Determination of 6-Mercaptopurine and Related Compounds by Phosphorescence Spectroscopy*

A. I. Al-Mosawi and J. N. Miller

Department of Chemistry, Loughborough University of Technology, Loughborough, Leicestershire, LE 11 3TU

and J. W. Bridges

Institute of Industrial and Environmental Health and Safety, University of Surrey, Guildford, Surrey, GU2 5XH

The phosphorescence properties of 6-mercaptopurine and ten related compounds have been studied in acidic, neutral and alkaline ethanolic glasses at 77 K, and adsorbed on thin layers at 77 K and at room temperature Nanogram amounts of most of the compounds can be detected at 77 K, and submicrogram amounts at room temperature A technique combining thin-layer chromatography and phosphorimetric scanning of the chromatograms has been used to determine trace amounts of the compounds in human blood plasma.

Keywords: *Mercaptopurine determination*; *thin-layer chromatography*; *phosphorimetry*; *room-temperature phosphorescence*

The widespread use of 6-mercaptopurine and azathioprine as immunosuppressive and anti-leukaemic agents has created a demand for the selective determination of these compounds and their metabolites at trace levels in biological fluids. The many methods used include paper[1] and thin-layer chromatography,[2,3] high-voltage[4] and thin-layer[5] electrophoresis, gas - liquid[6] and high-performance liquid chromatography[7] and mass spectrometry.[8] A number of luminescence methods have also been developed. Finkel[9] showed that 6-mercaptopurine could be determined fluorimetrically after oxidising deproteinised plasma samples with potassium permanganate to produce purine-6-sulphonate; the limit of detection was 1 μg ml^{-1}. The strong phosphorescence exhibited by many purine derivatives has been frequently reported (e.g., reference 10), and Aaron and Winefordner[11] showed that 6-methylmercaptopurine and 2-amino-6-methylmercaptopurine showed analytically useful phosphorescence. The same compounds were more recently shown to exhibit a weaker

TABLE I

MERCAPTOPURINES AND DERIVATIVES STUDIED

Compound No.	Name	Melting-point/°C	R_F*
1	2-Mercaptopurine	>325	0.37
2	6-Mercaptopurine	300	0.44
3	6-Mercaptoguanosine	226	0.51
4	6-Methylmercaptopurine	230	0.63
5	6-Mercaptopurine riboside	222	0.62
6	6-Mercaptopurine 2-deoxyriboside	214	0.41
7	6-Mercaptopurine riboside 5′-phosphate	>325	0.71
8	Azathioprine	254	0.66
9	2-Thioxanthine	>325	0.19
10	6-Thioxanthine	>325	0.21
11	2-Amino-6-mercaptopurine	>325	0.32

* On cellulose thin layers. Solvent: 0.1 M hydrochloric acid, except for 2-thioxanthine, for which where the solvent system was propan-2-ol - methanol - water - ammonia (60 + 20 + 20 + 1).

* Presented at the Meeting on "Research and Development Topics in Analytical Chemistry," Heriot-Watt University, Edinburgh, July, 1979.

room-temperature phosphorescence (RTP) when adsorbed on to chromatography paper from alkaline solution.[12] Maddocks and Davidson[13] reported the detection of picomole amounts of azathioprine, 6-mercaptopurine and seven related compounds after thin-layer chromatography on cellulose, using the luminescence developed when the chromatography plate was cooled to liquid nitrogen temperature. Wong and Maddocks[5] later used a similar technique to detect mercaptopurine derivatives after thin-layer electrophoresis on a variety of media. Neither of these papers reported spectroscopic data, and fluorescence and phosphorescence were apparently not distinguished.

This paper describes a detailed study of the luminescence properties of 11 mercaptopurine derivatives. The application of thin-layer phosphorimetry,[14] both at room temperature and at liquid nitrogen temperature, to their rapid determination at trace levels in deproteinised blood plasma is also described.

Experimental

The compounds studied are listed in Table I; all were obtained from Sigma Chemical Co. (Poole, Dorset). Each compound yielded a single spot when analysed by thin-layer chromatography (TLC) on cellulose thin layers (E. Merck, obtained through BDH Chemicals, Poole, Dorset) using 0.1 M hydrochloric acid as the normal developing solvent,[13] and low-temperature luminescence as the detection method. All other compounds were of the highest grades commercially obtainable.

Fluorescence measurements at room temperature were performed on a Fluoricord spectrofluorimeter (Baird-Atomic, Braintree, Essex) using silica cells of 10-mm path length. Phosphorescence studies in rigid glasses at 77 K were performed using a Fluorispec spectrofluorimeter (Baird Atomic) fitted with a silica Dewar flask and rotating cylinder phosphoroscope as previously described.[15] Thin-layer phosphorimetry was carried out using a specially designed accessory to the Fluoricord fluorimeter; this device and its operation have been described in detail in an earlier paper.[16] For room-temperature studies the thin-layer attachment was used in the same way, except that no liquid nitrogen was added to the sample drum. In all luminescence assays the limit of detection of a solute was defined as that concentration yielding a signal two standard deviations above the background signal. Absorption spectra were obtained using an SP 800 spectrophotometer (Pye-Unicam, Cambridge) at room temperature.

TABLE II

PHOSPHORESCENCE CHARACTERISTICS OF MERCAPTOPURINES IN ETHANOL GLASSES AT 77 K

	Neutral ethanol			Alkaline ethanol		
Compound No.	λ_{ex}/ nm	λ_p/ nm	Detection limit/ ng ml^{-1}	λ_{ex}/ nm	λ_p/ nm	Detection limit/ ng ml^{-1}
1	342	510	6	330	488	1
2	340	463	20	316	454	2
3	341	466	120	340	461	70
4	286	436	25	295	446	40
5	325	456	30	323	462	50
6	332	476	60	320	460	60
7	337	446	ND*	300	430	10^5
8	300	442	10^4	311	451	2600
9	295	458	10^4	295	452	10^5
10	344	480	50	349	508	50
11	356	484	40	330	479	50

* ND = not determined.

TLC of the mercaptopurine derivatives was performed on cellulose thin layers, and on high-performance thin-layer chromatography (HPTLC) plates coated with silica gel. Both types of thin layer were coated on aluminium foil (E. Merck, obtained through BDH Chemicals) to ensure good thermal contact when the plates were subsequently examined in the thin-layer phosphorimeter. Before the samples were applied all of the plates were completely

eluted with 96% ethanol (James Burroughs Ltd., London), and the top 1–2 cm of the plates cut off; the background luminescence of the oven-dried plates was thus substantially reduced. Samples of 0.1–1 μl were applied to the plates using disposable micropipettes (ICL Scientific, Fountain Valley, Calif., USA) and chromatography was performed in closed chambers. (The chromatography step was omitted in determinations of detection limits.) When the TLC step was complete the plates were air-dried and sprayed with ethanol. Thin-layer phosphorimetry at 77 K was then performed at once; room-temperature studies were performed after the plates had been dried overnight.

Pooled normal human blood plasma samples, obtained from Leicester Royal Infirmary, were spiked with the compounds under study. Deproteinisation was carried out using 10 volumes of cold ethanol, followed by centrifugation.

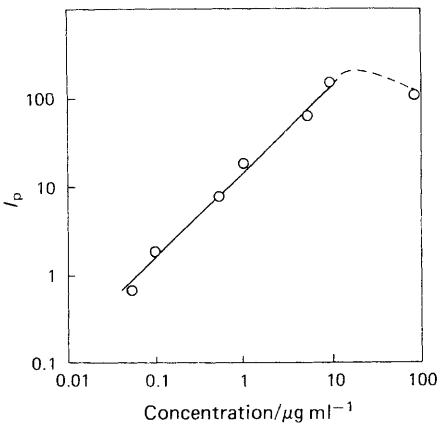


Fig. 1. Analytical growth graph for the phosphorimetric determination of 6-mercaptopurine riboside (compound 5) in ethanol glasses containing 0.1 M sodium hydroxide. Excitation and emission wavelengths, 323 and 462 nm, respectively.

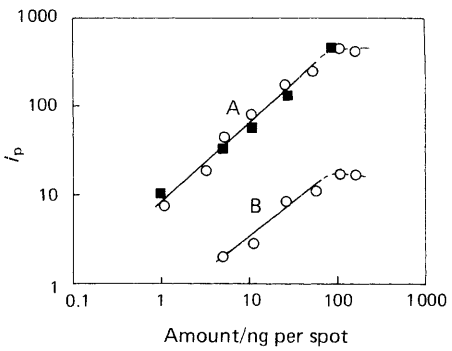


Fig. 2. Analytical growth graphs for the determination of 6-methylmercaptopurine (compound 4) by thin-layer phosphorimetry at (A) 77 K and (B) room temperature. At 77 K the graph was determined using pure solutions of the drug (○) and extracts of spiked blood plasma (■). Excitation and emission wavelengths, 286 and 436 nm, respectively.

Results

The ultraviolet absorption spectra of the 6-mercaptopurine derivatives were found to be pH dependent. Spectra obtained in neutral and acidic (0.1 M hydrochloric acid) ethanolic solutions were generally similar, but in ethanol containing 0.1 M sodium hydroxide shifts of absorption maxima and changes in molar absorbance were sometimes observed. 6-Mercaptopurine, for example, exhibited a blue shift of about 18 nm, and a slight decrease in molar absorbance, in alkaline solution. Such changes, similar to those found by other workers,[17] suggested the desirability of performing luminescence studies in acidic, alkaline and neutral solutions.

Room-temperature studies showed that only one compound, 2-amino-6-mercaptopurine, exhibited significant fluorescence. In alkaline ethanol, with excitation and fluorescence wavelengths of about 316 and 402 nm, respectively, this compound had a limit of detection of 7 μg ml^{-1}. The phosphorescence characteristics of all the compounds in ethanolic glasses at 77 K are given in Table II (in these conditions, compounds 3, 11 and, in alkaline ethanol, 4 exhibited relatively feeble fluorescence signals at 350–400 nm. The remaining compounds exhibited no fluorescence and could therefore be studied without the rotating cylinder phosphoroscope in the light beam). As expected from the absorption spectra, the phosphorescence spectra in neutral and acidic ethanolic solutions were very similar; only the data in neutral solution are therefore given in Table II. It is apparent that limits of detection of nanograms per millilitre can be obtained in many instances. For some compounds, alkaline conditions produced the best detection limits; for others, neutral or acidic solutions

were preferred. Analytical graphs were found to be linear over at least two orders of magnitude of concentration; the graph for 6-mercaptopurine riboside in alkaline ethanol is shown in Fig. 1.

When the compounds were adsorbed on to cellulose TLC plates at 77 K, the excitation and emission spectra were generally closely similar to those obtained in ethanolic glasses, and again very low levels of many of the compounds could be detected, particularly when the samples were applied as alkaline ethanolic solutions. Table III shows that amounts as low as 10 pg could be detected in deproteinised plasma samples. Similar results were obtained when pure solutions of the compounds were studied. The recovery of the solutes using the cold ethanol precipitation method was almost 100%; Fig. 2 shows typical analytical graphs. These detection limits were at least an order of magnitude better than could be obtained by visual observation of the TLC plates. Three of the compounds were also studied using silica gel HPTLC plates, and the limits of detection achieved were similar to those obtained using cellulose thin layers. In attempts to enhance the phosphorescence signals still further, ethanolic solutions of potassium iodide (1% *m*/*V*), lead tetraacetate (10% *m*/*V*) and thallium acetate (10% *m*/*V*) were investigated as spray reagents; none of these compounds produced a "heavy atom" enhancement on cellulose or silica gel thin layers and in most instances a quenching of the phosphorescence was observed.

TABLE III

LIMITS OF DETECTION OF MERCAPTOPURINES IN BLOOD PLASMA USING THIN-LAYER PHOSPHORIMETRY AT 77 K

	Limit of detection/ng per spot		
	Cellulose thin layers		
Compound No.	Neutral ethanol solvent	Alkaline ethanol solvent	Silica gel HPTLC layers, neutral ethanol solvent
1	45	2	ND*
2	3	0.05	0.04
3	4	0.1	0.2
4	1	1	1
5	2	5	ND
6	5	3	ND
7	30	25	ND
8	150	10	ND
9	50	50	ND
10	1	5	ND
11	0.1	5	ND

* ND = not determined.

Several mixtures of mercaptopurines were studied in order to test the precision and selectivity of the combined TLC - phosphorimetry method: all of these measurements were made at 77 K using cellulose thin layers. Fig. 3 shows the separation of four compounds on a cellulose TLC plate: again, the results obtained were the same whether the compounds were dissolved in ethanol or derived from spiked plasma samples. The coefficient of variation when the same TLC plate was scanned repeatedly for 30 min was 8%.

The room-temperature phosphorescence of all of the compounds was studied. Analytically useful signals were obtained only when cellulose was used as the adsorbent, with the highest intensities generally being obtained when alkaline ethanol was used as the solvent for sample application. Even then, the limits of detection (Table IV) were at least an order of magnitude inferior to those determined at 77 K. Room-temperature phosphorescence spectra generally exhibited higher band widths and less vibrational fine structure than spectra obtained at 77 K; the example of 6-mercaptopurine is shown in Fig. 4.

Discussion

The luminescence of purines has been investigated by a number of workers,[11,18,19] and it is well established that many purine derivatives exhibit strong phosphorescence signals in the

wavelength region 400–550 nm. The phosphorescence lifetimes and emission wavelengths indicate that π–π^* transitions are responsible. Some purines are also fluorescent, although the nature of the lowest singlet state has been a matter of controversy.[18] It has been established, however, that the lowest singlet excited state of purine itself is n–π^* in nature. In alkaline solution purines form anions by loss of a proton at the 9-position; the phosphorescence of the purine anion is red-shifted compared with the parent molecule, and fluorescence is also observed. Aaron and Winefordner[11] showed that 6-methylmercaptopurine and its 2-amino derivative could be detected phosphorimetrically at low levels (less than 1 ng ml^{-1}), and were more phosphorescent than purines lacking the mercapto group. This may be due to the "heavy atom" effect of the sulphur substituent. Indirect confirmation of this came from the results of Vo-Dinh *et al.*,[20] who found that an external heavy-atom perturbant (sodium iodide) did not enhance the phosphorescence of 6-methylmercaptopurine, when it was observed at room temperature adsorbed on filter-paper. The limit of detection under these conditions was inferior to that at 77 K, but was still of analytical value.

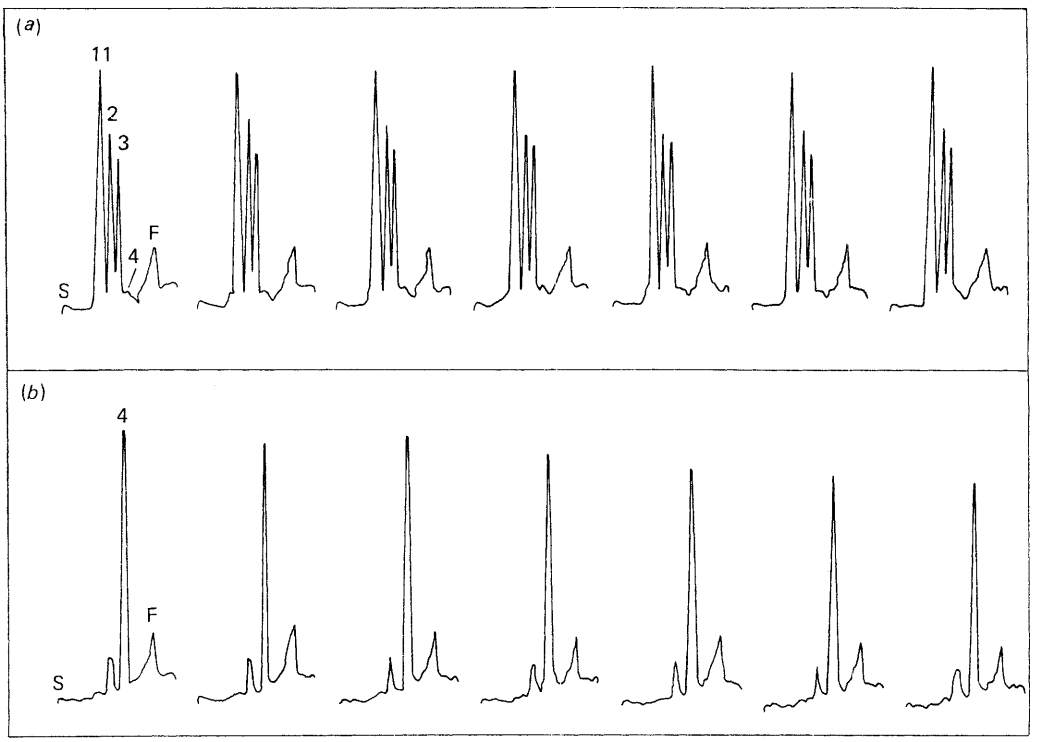
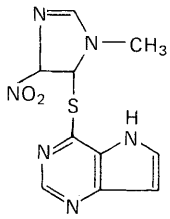


Fig. 3. Thin-layer phosphorimetry at 77 K of a mixture of compounds 2, 3, 4 and 11 on a cellulose thin layer. The developing solvent during the thin-layer chromatographic step was 0.1 M hydrochloric acid. Each of the 14 sequential scans took 1 min. S marks the point of sample application and F the solvent front; for R_F values see Table I. In (*a*) the excitation and phosphorescence wavelengths were 342 and 485 nm, respectively, and in (*b*) 320 and 448 nm, respectively.

In this work all of the compounds studied showed analytically useful phosphorescence, but few were found to be measurably fluorescent, again presumably because of the heavy atom effect on the inter-system crossing rate constant. Although the phosphorescence properties of the compounds are generally similar, there are considerable differences in phosphorescence intensity. The position and substitution of the thiol group in the purine system are clearly of importance. Compounds with this group at the 6-position are generally more strongly phosphorescent than 2-substituted purines: 6-mercaptopurine and 6-thioxanthine can be detected at lower levels than 2-mercaptopurine and 2-thioxanthine, respectively. Both 6-methylmercaptopurine and azathioprine have excitation and phosphorescence

maxima blue-shifted compared with the parent compound. Azathioprine is only very weakly phosphorescent, possibly because of its nitro group:



Azathioprine

The presence of a ribose group has, as expected, little effect (compounds **3**, **5** and **6**), but the phosphate group in 6-mercaptopurine riboside 5-phosphate also has a strong quenching effect. In agreement with earlier studies,[20] external heavy-atom effects could not be induced.

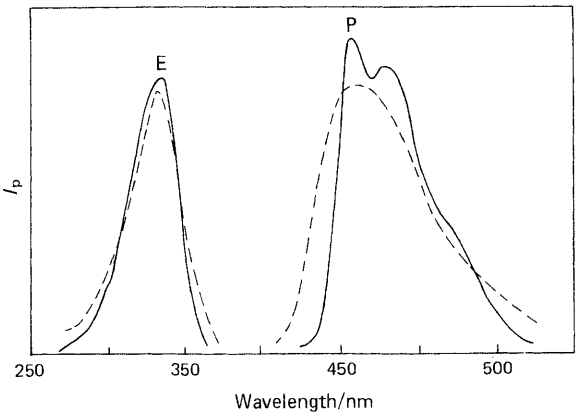


Fig. 4. Excitation (E) and phosphorescence (P) spectra of 6-mercaptopurine (compound 2) in a neutral ethanol glass at 77 K (solid line) and adsorbed on a cellulose thin layer at room temperature (broken line). The spectra obtained on a cellulose thin layer at 77 K were indistinguishable from those obtained in an ethanol glass.

It is apparent that thin-layer phosphorimetry at 77 K is a powerful method for determining 6-mercaptopurine and its metabolites. In many instances the limits of detection are very low, the drugs may be readily and completely recovered from deproteinised plasma and both the chromatographic and luminescence steps contribute to the selectivity of the method (Fig. 3). Further, the TLC step is rapid (especially using HPTLC plates) and utilises a simple, non-luminescent eluting solvent. Room-temperature phosphorescence was, as expected, weaker than the phosphorescence observed at 77 K. Nonetheless, it may be of some use where higher levels of the mercaptopurines are under study; the thin-layer phosphorimetry method is, of course, easier when no liquid nitrogen is required. The finding that cellulosic thin layers give optimum room-temperature phosphorescence effects is in agreement with the results of several previous workers (reviewed in reference 21).

This study has shown that the combination of thin-layer chromatography and phosphorimetry is a valuable analytical method, especially when several structurally similar phosphorescent compounds (*e.g.*, a drug and its metabolites) are to be analysed. It complements the numerous existing techniques that combine TLC and fluorimetry, and further work in the authors' laboratories is extending the method to the analysis of other groups of compounds.

TABLE IV

LIMITS OF DETECTION OF MERCAPTOPURINES IN PURE SOLUTION USING THIN-LAYER PHOSPHORIMETRY AT ROOM TEMPERATURE

Compound No.	Limit of detection/ng per spot	
	Neutral ethanol solvent	Alkaline ethanol solvent
1	NP*	25
2	28	4
3	20	20
4	6	5
5	NP	40
6	NP	20
7	NP	300
8	NP	50
9	NP	700
10	40	200
11	7	100

* NP = No phosphorescence detectable on cellulose thin layers.

We are grateful to the Medical Research Council for a Project Grant in support of this work.

References

1. Salser, J. S., and Balis, M. E., *Cancer Res.*, 1965, **25**, 539.
2. Loo, T. L., Luce, J. K., Sullivan, M. P., and Frei, E., *Clin. Pharmacol. Ther.*, 1968, **9**, 180.
3. Zimmerman, T. P., Chu, L.-C., Buggé, C. J. L., Nelson, D. J., Lyon, G. M., and Elion, G. B., *Cancer Res.*, 1974, **34**, 221.
4. Bennett, L. L., and Allen, P. W., *Cancer Res.*, 1971, **31**, 152.
5. Wong, P. C.-P., and Maddocks, J. L., *J. Chromatogr.*, 1978, **150**, 491.
6. Bailey, D. G., Wilson, T. W., and Johnson, G. E., *J. Chromatogr.*, 1975, **111**, 305.
7. Day, J. L., Tterlikkis, L., Niemann, R., Mobley, A., and Spikes, C., *J. Pharm. Sci.*, 1978, **67**, 1027.
8. Rosenfeld, J. M., Taguchi, V. Y., Hillcoat, B. L., and Kawai, M., *Anal. Chem.*, 1977, **49**, 725.
9. Finkel, J. M., *Anal. Biochem.*, 1967, **21**, 362.
10. Longworth, J. W., Rahn, R. O., and Shulman, R. G., *J. Chem. Phys.*, 1966, **45**, 2930.
11. Aaron, J. J., and Winefordner, J. D., *Anal. Chem.*, 1972, **44**, 2127.
12. Wellons, S. L., Paynter, R. A., and Winefordner, J. D., *Spectrochim. Acta, Part A*, 1972, **30**, 2133.
13. Maddocks, J. L., and Davidson, G. S., *Brit. J. Clin. Pharmacol.*, 1975, **2**, 359.
14. Gifford, L. A., Miller, J. N., Burns, D. T., and Bridges, J. W., *J. Chromatogr.*, 1975, **103**, 15.
15. Gifford, L. A., Hayes, W. P., King, L. A., Miller, J. N., Burns, D. T., and Bridges, J. W., *Anal. Chim. Acta*, 1972, **62**, 355.
16. Miller, J. N., Phillipps, D. L., Burns, D. T., and Bridges, J. W., *Anal. Chem.*, 1978, **50**, 613.
17. Breter, H.-J., and Zahn, R. K., *J. Chromatogr.*, 1977, **137**, 61.
18. Cohen, B. J., and Goodman, L., *J. Am. Chem. Soc.*, 1965, **87**, 5487.
19. Drobruk, J., and Augenstein, L., *Photochem. Photobiol.*, 1966, **5**, 13.
20. Vo-Dinh, T., Yen, E. L., and Winefordner, J. D., *Anal. Chem.*, 1976, **48**, 1186.
21. Vo-Dinh, T., and Winefordner, J. D., *Appl. Spectrosc. Rev.*, 1977, **13**, 261.

Received *September 10th*, 1979
Accepted *January 22nd*, 1980