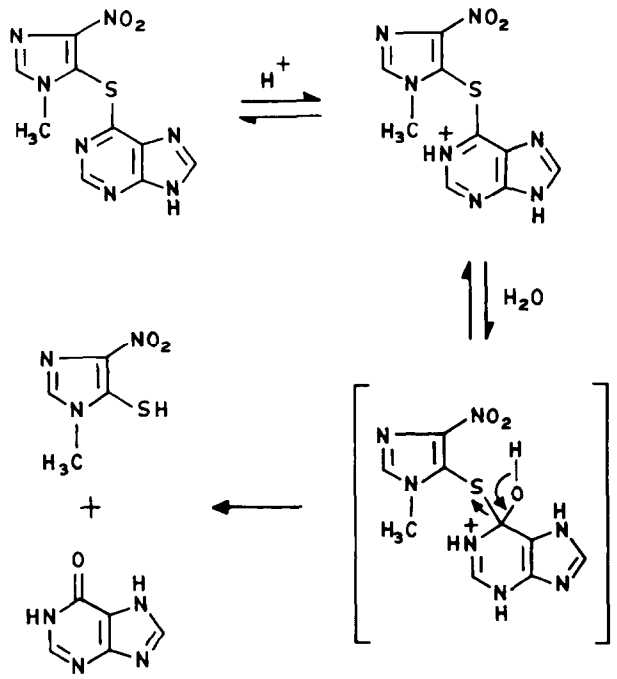
The characteristic HPLC profiles for degradation products formed during hydrolysis of azathioprine under alkaline and acidic conditions are shown in Figs. 1 and 2, respectively. It can be noted that the patterns of peak resolution in the two figures are distinctly different from each other. Thus the present study also suggests that azathioprine degrades in the two pH regions through separate reaction pathways.

Fig. 1A shows that, as previously known, 6-mercaptopurine is the initial and principal degradation product of azathioprine under alkaline conditions. When azathioprine is converted to 6-mercaptopurine, 1-methyl-4-nitro-5-hydroxyimidazole ought to be released as an associated degradant (Fell et al., 1979; Mitra and Narurkar, 1986). The peak *X* at R_T 1.04 in Figs. 1 A–C can be ascribed to this product but it is not established as an authentic sample of the compound was not available. It may be seen in Fig. 1B that with progress of reaction amount of 6-mercaptopurine decreases in the reaction mixtures and there is development of two peaks, one representing purine-6-sulphinate and other hypoxanthine. As the reaction proceeds further (Fig. 1C), purine-6-sulphinate is apparently lost and the amounts of hypoxanthine in the reaction solution increase. It clearly demonstrates that 6-mercaptopurine does not remain stable in neutral and alkaline solutions; it converts at high temperatures to hypoxanthine by a sequential reaction mediated by purine-6-sulphinate. Previous information in literature is that 6-mercaptopurine autoxidizes to purine-6-sulphinate in dilute alkali at room temperatures (Doerr et al., 1961).

The chromatogram in Fig. 2A confirms that 1-methyl-4-nitro-5-thioimidazole and hypoxanthine are formed as principal degradation products of azathioprine in acid solutions. The thioimidazole product is absent in Fig. 2B indicating that it is not stable under acidic conditions. Imidazole compounds of this type are known to be intrinsically unstable (Hofmann, 1951). They have been anticipated to convert to ring opened products not active in UV or to components which are retained within the HPLC column (Fell et al., 1979).

Considering that the reaction responsible for acid hydrolysis of azathioprine is essentially a proton-catalyzed reaction of undissociated drug (Mitra and Narurkar, 1986), a mechanism consistent with formation of hypoxanthine and 1-methyl-4-nitro-5-thioimidazole in acid conditions is depicted in Scheme I. It is postulated that water attack on the C-6 of purine nucleus protonated at N-1 is the rate-determining step in the reaction process.

Azathioprine is a thioether. The sulphur atom



Scheme I.

of thioethers is too large to allow effective overlap with a proton, therefore, generally no intermediate sulphonium compound is formed (Brandsma and Arens, 1967). The imidazole moiety of azathioprine is also not expected to take up protons as it is reported that protonation of nitroimidazoles takes place only in solutions of concentrated acids (Gallo et al., 1964). The likely preferred site for hydrogen attack is, therefore, the N-1 position in the purine nucleus. That 6-methylthiopurine, a close structural analogue of azathioprine, does not protonate in the imidazole ring but ionizes by gaining proton at N-1 is previously established (Albert, 1963).

The proposed mechanism in Scheme I is similar to one put forth by Albert (1969) for acid hydrolysis of 9-methyl-6-methylthio-8-azapurine. It is analogous to that accepted for the acid-catalyzed hydrolysis (bimolecular) of esters and amides (Ingold, 1953).

The significant general acid catalysis observed by Mitra and Narurkar (1986) between pH 3.5 and 6.5 is rationalized by consideration that the acidic component of buffer may transfer protons to N-1 of the purine nucleus, thereby increasing the susceptibility of the 1,6-double bond to nucleophilic attack by water.

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