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Note

Separation of purine analogues from nucleic acid derivatives on a thin layer of ECTEOLA-cellulose

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Azathioprine is an imidazole derivative of 6-mercaptopurine (6-MP) to which it is rapidly converted *in vivo*[1]. 6-MP is commonly used for treating leukaemia, while azathioprine is used extensively in the treatment of patients with organ transplants and auto-immune disorders. No methods are available for estimating therapeutic plasma levels of these drugs although nanogram quantities of both in aqueous solution may be measured microbiologically[2] and 6-MP may also be assayed fluorimetrically[3]. Separation of the analogues from physiological purines and pyrimidines is likely to be a pre-requisite to the application of either method of detection to the estimation of drug levels in human plasma.

Nucleic acid derivatives have been separated on a variety of thin layers including ECTEOLA-cellulose[4], PEI-cellulose[5], Sephadex-cellulose and Sephadex-silica gel[6], and Dowex 50 cation exchange resin[7]. Thin-layer chromatography of 6-MP or methylated derivatives has been reported on silica gel[8,9] and cellulose[10]. In this communication we present a technique for isolating both azathioprine and 6-MP from a mixture of purines and pyrimidines using a thin layer of the anion-exchange cellulose ECTEOLA.

EXPERIMENTAL

Azathioprine B.P. and mercaptopurine B.P. (6-MP) were gifts from Burroughs Wellcome and Co. (Dartford, Great Britain). All other purines and pyrimidines were obtained from Sigma (Kingston-upon-Thames, Great Britain). Stock aqueous solutions of 2 mg/ml of each nucleoside and nucleotide and 1 mg/ml of each base (0.5 mg/ml for azathioprine) were maintained. Azathioprine and 6-MP solutions were prepared freshly each day. Nucleosides and nucleotides, and both azathioprine and 6-MP, were dissolved initially in 0.1 *M* sodium hydroxide followed by an equal volume of 0.2 *M* hydrochloric acid. Xanthine and uric acid were dissolved in 0.05 *M* sodium hydroxide, and all other bases were prepared in 0.1 *M* hydrochloric acid.

ECTEOLA 300 (Macherey, Nagel & Co., Düren, G.F.R.) was obtained from Anderman and Co. (London, Great Britain). Fourteen grams were shaken vigorously with 70 ml of distilled de-ionized water for 30 sec and spread over five 20 × 20 cm glass plates in a layer of 250 μm. The plates were air-dried overnight before use.

Ten μl of azathioprine solution and 5 μl of every other stock solution were applied for each spot using a 5-μl ultra-micro sampler (Oxford Laboratories). This gave the equivalent of 5 μg of base and 10 μg of nucleoside or nucleotide. The plates were developed in a solvent system prepared by mixing, in order, 45 ml acetone, 10 ml 0.1 *M* sulphuric acid, and 45 ml ethyl acetate (system 1). Development to a height of 15 cm was normally complete in 45–50 min. The chromatography was carried out in a cool environment (10–15°) to prevent the development from being too rapid as this could result in streaking of certain components.

A second solvent system consisted of 20% acetone in distilled de-ionized water (system 2). Development with this system to a height of 15 cm took approximately 90 min. It was possible to reduce the amount of fluorescent impurity appearing at the solvent front (characteristic of ECTEOLA plates developed in aqueous systems) by developing the plates in distilled water and air-drying them prior to application of the samples.

Developed chromatograms were air-dried and viewed under an ultraviolet lamp (Camag). 6-MP and its 5′-riboside exhibited a green fluorescence at 366 nm and also fluoresced weakly at 254 nm. Azathioprine quenched fluorescence at both wavelengths while all other compounds quenched fluorescence at 254 nm only.

To determine the recovery of azathioprine and 6-MP from the chromatograms 10 μg of each were chromatographed (using either solvent system) and the developed spots were removed and eluted with 4 ml of acetone–0.1 *M* hydrochloric acid (8:2) by shaking mechanically for 15 min.

Areas of ECTEOLA of equivalent size but containing no sample were treated similarly to serve as blanks. The supernatant was evaporated at 55° under a stream of air to ensure removal of all trace of acetone (particularly critical with azathioprine due to the high UV absorbance of acetone) and the volume was then made up to 4 ml with 0.1 *M* hydrochloric acid. Standards were prepared by adding aliquots of drug solution equivalent to those applied to the chromatograms to 4 ml of 0.1 *M* hydrochloric acid. Drug concentration was determined spectrophotometrically at 281 nm for azathioprine and 325 nm for 6-MP.

RESULTS

Azathioprine and 6-MP were both well separated from physiological purine bases and nucleosides using solvent system 1, and adequately separated from pyrimidine derivatives. Nucleotides and uric acid remained at the origin. A typical chromatogram, photographed at 254 nm, is illustrated in Fig. 1. It should be noted that the 6-MP spots were considerably more intense at 366 nm, but photography at both wavelengths together was impracticable.

Separation of the two drugs from other purines using solvent system 2 was less adequate, azathioprine (R_F 0.47) being incompletely separated from adenine while 6-MP (R_F 0.36) overlapped with xanthine. However, this system did permit excellent separation from pyrimidine bases and nucleosides all of which had R_F values greater than 0.75.

The limit of detection of all components, including azathioprine and 6-MP, was estimated to be approximately 10 nmol. The recovery of azathioprine in eight experiments was 72 $\pm$ 10% and of 6-MP 86 $\pm$ 5% (mean $\pm$ S.D.).

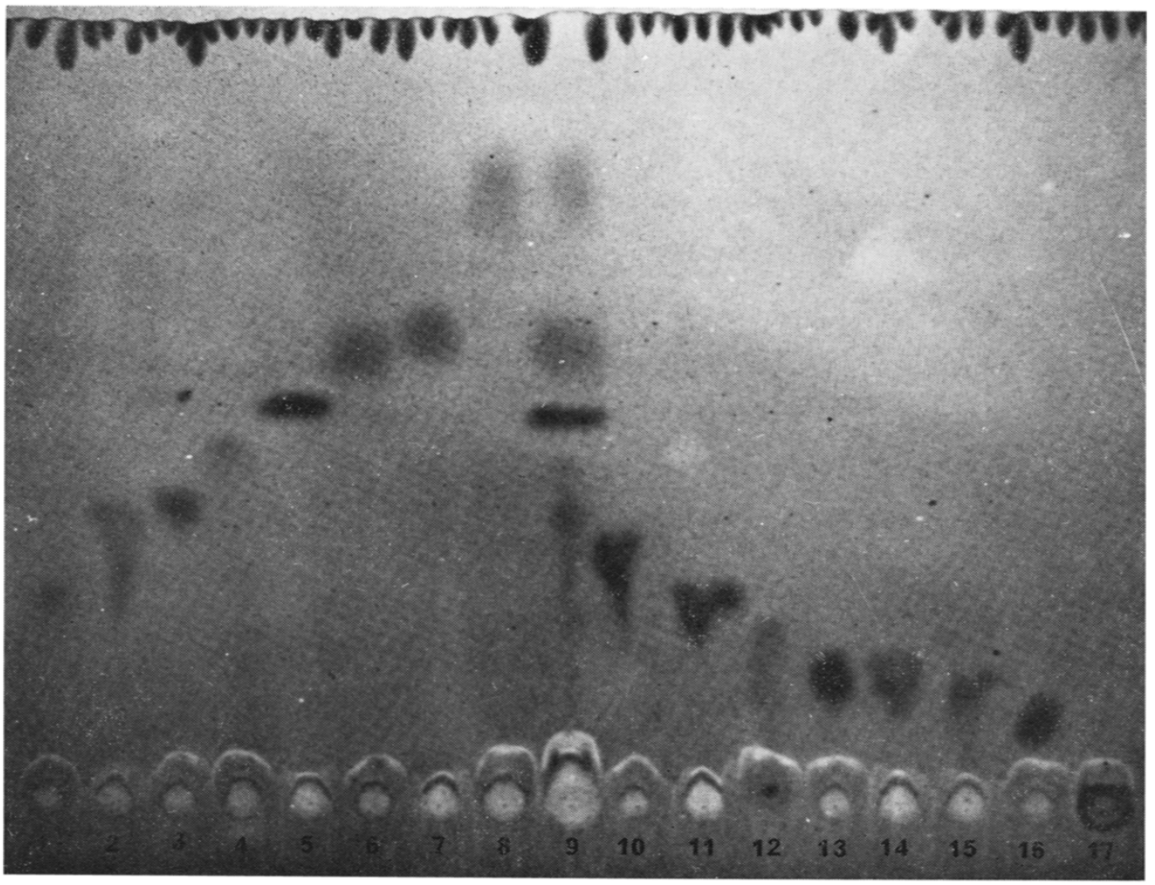

Fig. 1. Separation of azathioprine and 6-mercaptopurine (6-MP) from nucleic acid derivatives on ECTEOLA-cellulose in acetone–0.1 *M* sulphuric acid–ethyl acetate (45:10:45) (system 1). 1 = 6-MP riboside; 2 = adenine; 3 = uridine; 4 = 6-MP; 5 = uracil; 6 = thymidine; 7 = thymine; 8 = azathioprine; 9 = mixture 2–8; 10 = adenosine; 11 = hypoxanthine; 12 = xanthine; 13 = inosine; 14 = cytosine; 15 = guanine; 16 = guanosine; 17 = AMP + uric acid.

DISCUSSION

Although it is likely that ion-exchange mechanisms were involved with solvent system 2, the separations obtained using system 1 probably resulted from an interaction of partition and adsorption effects. Some ion-exchange activity might possibly have influenced the developments with system 1, however, as different patterns were observed when a non-ion-exchange cellulose (Eastman 13255) was used under identical conditions. Azathioprine ran with the solvent front while the R_F value for 6-MP (0.17) was considerably lower than on ECTEOLA (0.45).

The sensitivity of the UV detection was similar to that reported for nucleic acid derivatives separated on layers other than ECTEOLA-cellulose[7]. However, if the ECTEOLA thin-layer system were to be applied to the separation of nanogram quantities of azathioprine or 6-MP, then a more sensitive method of detection, such as the incorporation of radioactive markers, might be required.

The separation methods described in this communication could be used in studies concerning the metabolism of azathioprine and 6-MP and perhaps also as part of a method for estimating plasma levels in patients.

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