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STABILITY-INDICATING ASSAY FOR AZATHIOPRINE AND 6-MERCAPTOPURINE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid method based on reversed-phase high-performance liquid chromatography is described for the separation and quantitation of azathioprine, 6-mercaptopurine and their potential contaminants and degradants present in dosage forms. Procedures based on simple dissolution or extraction in 0.02 *M* NaOH give quantitative precision of 1% relative standard deviation at the macro-level and 2% relative standard deviation for the micro-assay of analytes.

The method is stability-indicating and has been used for preliminary studies of the degradation of azathioprine in 0.9% sodium chloride injection, subjected to thermal or ultraviolet irradiation treatment.

The content uniformity of dosage forms of azathioprine and 6-mercaptopurine has been measured. Only azathioprine injection as the freeze-dried sodium salt was found to contain detectable amounts of contaminants 6-mercaptopurine, 1-methyl-4-nitro-5-chloroimidazole and hypoxanthine.

INTRODUCTION

Azathioprine [6-(1-methyl-4-nitroimidazol-5-ylthio)-purine] (I) is widely used for facilitating the survival of tissue and organ transplants. It is commercially available as the freeze-dried sodium salt (Imuran® injection; Wellcome Foundation, Dartford, Great Britain) and in tablets as the neutral product. *In vivo* I is transformed[1–3] to the active moiety 6-mercaptopurine (II), which itself is commercially available in tablet form (Puri-Nethol®; Wellcome Foundation) and may be administered as an antineoplastic agent, especially in acute leukaemia in children[4].

The analysis of azathioprine in pharmaceutical dosage forms presents a major challenge due to the complexity of impurities and degradation products potentially

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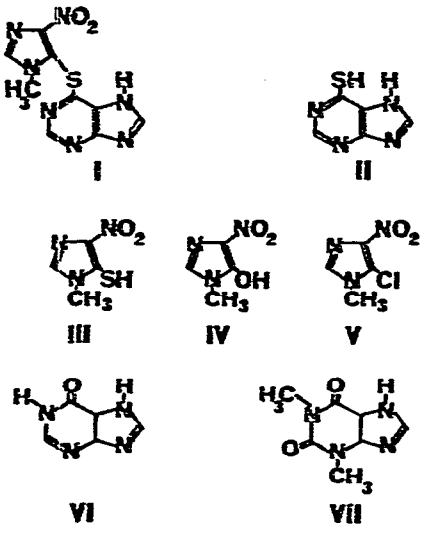


I azathioprine
II 6-mercaptopurine
III 1-methyl-4-nitro-5-thioimidazole
IV 1-methyl-4-nitro-5-hydroxyimidazole
V 1-methyl-4-nitro-5-chloroimidazole
VI hypoxanthine
VII theophylline

co-present. The principal *in vitro* degradation product of I is II, which is limited by the official British[5] and American[6] compendia to less than 1% (w/w) by thin-layer chromatography. Although potentially present as trace impurities, the associated degradants 1-methyl-4-nitro-5-thioimidazole (III) and the 5-hydroxyimidazole (IV), and the synthetic precursors 1-methyl-4-nitro-5-chloroimidazole (V) and hypoxanthine (VI) are not officially controlled in azathioprine or its pharmaceutical formulations. In 6-mercaptopurine tablets the only major contaminant appears to be the synthetic precursor VI, for which the British Pharmacopoeia is the only compendium to specify a limit (1%, w/w), based on a relatively non-specific ultraviolet (UV) absorbance ratio test[5].

Published analytical methods for I either alone or in the presence of its degradant II have been based on UV spectrophotometry[5], non-aqueous titration[6], colorimetry[7,8], ion-exchange chromatography[9] and polarography[6,7]. Methods applicable to either I or II have utilised chloramine-T titration[10], separation on DEAE-cellulose followed by UV spectrophotometry[11], thin-layer chromatography[7,8,12], strong anion-exchange high-performance liquid chromatography (HPLC) with gradient elution[13], strong cation-exchange HPLC[14] and a microbiological procedure[15]. Pharmacopoeial methods[5,6] for the control of II differ according to whether it is present at the trace level (when thin-layer chromatography is prescribed) or whether it is the pure drug (non-aqueous titrimetry) or in tablet form (UV spectrophotometry). Other methods specifically for the analysis of II include colorimetry[16], column chromatography followed by UV spectrophotometry[17], fluorimetry[18], gas–liquid chromatography[19], strong anion-exchange gradient-elution HPLC[20–22] and a radio-tracer method[23] for *in vivo* studies.

Few of the published methods for I consider the problem of potential interference by contaminants II–VI in pharmaceutical dosage forms and none enables all six components to be controlled. Recent work by Czarnecki[8] on the colorimetric assay of I points to an indirect method for the colorimetric control of V extracted by benzene from tablets. However this possible approach would require further valida-

tion. Czarnecki proposes thin-layer and paper chromatographic methods for detecting trace amounts of II and V in I.

Specific and sensitive quantitative procedures are required for the assay of azathioprine and its potential contaminants II–VI, and for 6-mercaptopurine and VI, especially for quality control and for stability studies on I and II in their respective dosage forms. In this latter context, a recent report by Neil[24] indicates that little is known about the stability of azathioprine when administered in intravenous fluids that contain dextrose, sodium chloride or both, although the manufacturers, The Wellcome Foundation, certainly have a considerable body of hitherto unpublished data on the stability of I in its normal dosage forms[25].

The present work reports the development of simple, precise and stability-indicating assay procedures by reversed-phase HPLC for the rapid analysis of I and II in their dosage forms, and for their anticipated trace impurities and degradation products. Preliminary examination of the stability of I in isotonic saline injections subjected either to elevated temperature or to UV irradiation at room temperature, has for the first time confirmed the identity of the principal degradation products of azathioprine.

EXPERIMENTAL

Reagents and materials

HPLC grade methanol (Rathburn Chemicals, Walkerburn, Great Britain) was used as received. Water was glass-distilled. Glacial acetic acid and ammonium carbonate were reagent-grade (BDH, Poole, Great Britain). Sodium hydroxide and potassium dihydrogen phosphate were Analar grade (Hopkin and Williams, Chadwick Heath, Great Britain). All eluent mixtures were filtered through a Millipore® 0.45-μm MF filter using an all-glass apparatus, before degassing for 10 min in an ultrasonic bath under reduced pressure.

Azathioprine powder (melting point range 237–238°), the putative degradants 1-methyl-4-nitro-5-thioimidazole and 1-methyl-4-nitro-5-hydroxyimidazole, and the synthetic precursors 1-methyl-4-nitro-5-chloroimidazole and hypoxanthine were generously provided by The Wellcome Foundation, and used as received. 6-Mercaptopurine powder (melting point range 298–301°) and theophylline were obtained from Sigma (St. Louis, Mo., U.S.A.).

Azathiopurine (Imuran®) tablets and injections, and 6-mercaptopurine (Puri-Nethol®) tablets were kindly supplied by The Wellcome Foundation, and stored in the dark until used. Infusion bags (100 ml) of 0.9% sodium chloride injection USP were obtained from Travenol Labs. (Thetford, Great Britain).

Standard solutions

Azathioprine and compounds II–VI are poorly soluble in water but are soluble in 0.02 *M* NaOH. At ambient temperatures a 1.0 mg/ml solution of I in 0.02 *M* NaOH was stable chromatographically for 8 days in the dark and for 4 days when exposed to diffuse daylight. All standard solutions were stored in light-resistant containers and discarded after two days. In 0.04 *M* and higher concentrations of NaOH, degradation of azathioprine was apparent chromatographically after 2 days.

Before selection of an internal standard for quantitative purposes, a solution

of 1.0 mg/ml of I and 0.5 mg/ml of II in 0.04 *M* NaOH was heated at 60° for 48 h, exposed to daylight for 8 h and 1.5 μl injected into the reversed-phase HPLC system developed. Apart from I and II, three other component peaks were detected (at 240 nm) and accordingly theophylline (VII) was selected as the internal standard, since it was well-resolved from all known or putative degradant and contaminant peaks. Caffeine and theobromine were also considered as internal standards, but although well resolved from the major peaks, they interfered with degradant and contaminant peaks of interest. For developing and optimising the separation system a standard test mixture A in 0.02 *M* NaOH consisting of 1.0 mg/ml of I, 0.5 mg/ml of II and 1.5 mg/ml of the internal standard was employed.

For quantitative analysis two sets of stock standards were freshly prepared in 0.02 *M* NaOH every two days, one for the macro-level assay of I and II, the other for the micro-assay of II, V and VI in dosage forms of I and for VI present in dosage forms of II. The stock solutions in 0.02 *M* NaOH were: azathioprine, 4 mg/ml; 6-mercaptopurine, 2 mg/ml (macro-assay); 20 μg/ml (micro-assay); theophylline, 3 mg/ml (macro-assay); 150 μg/ml (micro-assay); 1-methyl-4-nitro-5-chloroimidazole, 20 μg/ml; hypoxanthine, 20 μg/ml. Solutions of contaminants III and IV were prepared in 0.02 *M* NaOH for identification purposes as required.

Equipment

The liquid chromatograph was assembled in the laboratory from commercially available units, comprising a constant flow LC pump and an interconnecting decilinear solvent programmer system (Applied Chromatography Systems, Luton, Great Britain), and a variable-wavelength UV monitor with an 8-μl flow cell (CE-212, Cecil Instruments, Cambridge, Great Britain) operated at 240 nm. The 100 × 5 mm I.D. stainless-steel column (Shandon-Southern Instruments, Cheshire, Great Britain) was slurry-packed with a microparticulate bonded reversed-phase packing (5-μm ODS-Hypersil; Shandon-Southern Instruments) by the upward displacement technique of Knox[26]. Sample introduction was by SGE 5-μl high-pressure syringe (Scientific Glass Engineering, London, Great Britain) and septum port injector (Shandon-Southern Instruments). The chromatograph was flushed clean at the end of each day with methanol–water (60:40, v/v) to prevent salt deposition in the system. Using the same eluent, the column performance was checked daily with a test mixture of phenol and *p*-cresol, the average number of theoretical plates (*N*) being over 40,000 plates/m. UV irradiation of test samples was performed at ambient temperatures with a water-cooled medium-pressure Hanovia mercury lamp.

HPLC procedure

Choice of packing material. Previous work[27] with microparticulate bonded reversed-phase packing materials had shown promise. Experience in this laboratory with the "fully-capped" 5-μm ODS-silica (ODS-Hypersil) had shown excellent quantitative reliability and led to the choice of this packing material for the present work.

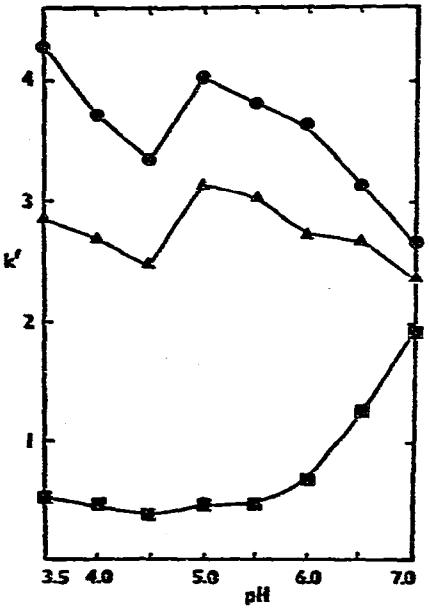
Choice of mobile phase. Initially the mobile phase used was methanol–water (20:80, v/v; pH 5.2) when no useful separation of 1 μl of a test mixture of I (1.0 mg/ml) and II (0.5 mg/ml) was observed. At pH 5.2 both I ($pK_a = 8.2$) and II ($pK_a = 7.7$) are almost completely ionised in the eluent and therefore unlikely to partition into the lipophilic bonded stationary phase.

Operation at the alkaline pH necessary to suppress ionisation (*ca.* pH 10) is inadvisable, both because of the questionable stability of the packing material above pH 9 and because of the possible instability of I. Therefore it was decided to examine a reversed-phase ion-pair system. Acetate anion was selected as a potential pairing ion and incorporated in the same eluent, using glacial acetic acid (pK_a 4.6) at concentrations from 0.25 to 1.0% (v/v). The pH was adjusted to pH 4.5 by addition of solid ammonium carbonate. At 0.5% (v/v) acetic acid and above, good resolution of I and II was obtained. The phase capacity ratios (k') for I, II and the internal standard VII fell by 5–10% to a plateau as acetic acid concentration increased. At low acetate levels the peaks showed marked tailing and chromatographic variability. A concentration of 0.5% (v/v) acetic acid was therefore selected for further work.

Potassium dihydrogen phosphate (25 m*M*) was selected as a flexible buffer system to examine the effect of pH in the range 3.5–8.0, adjusted by drop-wise addition of 1 *M* NaOH to the eluent methanol–buffer–glacial acetic acid (20:79.5:0.5, v/v/v).

Figs. 1 and 2 illustrate the relationship of k' and N with pH for I, II and VII each point being the average of 5 injections of 1.5 μl of test mixture A. Although the pH profile of k' for I and VII is different from that for II, all three components exhibit their highest chromatographic efficiency at pH 4.5 (*ca.* 40,000 plates/m).

Below pH 4.0 and above pH 5.5 the three peaks show marked tailing, reflecting perhaps the lower acetate anion concentration at one extreme, and lower analyte



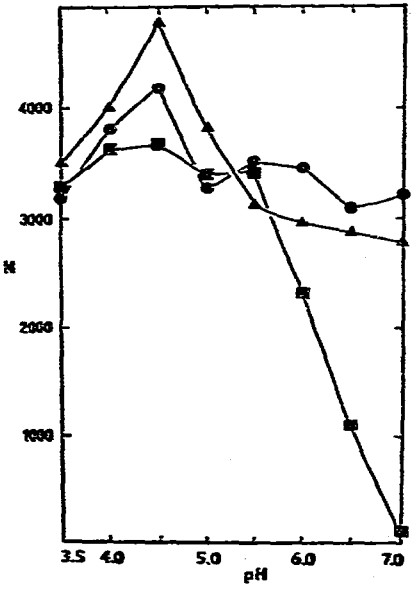
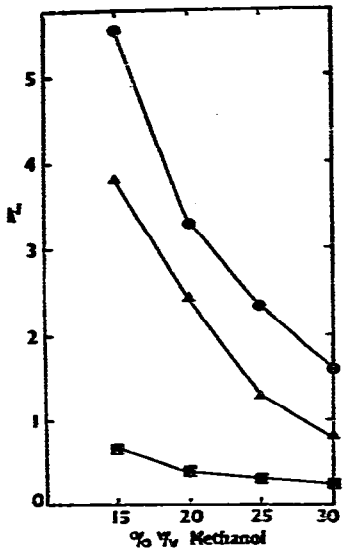


Fig. 1. Variation of k' with pH for azathioprine (●), 6-mercaptopurine (■) and theophylline (▲). Chromatographic conditions as in text.

Fig. 2. Variation of the number of theoretical plates N with pH for azathioprine (●), 6-mercaptopurine (■) and theophylline (▲). Column: 100 × 5 mm I.D. slurry-packed with 5-μm ODS-Hypersil. Chromatographic conditions as in text.

ionisation at the other. At pH 7.5 the separation breaks down completely, 6-mercaptopurine being firmly retained on the column.

The concentration of organic modifier was optimised by using the deci-linear solvent programmer ("Scout") system to vary the methanol concentration from 15 to 30% (v/v) in the 25 m*M* phosphate–glacial acetic acid eluent, adjusting to pH 4.50. As shown in Fig. 3 (each point being the average of three injections), k' decreases as the methanol concentration increases and effectively competes with the bonded stationary phase for the solutes. At 15% (v/v) methanol band-spreading was unacceptable and analysis times long, whereas at 30% (v/v), resolution of I and the internal standard VII was incomplete. When all the potential degradants and contaminants of I were chromatographed with internal standard as a seven component mixture, an eluent composition corresponding to 20% (v/v) methanol gave good resolution (Fig. 4) and corresponded to the maximum number of theoretical plates for the system. The k' values observed under these conditions are summarised in Table I.



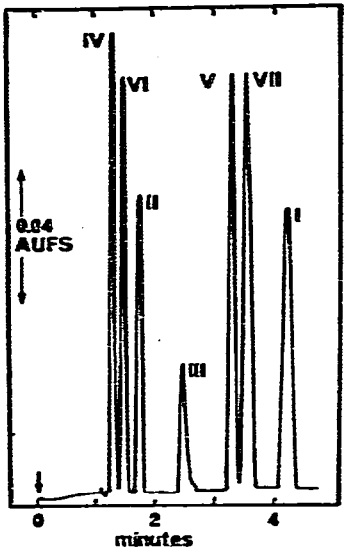


Fig. 3. Variation of k' with concentration (%, v/v) of methanol for azathioprine (●), 6-mercaptopurine (■) and theophylline (▲). Chromatographic conditions as in text.

Fig. 4. Chromatogram of a synthetic mixture of standards I–VI at 1.0 mg/ml and VII at 1.5 mg/ml in 0.02 *M* NaOH. Column, 5 μm ODS-Hypersil, 100 × 5 mm I.D.; mobile phase, methanol–potassium dihydrogen phosphate–glacial acetic acid (20:79.5:0.5, v/v/v) at pH 4.50; flow-rate, 1.5 ml/min; detector wavelength, 240 nm at 0.2 AUFS; injection volume, 1.5 μl. Peaks: I = azathioprine; II = 6-mercaptopurine; III = 1-methyl-4-nitro-5-thioimidazole; IV = 1-methyl-4-nitro-5-hydroxyimidazole; V = 1-methyl-4-nitro-5-chloroimidazole; VI = hypoxanthine; VII = theophylline (internal standard).

The eluent composition finally selected for the separation of components I–VII was methanol–25 m*M* potassium dihydrogen phosphate–glacial acetic acid (20:79.5:0.5, v/v/v) adjusted to pH 4.50. An eluent flow-rate of 1.5 ml/min gives good chromatographic efficiency (>40,000 plates/m) for components I–VII and an analysis

TABLE I

PHASE CAPACITY RATIOS AND DETECTION LIMITS

k' at a flow-rate of 1.5 ml/min and detection limits (ng): $\lambda = 240$ nm, 0.02 aufs. Chromatographic conditions as in text.

No.	*Compound*	k'	*Detection limit (ng)*
IV	1-Methyl-4-nitro-5-hydroxyimidazole	0.10	0.3
VI	Hypoxanthine	0.28	0.4
II	6-Mercaptopurine	0.38	0.4
III	1-Methyl-4-nitro-5-thioimidazole	1.77	1.0
V	1-Methyl-4-nitro-5-chloroimidazole	2.13	0.4
VII	Theophylline (internal standard)	2.48	0.5
I	Azathioprine	3.34	0.5

time of 4.5 min, which is satisfactory for routine quality control and stability studies.

In preliminary work it was observed that, after equilibrating the analytical column with fresh eluent at the start of a series of assays, the first two or three injections of test mixture A gave poor resolution of I, II and VII, whereas after five or more injections good resolution was observed, with a relative standard deviation in k' of $\pm 0.88\%$ for each of the peaks. This "equilibration" phenomenon was observed throughout the course of the present work, and perhaps reflects the availability of residual uncapped polar silanol sites, which affect the chromatography until occupied by ionised, polar molecules of azathioprine and 6-mercaptopurine.

Choice of detection wavelength. Whereas in acidic conditions azathioprine has a $\lambda_{max.}$ at 281 nm and a broad $\lambda_{min.}$ at 246 nm, the $\lambda_{max.}$ for 6-mercaptopurine is 323 nm and for theophylline is 273 nm. A compromise wavelength of 240 nm was selected at which all components of interest absorbed sufficiently. At a detector range of 0.02 a.u.f.s. there was adequate sensitivity for components II–VI to be quantitated, as illustrated in Table I which summarises the detection limits.

Quantitative procedure

The internal standard, theophylline (VII), was incorporated in standards and test solutions by quantitative dilution at a level (1.5 mg/ml for macro- and 15 μg/ml for micro-assay) which gave peak heights comparable with the anticipated peak deflections for test analytes. Since all peaks observed were sharp and well-resolved, quantitation was performed using peak heights for simplicity. In the micro-assay procedure, peak heights were measured to the baseline extrapolated from the leading edge of the peak. Analytical curves of peak height ratios (with respect to internal standard) against analyte concentration were rectilinear and passed through the origin, the relevant statistical data being summarised in Table II. As shown in Figs. 5 and 6, the concentration of test samples was adjusted to correspond with the top of the calibration graph in each case, since this is the zone of highest relative precision. The technique used for routine quantitative measurements was the so-called "single-point bracketting" procedure, where a group of test samples is preceded and followed by a standard mixture of comparable concentration, the average of the standards being used to calculate the test concentrations.

Samples of azathioprine injection (nominally 50 mg freeze-dried sodium salt in absence of a preservative) were easily dissolved in 25.00 ml of 0.02 *M* NaOH. Two

TABLE II

QUANTITATIVE PERFORMANCE OF ASSAY

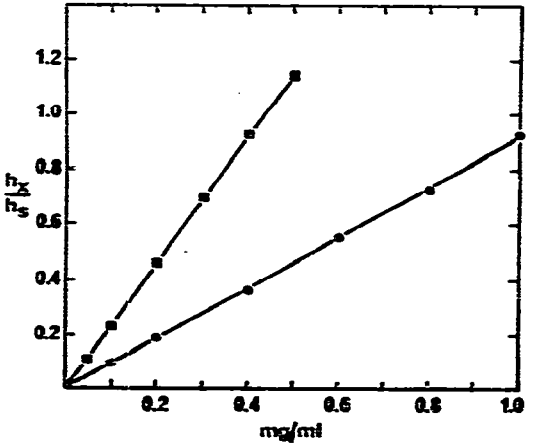
Internal standard: theophylline at 1.5 mg/ml for macro-assay at 0.2 aufs, and 15 μg/ml for micro-assay at 0.02 aufs. Chromatographic conditions as in text.

No.	*Compound*	*Regression equation*	*Degrees of freedom*	*Mean concentration ± confidence limits (P = 0.95)*
I*	Azathioprine	$y = 0.912x + 0.0021$	21	1.000 ± 0.008 mg/ml
II*	6-Mercaptopurine	$y = 2.291x - 0.0001$	21	1.000 ± 0.011 mg/ml
II**	6-Mercaptopurine	$y = 154.2x - 0.0008$	21	10.00 ± 0.18 μg/ml
V**	1-Methyl-4-nitro-5-chloroimidazole	$y = 186.0x + 0.0021$	15	10.00 ± 0.22 μg/ml
VI**	Hypoxanthine	$y = 255.1x + 0.0032$	15	10.00 ± 0.10 μg/ml

* Macro-assay.
** Micro-assay.

10.00-ml aliquots were combined, respectively, with 10.00 ml of stock 3.00 mg/ml internal standard (for macro-assay); or with 2.00 ml of stock 150 μg/ml internal standard (for micro-assay) in a 20.00-ml volumetric flask and made up to volume with 0.02 *M* NaOH. Samples of azathioprine tablets (nominally 50 mg) were individually crushed with 0.02 *M* NaOH, filtered and brought to 25.00 ml with 0.02 *M* NaOH. Two 10.00-ml aliquots were treated exactly as for the freeze-dried powder. Samples of 6-mercaptopurine tablets were treated exactly as for azathioprine tablets, except that after filtering, each sample was brought to 50.00 ml with 0.02 *M* NaOH.

A 1.5-μl volume of each extract was subjected to HPLC assay in replicate and the relative standard deviation (R.S.D.) of the contents of injection vials and tablets calculated.



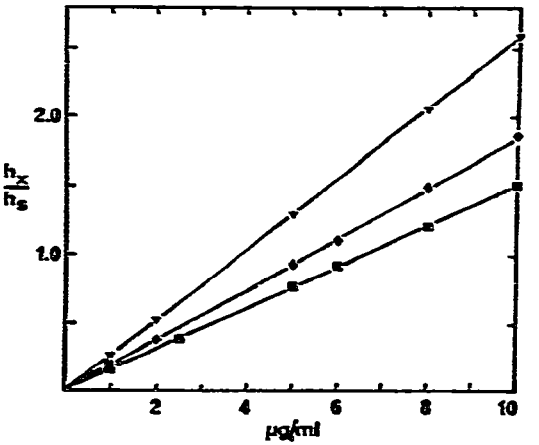


Fig. 5. Analytical curves for azathioprine (●) and 6-mercaptopurine (■) peak height ratios. Internal standard: theophylline at 1.5 mg/ml; detector sensitivity: 0.2 AUFS.

Fig. 6. Analytical curves for micro-assay of 6-mercaptopurine (■), 1-methyl-4-nitro-5-chloroimidazole (◆) and hypoxanthine (▼) peak height ratios. Internal standard: theophylline at 15 μg/ml; detector sensitivity: 0.02 AUFS.

Stability studies

A preliminary study on the stability of azathioprine in saline infusion fluid was performed by adding the contents of two vials of Imuran, nominally equivalent to 100 mg, to the contents of a 100-ml bag of 0.9% sodium chloride. This dosage regime corresponds approximately to that used in clinical practice. Each infusion bag was assayed immediately to establish the initial concentration of I (*ca.* 1 mg/ml). Individual bags were maintained in ambient room light in water baths at room temperature or 85°, or were subjected to radiation from a Hanovia mercury lamp at ambient temperature. Over a period of 5 h, samples of 2.3 ml were withdrawn at 30-min intervals, cooled, 2.00 ml added to 2.00 ml of internal standard stock solution (3 mg/ml) and assayed for I content by HPLC.

RESULTS AND DISCUSSION

As illustrated in Fig. 4, azathioprine and 6-mercaptopurine were well resolved from all their known or potential impurities, thus enabling the assay to be used for stability studies. The quantitative performance of the assay at both the macro- and the micro-level was excellent (Table III). The procedures are suitable for routinely controlling the major and minor components in these dosage forms. Although manual injection was employed for this work, the addition of a loop valve or automatic injector would facilitate repetitive analysis, and may enable the internal standard to be omitted from the system. The chromatographic efficiency of the system is sufficiently high to accommodate the slight loss of column efficiency normally encountered with loop-valve injector systems.

In a separate experiment the volume of test mixture A injected in duplicate was varied from 0.75–6.0 μl and the effect on the peak height ratio (to VII) of I and II examined. The R.S.D. of data for I (mean 0.917) was $\pm$0.39%, with no apparent functionality. The data for II, however, showed a sharp fall in peak height ratio (from 1.19 to 1.14) at 1.5 μl, followed by a progressive rise to 1.26, 10% above the minimum value. While the data for I is as expected, that for II gives rise for concern and indicates that, even when using an internal standard, reproducibility of injection technique is necessary for precise results. It is possible that this sharp change in relative instrumental response to II may reflect differential chromatographic properties of II and VII, or it may be related to the fact that in both cases the UV detector is required to measure absorbances on the slope of their respective absorption curves.

As recorded in Table I, the sensitivity of the proposed micro-assay procedure is such that less than 33 μg of any of the known potential degradants or contaminants could be detected in a 50-mg tablet or injection vial. This corresponds to a detection limit of 0.066% (w/w), some 15-fold less than the compendial[5,6] 1% (w/w) limits applied to II in I and VI in II. If necessary, the detection limit could be reduced 6-fold by injecting 10 μl of sample, without appreciable loss of performance. In the case of II, with a strong absorption peak at 323 nm, sensitivity could be further increased by adjusting the variable wavelength detector to 323 nm, although another internal standard with suitable absorbing properties would then be required.

The "recoveries" of I in Imuran® vials and tablets and of II in Puri-Nethol® tablets, are expressed as a percentage of the nominal content (Table III). The content uniformity of I in Imuran® tablets and of II in Puri-Nethol® tablets was better than

TABLE III

ASSAY DATA FOR AZATHIOPRINE AND 6-MERCAPTOPURINE IN DOSAGE FORMS

Assay values calculated with respect to "bracketting" standards (see text) are presented as the mean $\bar{x}$ for the total amount in each of n samples, each analysed in duplicate.

Sample	*Compound*	*Nominal content (mg)*	*n*	$\bar{x}$ *(mg)*	*Recovery (%)*	*Standard deviation (mg)*	*Relative standard deviation (%)*
Imuran® injection Batch No. 99012	Azathioprine	50	5	48.55	97.1	1.26	2.6
Imuran® tablets Batch No. TM8480	Azathioprine	50	5	50.65	101.3	0.49	0.97
Puri-Nethol® tablets	6-Mercaptopurine	50	5	50.02	100.0	0.24	0.48
Azathioprine standard	Azathioprine powder Batch No. BX252	1.00 mg/ml	10	—	—	0.0099 mg/ml	0.99
6-Mercaptopurine standard	6-Mercaptopurine powder Batch No. 82C1130	1.00 mg/ml	10	—	—	0.0027 mg/ml	0.27
6-Mercaptopurine standard	6-Mercaptopurine powder	10.0 μg/ml	10	—	—	0.0092 μg/ml	0.92

TABLE IV

ASSAY DATA FOR IMPURITIES IN AZATHIOPRINE AND 6-MERCAPTOPURINE DOSAGE FORMS

Assay values are presented as the mean $\bar{x}$ for the total amount in each of 5 samples analysed in duplicate at 0.02 aufs and are expressed as a percentage (w/w) of the mean content found for the principal drug component (Table III)

No.	*Impurity*	*Dosage forms*					
		Imuran® injection		*Imuran® tablets*		*Puri-Nethol® tablets*	
		$\bar{x}$ *(mg)*	*% (w/w)*	$\bar{x}$ *(mg)*	*% (w/w)*	$\bar{x}$ *(mg)*	*% (w/w)*
II	6-Mercaptopurine	0.37	0.76	<0.013	<0.026	50.02	100.0
III	5-Thioimidazole	<0.033	<0.066	<0.033	<0.066	—	—
IV	5-Hydroxyimidazole	<0.010	<0.020	<0.010	<0.020	—	—
V	5-Chloroimidazole	0.065	0.13	<0.013	<0.026	—	—
VI	Hypoxanthine	0.32	0.66	<0.013	<0.026	<0.013	<0.026

$\pm 1\%$ ($n = 5$), expressed as relative standard deviation, which is comparable with the intrinsic variability of the macro-assay itself on pure standards of I and II (Table III). No impurities were detectable in these dosage forms. By contrast, the batch of Imuran® injection samples contained detectable quantities of II, V and VI (Table IV and Fig. 7) and showed a variation in content uniformity of $\pm 2.6\%$ ($n = 5$). It seems likely that the sodium salt of I is less stable than the base itself, the principal degradant being II, present at less than the 1% (w/w) pharmacopoeial limit prescribed.

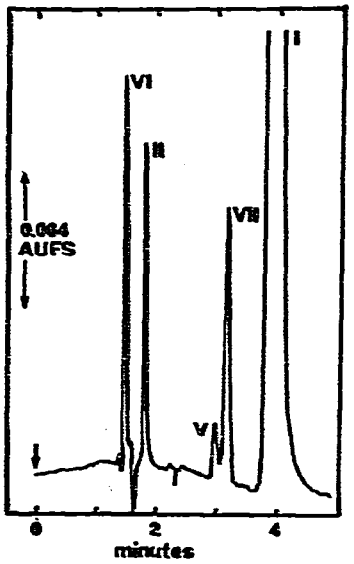


Fig. 7. Chromatogram of a solution of Imuran injection in 0.02 *M* NaOH, containing internal standard (theophylline) at 15 μg/ml. Column, 5 μm ODS-Hypersil, 100 × 5 mm I.D.; mobile phase, methanol–potassium dihydrogen phosphate–glacial acetic acid (20:79.5:0.5, v/v/v) at pH 4.50; flow-rate, 1.5 ml/min; detector wavelength, 240 nm at 0.02 AUFS; injection volume, 1.5 μl. Peaks: I = azathioprine; II = 6-mercaptopurine; V = 1-methyl-4-nitro-5-chloroimidazole; VI = hypoxanthine; VII = theophylline.

The presence of small amounts of synthetic precursors of V and VI would indicate the need to bring these contaminants under pharmacopoeial control. The identity of each contaminant in this and subsequent experiments was confirmed chromatographically by injecting a mixture of the sample and each putative degradant in turn. Rather surprisingly, in view of the significant level of the 6-mercaptopurine breakdown product, no detectable quantities of the associated imidazole degradants III and IV could be found in this batch. These compounds are known to be intrinsically unstable[28] and the possibility of secondary reaction mechanisms converting these degradants to ring-opened products not active in the UV, or to components which are retained on column, may be anticipated.

As shown in Fig. 8, the particular batch (No. 98436) of Imuran injections

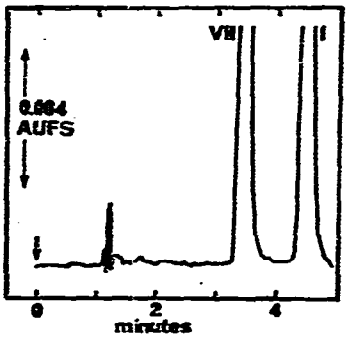


Fig. 8. Chromatogram of initial sample from 0.9% sodium chloride injection with azathioprine added at 1 mg/ml as the freeze-dried sodium salt. Chromatographic conditions as in Fig. 7. Peaks: I = azathioprine; VII = theophylline (1.5 mg/ml). Injection volume, 4 μl.

TABLE V

ASSAY DATA FOR STABILITY OF AZATHIOPRINE IN 0.9% SODIUM CHLORIDE INJECTION

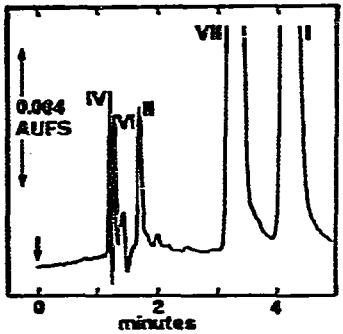
Time (min)	*Thermal degradation**		*UV-irradiation***		*Ambient temperature, diffuse daylight****	
	Azathioprine concentration (mg/ml)	*% remaining*	*Azathioprine concentration (mg/ml)*	*% remaining*	*Azathioprine concentration (mg/ml)*	*% remaining*
0	0.970	100	0.972	100	0.981	100
30	0.966	99.6	0.965	99.3	0.980	99.9
60	0.957	98.7	0.963	99.1	0.978	99.7
90	0.949	97.8	0.951	97.8	0.972	99.1
120	0.928	95.7	0.943	97.0	0.975	99.4
180	0.916	94.4	0.937	96.4	0.973	99.2
240	0.909	93.7	0.937	96.4	0.980	99.9
300	0.904	93.2	0.931	95.6	0.977	99.6

* 100-ml injection containing azathioprine at 1 mg/ml and incubated at 85° in diffuse daylight.

** 100-ml injection containing azathioprine at 1 mg/ml and subjected to UV irradiation by a medium-pressure Hanovia mercury lamp (water cooled).

*** 100-ml injection containing azathioprine at 1 mg/ml and incubated at ambient temperature in diffuse daylight.

used for the preliminary stability study in isotonic saline injections contained no detectable quantities of components II–VI, a convenient but unexpected finding. At room temperature, no detectable degradation of I was observed in diffuse daylight after 5 h. After 19 h the loss of I was 1.13%. This data confirms the stability of azathioprine used as an additive under normal clinical conditions in this injection at pH 9.51. At 85° the content of I fell by 6.8% after 5 h (Table V) with a characteristic profile for the degradants II–VI (Fig. 9) featuring a higher content of component IV than VI. Under UV irradiation a loss of 4.4% of I occurred after 5 h at room temperature (Table V) and a characteristic degradant profile was generated (Fig. 10), where component VI was dominant.



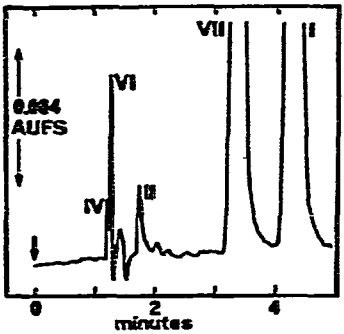


Fig. 9. Chromatogram of 5-h thermally degraded sample of 0.9% sodium chloride injection containing azathioprine (added as the freeze-dried sodium salt) after incubation at 85°. Chromatographic conditions as in Fig. 7. Peaks: I = azathioprine; II = 6-mercaptopurine; IV = 1-methyl-4-nitro-5-hydroxyimidazole; VI = hypoxanthine; VII = theophylline (1.5 mg/ml). Injection volume, 4 μl.

Fig. 10. Chromatogram of 5-h UV-irradiated sample of 0.9% sodium chloride injection containing azathioprine (added as the freeze-dried sodium salt) after irradiation by a medium-pressure Hanovia mercury lamp. Chromatographic conditions as in Fig. 7. Peaks: I = azathioprine; II = 6-mercaptopurine; IV = 1-methyl-4-nitro- 5-hydroxyimidazole; VI = hypoxanthine; VII = theophylline (1.5 mg/ml). Injection volume, 4 μl.

These degradation patterns, neither of which followed first or second order kinetics, were generated under extreme conditions of temperature or radiation. To this extent they are uncharacteristic of the normal clinical usage of Imuran as an infusion fluid additive. These studies do however confirm the necessity to store azathioprine preparations in cool, light-resistant containers. Further work on the stability of I in infusion fluids could be carried out in buffered solutions over the range pH 2–12, it being likely that the mechanism of degradation may be influenced by hydrogen ion concentration.

The micro-assay for I and II is sufficiently sensitive and specific for deproteinised serum samples of I and II to be readily assayed for pharmacokinetic studies by this rapid and flexible HPLC procedure.

CONCLUSIONS

The potential contribution of HPLC to the rapid and precise quantitation of two antineoplastic drugs and their associated contaminants is illustrated by the

present assay, based on chemically bonded, reversed-phase microparticulate packing material.

The assay procedures developed are applicable to quality control and stability studies of azathioprine and 6-mercaptopurine in their dosage forms. Preliminary stability studies on azathioprine have, for the first time, confirmed the identity and relative distribution of the principal products of thermal and photolytic degradation. With little modification this sensitive and specific separation method may be used for clinical studies.

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