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SEPARATION OF THIOPURINE DERIVATIVES BY THIN-LAYER ELECTROPHORESIS USING LOW-TEMPERATURE FLUORESCENCE DETECTION

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SUMMARY

A method using thin-layer electrophoresis to separate thiopurine derivatives is described. Of the thin-layer media studied only Ecteola-cellulose and silica gel have been found to give satisfactory separations. Picomole quantities of the separated compounds have been made detectable by low-temperature fluorescence using liquid nitrogen. The sensitivity limit of this low-temperature technique for various purine derivatives is reported.

INTRODUCTION

The separation and detection of thiopurine derivatives has occupied a central role in the study of metabolism of the immunosuppressive drugs, azathioprine and 6-mercaptopurine, used for the prevention of organ transplant rejection and as treatment in leukaemia. To date, the common techniques used for separating the thiopurine metabolites are high-voltage paper electrophoresis[1] various types of chromatography such as thin-layer chromatography (TLC)[2], paper chromatography[3], high-pressure liquid chromatography[4] and column chromatography using Dowex resin[5]. This communication describes a low-voltage thin-layer electrophoresis (TLE) method which has so far been little used in the separation of thiopurine analogues. It provides a useful complementary method to TLC. The equipment involved is relatively simple and inexpensive.

In this report the use of a low-temperature fluorescence detection method is also described. It has been known for some time that low temperature enhances both fluorescence and phosphoresence of purine derivatives. It was first reported by Steele and Szent-Gyorgyi[6] and later by Randerath[7] and Maddocks and Davidson[8]. They reported that the sensitivity of UV detection at low temperature was many times better than that at room temperature and they applied this technique to the detection

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of purine derivatives after separation by TLC. Despite these earlier reports, this technique is still not widely known. Experience and experimental data on its merits and limitations in the detection of thiopurines are insufficient. This communication describes the authors' experience with this method and reports its sensitivity limits in various thin layer media.

MATERIALS AND METHODS

Azathioprine and 6-mercaptopurine were gifts from Burroughs Wellcome and Co. (Dartford, Great Britain). All other thiopurine derivatives were obtained from Sigma (London, Great Britain). Chemicals used for constituting buffers were from BDH (Poole, Great Britain). They were all of the highest grade commercially available. Radioactive [^{35}S] azathioprine was obtained from Radiochemical Centre (Amersham, Great Britain) and the scintillation fluid, Unisolve 1, from Koch-Light (Colnbrook, Great Britain).

Plastic-backed TLC sheets were supplied by Eastman-Kodak (Rochester, N.Y., U.S.A.): Ecteola-cellulose (sheet No. 6064, 160 μm thick), silica gel (sheet No. 6061 without acrylic binder, 100 μm thick), alumina (sheet No. 6062, 100 μm thick) and polycarbonate (sheet No. 6067, 60 μm thick).

Reagent solutions

The following solutions were used: triethanolamine buffer 0.7% (v/v) adjusted to pH 9.5 with glacial acetic acid; pyridine buffer 5% (v/v) adjusted to pH 6.0 with glacial acetic acid; standard solutions of thiopurines freshly prepared for each experiment by dissolving in 0.05 *N* NaOH to a final concentration of 20 mmol/l.

Procedure

To obtain the optimal separation of the major metabolites of azathioprine electrophoresis was performed on either silica gel thin layer with triethanolamine–acetic acid (pH 9.5) as electrophoresis buffer or on Ecteola-cellulose layer with pyridine–acetic acid (pH 6.0) as buffer (see Results).

Samples (4 μl) were applied with a Hamilton microdispenser onto the thin layers (20 $\times$ 20 cm), 4 cm from the edge. They gave a spot size of about 0.9 $\pm$ 0.1 cm in diameter. After the samples had been dried in a current of warm air, the thin-layer sheet was sprayed with the buffer solution used for electrophoresis until it was just wet (about 20 ml). It was then transferred to the horizontal electrophoresis chamber (Deluxe Electrophoresis Chamber; Gelman, Northampton, Great Britain). The chamber consisted of four compartments, two central electrode compartments with platinum electrodes running through the whole length, and two buffer compartments on the outside. The central partition separated the anode from the cathode compartments while two lateral adjustable bridges partially partitioned the electrode from the buffer compartments. All compartments were filled with buffer. The thin-layer sheet, resting horizontally between the two bridges, was then connected to the buffer compartments by means of Whatman No. 1 chromatography paper presoaked in the buffer. An air-tight lid was placed on top and electrophoresis was started.

The whole procedure was carried out at 300 V at 4° C for 3 h for the silica gel layer and 3.5 h for the Ecteola-cellulose.

After electrophoresis, the thin layer was air-dried and dipped into an ammonia fume chamber for 30 sec. It was then fixed with two plastic covered magnetic strips onto a steel plate wrapped with black paper in the centre of a polystyrene box completely covered with non-fluorescent paper. Beneath the plate was a well, designed to hold liquid nitrogen. Just above the box was a ventilation hole. Liquid nitrogen (77 °K) was poured onto the thin-layer sheet until the well beneath it was just overflowing. With a UV lamp (Camlab; Camag, Cambridge, Great Britain) sitting on top of the box at a distance of 13 cm from the steel plate, the thin layer was viewed under UV light at both 254 and 366 nm. When the mist cleared, the colour and position of the fluorescent spots were noted.

RESULTS

Of the four kinds of thin-layer media investigated only Ecteola-cellulose and silica gel gave reasonably compact spots without much tailing. Tailing was most marked with alumina while diffusion of spots was greatest with polycarbonate layer which also had the least satisfactory detection sensitivity (see below). For these reasons the method of TLE was only assessed with the silica gel and cellulose media.

Separation of thiopurine derivatives at different pH values

The following pH values with volatile buffers were studied; pH 1.6 formic acid

TABLE I

RELATIVE ELECTROPHORETIC MOBILITIES OF THIOPURINE DERIVATIVES ON THIN-LAYER SHEETS OVER A RANGE OF pH VALUES

Aza = azathioprine; 6MP = 6-mercaptopurine; 6MPRP = 6-mercaptopurine riboside phosphate; 6MPR = 6-mercaptoriboside; TA = thiouric acid; 6mMPR = 6-methyl mercaptopurine riboside; 6mMP = 6-methyl mercaptopurine; 2a6mMP = 2-amino-6-methyl mercaptopurine; 2h6MP = 2-hydroxy-6-mercaptopurine; 6MG = 6-mercaptoguanosine. The electrophoretic mobilities were calculated as ratios relative to azathioprine as standard. All compounds migrated towards the anode except those marked with −sign which migrated towards the cathode. The data were averages of duplicates. In no case was the variation between duplicates greater than 0.2.

pH *Silica gel*	*Aza*	*6MP*	*6MPRP*	*6MPR*	*TA*	*6mMPR*	*6mMP*	*2a6mMP*	*2h6MP*	*6MG*
1.6	−1.0	−1.8	−0.7	−1.3	−1.1	−1.7	−1.1	−2.6	−1.1	−1.8
3.0	−1.0	−1.5	−0.5	−1.6	−0.9	−1.1	−1.3	−1.6	−1.3	−1.4
4.0	1.0	1.4	1.7	1.4	1.8	1.0	1.1	0.8	1.2	1.2
6.0	1.0	1.2	1.5	1.2	1.8	1.2	1 0	0 9	1.1	1.0
8.0	1.0	1.5	2.0	1.3	1.9	0.5	0.9	0.5	1.8	1.0
9.5	1.0	1.9	3.3	1.5	3.0	0.9	0.6	0.5	2.7	1.4
Ecteola-cellulose										
1.6	−1.0	−1.2	−0.6	−1.1	−0.6	−1.5	−1.1	−2.1	−0.6	−1.3
3.0	−1.0	−0.6	−0.6	−0.9	−0.4	−0.8	−1.0	−1.0	−0.5	−0.7
4.0	1.0	0.7	1.3	1.0	0.7	0.8	—	0.6	—	0.6
6.0	1.0	0.7	2.6	0.9	2.0	0.7	1.0	0.6	0.8	0.9
8.0*	1.0	0.8	1.5	1.0	1.0	0.4	0.3	0.1	1.1	0.6
9.0*	1.0	0.7	1.9	1.0	0.8	0.5	0.3	0.1	0.8	—

* Spot tailing occurred at these pH values, more marked at 9.0 especially for 6MG.

8% (v/v), pH 3.0 formic acid 2% (v/v) adjusted with ammonia, pH 4.0 formic acid 2% (v/v) adjusted with ammonia, pH 6.0 pyridine 5% (v/v) adjusted glacial acetic acid, pH 8.0 ammonium carbonate (50 mmol/l) adjusted with carbon dioxide, pH 9.0 ammonium bicarbonate (50 mmol/l) adjusted with ammonia and pH 9.5 triethanolamine 0.7% (v/v) adjusted with acetic acid.

The results are listed in Table I. The optimal separation of the major metabolites of azathioprine, 6-mercaptopurine[9], its riboside and ribotide[10] and thiouric acid[11] was achieved at pH 9.5 with silica gel (Fig. 1) and at pH 6.0 with Ecteola-cellulose (Fig. 2). In both cases 6-mercaptopurine riboside phosphate, the active metabolite of both azathioprine and 6-mercaptopurine, migrated ahead of all the others and was easily identifiable. On the whole silica gel plates gave a better separation and better spot shape than Ecteola-cellulose plates which showed streaking of spots at pH above 8.

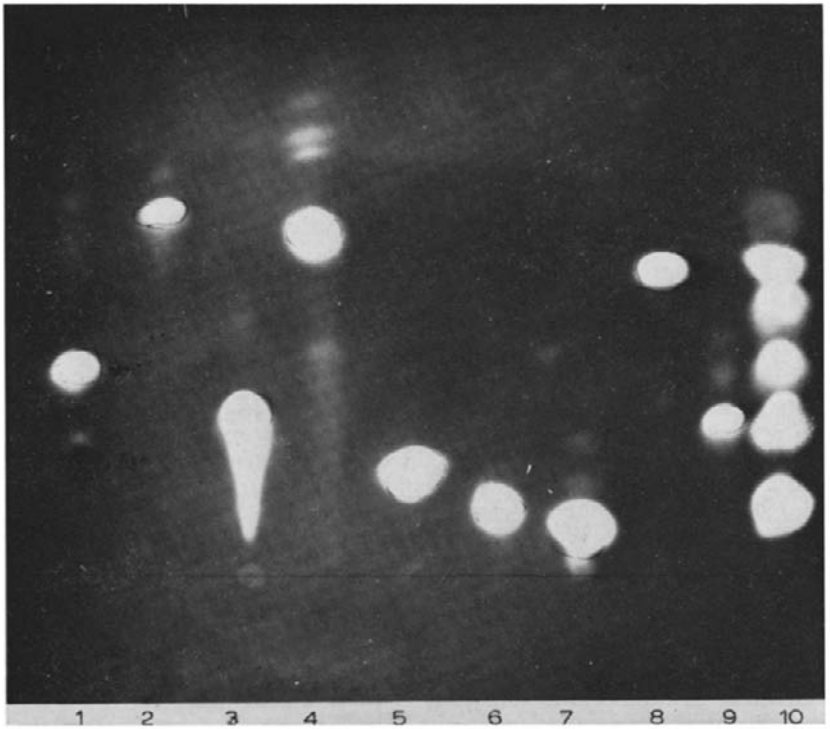

Fig. 1. Separation of thiopurine derivatives on silica-gel thin layer after electrophoresis. 1 = 6MP + Aza; 2 = 6MPRP; 3 = 6MPR; 4 = TA; 5 = 6mMPR, 6 = 6mMP; 7 = 2a6mMP; 8 = 2h6MP; 9 = 6MG; 10 = 1–9 together. Abbreviations are the same as those used in Table I. The sheet was photographed under UV at 254 nm in presence of liquid nitrogen. For conditions of electrophoresis see text.

Assessment of the method with [^{35}S] azathioprine

The percentage recovery of materials was studied by comparing the radioactivity of [^{35}S] azathioprine spots on the thin layer before and after electrophoresis over a range of azathioprine (0.8–80 nmoles). The between-plate precision of the method was assessed by performing duplicate analysis over the same range of azathio-

prine. For comparison, the TLC method using Ecteola-cellulose medium[12] with butanol–acetic acid–water (12:3:5) as developing solvent was also studied.

As indicated in Table II there was little difference in the recovery between the three methods (by paired *t* test of significance). They all ranged between 80 and 101 %. All three methods showed an increase in precision with increasing levels of azathioprine, although the silica gel method was, on the whole, slightly more reproducible than the other two.

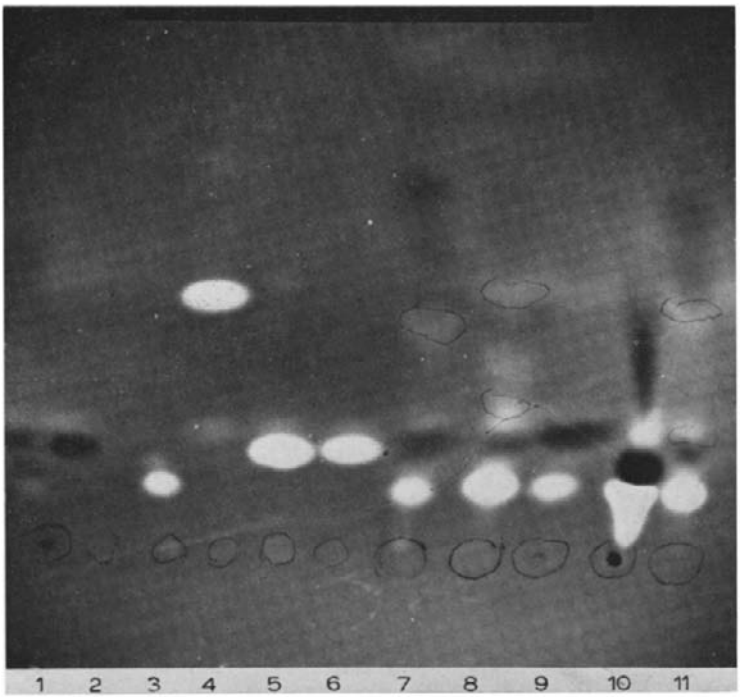

Fig. 2. Separation of metabolites of azathioprine from human blood cells on Ecteola-cellulose after electrophoresis. 1 = Control whole blood with azathioprine introduced after incubation; 2 = azathioprine standard; 3 = 6MP standard; 4 = 6MPRP standard; 5 = 6MPR standard; 6 = 6mMPR standard; 7 = inside erythrocytes after 5-h incubation at 37 °C; 8 = medium bathing erythrocytes after 5-h incubation; 9 = platelet-rich plasma after 5 h; 10 = medium bathing lymphocytes after 5 h; 11 = inside lymphocytes after 5-h incubation. The photograph was taken under UV at both 254 and 366 nm in presence of liquid nitrogen. Abbreviations are the same as those used in Table I.

Sensitivity of the low-temperature (77 °K) fluorescence detection

Tables III and IV show the fluorescence colour and the sensitivity limits of purine derivatives which had been treated with ammonia fumes for 30 sec. The thiopurines fluoresced more at 366 nm than at 254 nm with the exception of the methylated thioderivatives which, like the physiological purines studied, were, at the concentrations used, identifiable only at 254 nm. These physiological purines were hardly fluorescent, they largely quenched UV light at 254 nm and much higher quantities were needed for detection than in the case of thiopurines.

TABLE II

PRECISION AND PERCENTAGE RECOVERY OF THE THIN-LAYER ELECTROPHORESIS METHODS

$\bar{x}$, mean value of azathioprine; σ, standard deviation; C.V., coefficient of variation. Numbers of data are presented in parentheses. The standard deviation of the intersheet precision was obtained from duplicate analysis using the formula $\sqrt{\Sigma d^2/(n-1)}$, where d is the difference between duplicates and n the number of data. The radioactivity of [^{35}S]azathioprine was measured by the Intertechnique SL30 Automated Liquid Scintillation Counter after transferring fine scrapings of azathioprine spots into vials containing 10 ml scintillation fluid, Unisolve 1, followed by 4 ml deionised water.

[^{35}S]Azathioprine (nmole)	Cellulose electrophoresis		Silica gel electrophoresis		Cellulose chromatography	
	Intersheet precision	Average recovery (%)	Intersheet precision	Average recovery (%)	Intersheet precision	Average recovery (%)
0.8–4.0	$\bar{x} = 2.2(10)$ $\sigma = 0.4$ C.V. = 19%	101(10)	$\bar{x} = 2.3(8)$ $\sigma = 0.3$ C.V. = 14%	93(8)	$\bar{x} = 1.9(10)$ $\sigma = 0.3$ C.V. = 15%	90(10)
4.0–20	$\bar{x} = 8.8(10)$ $\sigma = 1.4$ C.V. = 15%	80(10)	$\bar{x} = 10.1(10)$ $\sigma = 0.9$ C.V. = 8.8%	87(10)	$\bar{x} = 11.4(10)$ $\sigma = 2.3$ C.V. = 20%	88(10)
20–80	$\bar{x} = 44.5(8)$ $\sigma = 3.4$ C.V. = 7.6%	81(8)	$\bar{x} = 44.2(8)$ $\sigma = 3.3$ C.V. = 7.5%	84(8)	$\bar{x} = 45(8)$ $\sigma = 2.7$ C.V. = 6%	84(8)

TABLE III

SENSITIVITY LIMIT FOR DETECTION OF PURINE DERIVATIVES IN THIN-LAYER MEDIA BY LOW-TEMPERATURE (77 °K) FLUORESCENCE TECHNIQUE (UV AT 336 nm)

All spot sizes were 0.9 ± 0.1 cm in diameter. Colours of fluorescence are given in parentheses: B = blue; D = dark; G = green; O = orange; V = violet; Y = yellow. For abbreviations see Table I.

Purine	Silica gel (pmole)	Ecteola-cellulose (pmole)	Alumina (pmole)	Polycarbonate (nmole)
Aza	1500 (O)	200 (O)	2000 (O)	4.0 (D)
6MP	800 (B)	10 (G)	800 (B)	8.0 (B)
6MPR	320 (B)	160 (B)	800 (B)	0.8 (B)
6MPRP	600 (YG)	300 (B)	1600 (B)	2.0 (G)
TA	60 (YG)	60 (Y)	65 (Y)	80 (D)
2a6mMP	100 (B)	25 (BV)	80 (BG)	60 (B)
2h6MP	40 (G)	20 (BG)	40 (G)	70 (D)
6MG	80 (G)	50 (G)	70 (G)	60 (D)

In nearly all cases Ecteola-cellulose was shown to be the most sensitive medium while polycarbonate was the least satisfactory of the four.

The Ecteola-cellulose electrophoresis technique was therefore used in an *in vitro* study of metabolism of azathioprine in human blood cells (see Fig. 2). The metabolites correlated well with those separated by TLC with Ecteola-cellulose using butanol–acetic acid–water as developing solvent. They suggested the formation of 6-mercaptopurine riboside and ribotide inside red blood cells after 1 h of incubation at 37 °C, trace formation of the ribotide inside lymphocytes after 5 h, and the presence of the 6-methylated mercaptopurine riboside in platelet-rich plasma after 2 h.

TABLE IV

SENSITIVITY LIMIT FOR DETECTION (nmoles) OF PURINE DERIVATIVES IN THIN-LAYER MEDIA BY LOW-TEMPERATURE (77 °K) FLUORESCENCE TECHNIQUE (UV AT 254 nm)

All spot sizes were 0.9 ± 0.1 cm in diameter. Colours of fluorescence are given in parentheses: B = blue; D = dark; G = green; O = orange; V = violet; Y = yellow. IA = inosinic acid; I = inosine; Hy = hypoxanthine. For other abbreviations see Table I.

Purine	*Silica gel*	*Ecteola-cellulose*	*Alumina*	*Polycarbonate*
6mMPR	1.2 (B)	0.12 (B)	0.28 (B)	60 (B)
6mMP	1.0 (BG)	0.03 (BG)	0.12 (BG)	50 (G)
IA	20 (D)	2.0 (D)	15 (D)	40 (D)
I	2.0 (D)	0.4 (D)	1.0 (D)	20 (D)
Hy	4.0 (D)	0.8 (DV)	1.0 (DV)	20 (D)
AMP	2.0 (DV)	2.0 (DV)	1.0 (DV)	10 (DV)
ADP	4.0 (DV)	2.0 (DV)	1.0 (DV)	5.0 (DV)
ATP	2.0 (DV)	2.0 (DV)	1.0 (DV)	5.0 (DV)
GMP	2.0 (V)	1.5 (V)	2.0 (V)	15 (DV)

DISCUSSION

TLE offers a useful alternative to TLC in the separation of thiopurine metabolites. It has certain advantages over TLC. For a reasonable separation of the major thiopurine metabolites of azathioprine only about 3 h is needed compared with the 4–10 h required for TLC with organic solvents. Unlike TLC, it does not require preliminary desalting of the samples and it has a sharpening effect on the spot shape. In terms of precision and recovery rate the two techniques are comparable.

It should noted that the precision data in Table II are, in fact, the combined effect of two procedures, *viz.* TLE and liquid scintillation counting. The precision of the liquid scintillation counting in the range of azathioprine studied has been found, by repeated analysis, to have a coefficient of variation of 4.8 % (with 10 pairs of data) and hence any increase above that value in the combined procedure can be ascribed to TLE.

Low-voltage is preferred to high-voltage electrophoresis because the equipment involved is less expensive and less complicated. Furthermore no elaborate cooling system is necessary. However, a longer electrophoresis time is needed and the spot shape obtained is more diffuse than in high-voltage electrophoresis.

The disadvantage of silica gel being less sensitive in thiopurine detection than Ecteola-cellulose is offset by its ability to give better separation and better spot shape of thiopurines. Also, it is marginally more precise and requires slightly shorter period for separation. It is therefore the medium of choice for TLE if sensitivity is not critical. However, if a two-dimensional TLE–TLC is to be done, Ecteola-cellulose is then a better alternative as it is, in our hands, a much better medium than silica gel in TLC both in terms of speed and sensitivity.

Low-temperature fluorescence provides a very satisfactory detection method for thiopurines, probably the best on thin-layer plates. Ammonia fumes are used to increase sensitivity as many purines fluoresce better at alkali pH[7]. With this method picomole quantitities are readily discernable compared with the micro–nanomole

quantities required for UV detection at room temperature[7]. It is a more specific method for thiopurines than the room temperature technique as many of them fluoresce strongly at low temperature with different colours at 366 nm and as they have a much lower threshold for detection than any of the physiological purines studied (which occur mainly as quenching spots at 254 nm). However, this method is not quantitative. The main technical problem in quantitation is to design a cooling system using liquid nitrogen which avoids the formation of mist so that densitometry can be undertaken. A disadvantage of the method is that it requires the use of expensive plastic-backed thin-layer plates since glass plates crack in the presence of liquid nitrogen.

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