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SEPARATION OF THIOLS AS PHENYL MERCURY DERIVATIVES BY THIN-LAYER CHROMATOGRAPHY

I. AZATHIOPRINE AND 6-MERCAPTOPURINE METABOLITES

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SUMMARY

A method is described for converting thiol metabolites of azathioprine and 6-mercaptopurine into phenyl mercury derivatives. Separation of these derivatives was performed by chromatography on silica thin layers and they were detected by low temperature (−196°) fluorescence. The parent compounds were regenerated on the chromatogram by spraying with 2 *N* HCl and these were detected by low temperature fluorescence. Mercury was also detected in spots by spraying with dithizone. The method provides a simple solution to the problem of oxidation of thiol compounds during isolation procedures.

INTRODUCTION

Azathioprine is one of the most commonly used immunosuppressive drugs particularly in patients with organ transplants. Its metabolite 6-mercaptopurine is useful in the treatment of leukaemia. Little is known of the metabolism of these drugs in patients owing to a lack of sufficiently sensitive analytical techniques. We have developed methods for separating these drugs and their metabolites by thin-layer chromatography (TLC) and electrophoresis, together with detection using low-temperature fluorescence[1–3]. However, our mass-spectrometry studies of azathioprine and 6-mercaptopurine metabolites have been hampered by the instability of these compounds during isolation procedures. In particular, the thiol group is easily oxidised[4–6] and we have found it difficult to isolate pure compounds. Indeed, thiol metabolites of azathioprine may even be oxidised on thin-layer chromatograms viewed under UV light as this accelerates oxidation[7,8].

To overcome the problem of the instability of thiols we have devised TLC systems for isolating these compounds as phenyl mercury derivatives, thus protecting the thiol group from oxidation.

MATERIALS AND METHODS

The following metabolites were studied as phenyl mercury derivatives: 6-mercaptopurine[9] (6MP) was a gift from Burroughs Wellcome, Dartford, Great Britain; 6-mercaptopurine riboside (MPR), 6-mercaptopurine riboside-5′ phosphate[10] (MPRP) and 6-thioguanine[11] (6TG) were obtained from Sigma, St. Louis, Mo., U.S.A.; 6-thiouric acid[12] (6TU) and 2-hydroxy 6-mercaptopurine[13] (2OH 6MP) were supplied by Calbiochem, Los Angeles, Calif., U.S.A., and Aldrich, Milwaukee, Wisc., U.S.A., respectively; 8-hydroxy-6-mercaptopurine was synthesised by the method of Robins[14] and 5-mercapto-1-methyl-4-nitroimidazole (NMMI) from azathioprine by the method of Chalmers *et al.*[15].

Silica gel 60 F 254, size 20 × 20 cm with a layer thickness of 0.2 mm on plastic sheets and silica gel 60 PF 254 for preparative chromatography were obtained from Merck, c/o BDH, Poole, Great Britain. Dimethyl sulphoxide (DMSO) AR was obtained from BDH.

Phenylmercuric acetate (PMA) 0.3% solution was prepared by adding a weighed amount to boiling water and vigorously stirring. The solution was stable for several weeks when stored in a dark glass bottle.

Dithizone (diphenyl thiocarbazone, obtained from BDH) was dissolved in chloroform to give a saturated solution.

The following mobile phases were used for TLC: A = acetic acid–ethanol (1:9); B = ammonia–butanol–water (1:60:39) and C = heptane–ethanol–chloroform (1:1:1 with 1.5% water).

Apparatus

UV lamp (Minerva light UV SL 25) obtained from Ultra Violet Products, Calif., U.S.A., was used for studying chromatograms. For UV spectroscopic studies the SP500 and SP1800 spectrophotometers of Pye Unicam, Cambridge, Great Britain, were used.

Low temperature fluorescence

Detection of low temperature fluorescence[2] was carried out in a polystyrene box which had been completely covered with non-fluorescent black paint. The floor of the box had a central well to hold liquid nitrogen and two sides had a series of holes just above the floor through which "clouds" of ice particles could be pumped out through brass tubes connected to a suction pump. Chromatograms were fixed to a black steel plate by two plastic-covered strip magnets and placed in the box over the well. Liquid nitrogen was then poured onto the plate until the well beneath was just overflowing. Viewing of chromatograms was performed in the dark under a UV lamp at 254 and 366 nm.

Preparation of phenyl mercury derivatives

The base, nucleoside or nucleotide (3–4 mg) was dissolved in the least volume of 0.1 *N* NaOH. To each solution 0.3% PMA was added dropwise with mixing until there was no further precipitation. After centrifugation at 2000 *g* for 10 min, the aqueous phases were rejected and the precipitates washed with distilled water and centrifuged, three times. The residues were dried by a stream of air at 30° and then

dissolved in 0.5 ml of DMSO. A control solution was prepared by evaporating the same volume of 0.1 *N* NaOH and PMA and dissolving the residue in 0.5 ml of DMSO.

Thin-layer chromatography

Freshly prepared solutions of the phenyl mercury derivatives and control solution were applied to precoated silica gel 60 F 254 using a Pasteur pipette with a very fine outlet. At least 6 applications (approx. 2–3 μl each) of each derivative were applied at the origin with drying in a stream of air between applications. Chromatograms were developed with three mobile phases at room temperature. It took 1 h to develop the chromatogram using the heptane–chloroform–ethanol–water system, 3 h for the acetic acid–ethanol system and 6 h for the ammonia–butanol–water system. Chromatograms were dried in a gentle air stream at room temperature and studied under UV light at 254 and 366 nm at room temperature and at low temperature (—196°).

Conversion of phenyl mercury derivatives to parent thiols

This was done by spraying the chromatograms with 2 *N* HCl. After drying they were again studied at room and low temperature under UV light.

Detection of mercury

Dry chromatograms were sprayed with 0.1 *N* acetic acid[16] followed by a saturated solution of dithizone in chloroform in a fume cupboard.

Recovery of 6-phenyl mercury thiopurine

The 6-phenyl mercury thiopurine was prepared as previously and purified by preparative layer chromatography using silica (0.5 mm) on a pyrex glass plate and ammonia–butanol–water (1:60:39) as the mobile phase. It was eluted from the silica with DMSO. The supernatant was evaporated overnight in a dry block at 30° under a gentle stream of air. The purified derivative was redissolved in DMSO to give a concentration of 2 mg/ml. Five replicate samples, each of 100 μl, were applied to precoated silica sheets. A control of DMSO was also applied and the chromatogram developed with the ammonia–butanol–water (1:60:39) system. The amounts of 6-phenyl mercury thiopurine recovered by scraping off the spots and eluting with DMSO, were measured spectrophotometrically at 303 nm with reference to a standard curve. Equivalent areas containing no sample were scraped off the chromatogram, eluted with DMSO and used as blanks.

RESULTS

Thin-layer chromatography

Phenyl mercury derivatives were detected under UV light at 254 and 366 nm at —196° as shown in Table I. They were mainly seen as blue or dark spots against the fluorescent background of the plate at 254 nm. At 366 nm the spots fluoresced rather weakly but after spraying with 2 *N* HCl the fluorescence became intense (Fig. 1). In some cases a change in colour of the fluorescence was observed after conversion to the parent compounds. For example, at 365 nm 6-thiouric acid always had a purple fluorescence at low temperature whereas its phenyl mercury derivative had a red

TABLE I

R_F VALUES AND COLOUR OF SPOTS IN UV LIGHT AT −196° OF AZATHIOPRINE AND 6-MP METABOLITES AS PHENYL MERCURY DERIVATIVES

A = acetic acid–ethanol (1:9); B = ammonia–butanol–water (1:60:39); C = heptane–ethanol–chloroform (1:1:1) + 1.5% water.

Metabolite	*A*				*B*				*C*			
	R_F	*Colour*			R_F	*Colour*			R_F	*Colour*		
		254 nm	*366 nm*	*366 nm HCl spray*		*254 nm*	*366 nm*	*366 nm HCl spray*		*254 nm*	*366 nm*	*366 nm HCl spray*
6-MP	0.59	dark	green	green	0.63	–	blue/green	green	0.58	dark	green	green
6-TU	0.30	–	red	purple	0.37	–	red	purple	0.04	–	red	purple
8-OH 6-MP	0.69	blue	blue	green	0.59	blue	green	green	0.68	dark	blue	green
2-OH 6-MP	0.50	–	green	green	0.40	–	purple	green	0.47	–	blue	green
6-TG	0.52	–	blue	green	0.59	–	green	green	0.60	dark	green	green
NMMI	0.51	dark	–	red	0.55	dark	dark	red	0.66	dark	dark	red
MPR	0.58	blue	green	green	0.47	blue	blue	green	0.54	dark	green	green
MPRP	0.08	–	blue	green	0.00	–	blue	green	0.02	dark	blue	green

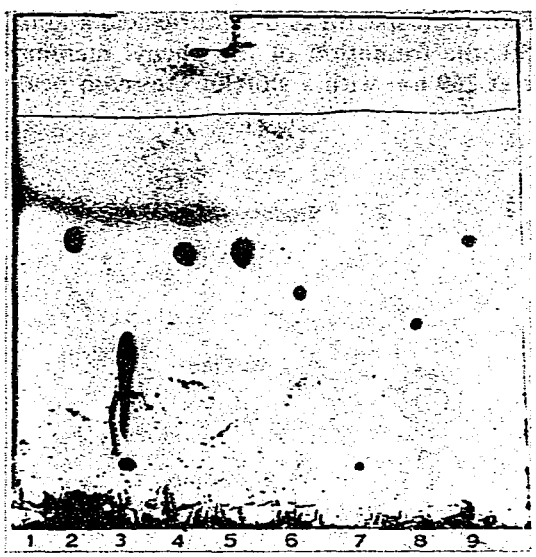


Fig. 1. Separation of azathioprine and 6-MP metabolites as phenyl mercury derivatives on a silica gel thin layer with ammonia–butanol–water (1:60:39) solvent system. Detection by UV light (366 nm) at −196° after spraying with 2 *N* HCl. 1 = PMA; 2 = 6-MP; 3 = 6-TU; 4 = 6-TG; 5 = 8-OH 6MP; 6 = MPR; 7 = MPRP; 8 = 2-OH 6 MP; 9 = NMMI.

fluorescence with all three solvent systems. Most of the phenyl mercury derivatives ran well in all three solvent systems with the exception of the MPRP derivative which remained close to the origin.

After spraying with dithizone all spots including the control developed a deep red colour indicating the presence of mercury. Although solvent system B provided spots with a high fluorescence, the best separation was obtained with solvent system C.

UV spectroscopy

The UV absorption spectra of the metabolites and their phenyl mercury derivatives studied are shown in Table II. Shifts in absorption maxima were observed

TABLE II

UV ABSORPTION MAXIMA OF AZATHIOPRINE AND 6MP METABOLITES AND THEIR PHENYL MERCURY DERIVATIVES IN DMSO

Metabolite	$\lambda_{max.}$	$\lambda_{max.}$ *of derivative*
6-MP	357	295, 303
6-TU	269, 365	357
8-OH 6-MP	308, 357	308
2-OH 6-MP	275, 348	351
6-TG	270, 366	276, 326*
NMMI	334, 436	295*, 380*
MPR	328	298
MPRP	327	305

* Broad peak.

in most cases. Figs. 2 and 3 show the UV absorption spectra of 6-thiouric acid and its phenyl mercury derivative respectively. With the fotmation of the phenyl mercury derivative there is a loss of the minor peak at 269 nm with a shift of the main peak from 365 to 357 nm.

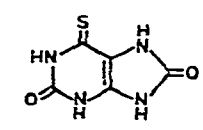


Fig. 2. UV absorption scan of 6-thiouric acid.

Fig. 3. UV absorption scan of 6-phenyl mercury thiouric acid.

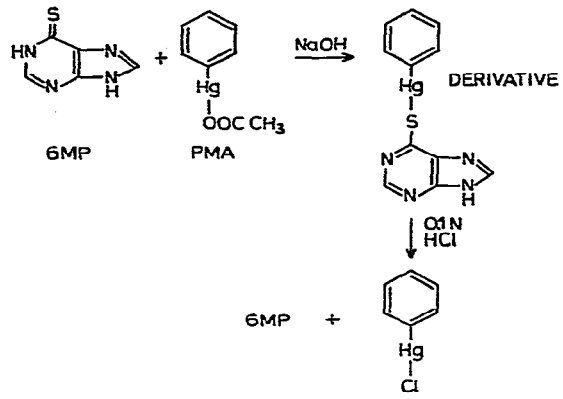
Recovery of 6-phenyl mercury thiopurine

The recovery of 6-phenyl mercury thiopurine after TLC using solvent system B was 70 ± 3.8% (mean ± S.D.).

DISCUSSION

Ease of oxidation appears to be a universal problem with thiol compounds[4–6,17] and may cause difficulty in their structural determination and quantitative assay. It is particularly important with the isolation of thiopurines as these compounds have a poor solubility in most solvents but dissolve well in alkali in which oxidation increases with pH.

The preparation of phenyl mercury derivatives to protect the thiol group from oxidation has several advantages. They are rapidly prepared, are extremely stable and the phenyl mercury group may be easily removed by reaction with chloride ions to reform the parent thiol compound. For example, with 6MP the following reaction takes place:



An additional advantage of preparing phenyl mercury derivatives is that they may be much more soluble in organic solvents than the original thiol compounds. More non-polar solvent systems may be used in isolation procedures from biological material resulting in increased selectivity of extraction.

Low temperature fluorescence as a method of detecting thiopurines is remarkably sensitive. It is 1000-fold more sensitive than detection under UV light at room temperature and as little as 1.5 ng of 6-mercaptopurine may be detected with the naked eye by this method[2].

Quantitative analysis of thiols after TLC may be performed using spectrophotometry or for greater sensitivity the mercury content of the derivative could be measured by atomic absorption spectrophotometry. Also, PMA with a radioactive label might be used if very high sensitivity is required.

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