The aggregation curve due to collagen changed when ethylene glycol was added to plasma, and the time lag characteristic of this aggregant disappeared (Fig. 1).

In Table I, the action of indolecarbohydrazides in platelet aggregation is represented with reference to the aggregant adenosine diphosphate (2×10^{-5} *M*), epinephrine (10^{-4} *M*), and collagen (0.2 mg/ml). Platelet-rich plasma was incubated with the compounds for 90 sec at 37°. Inhibition of aggregation at 5×10^{-4} *M* of the compounds is significant (Table I).

When the incubations were carried out for 5 min at 37°, obvious differences were observed in the antiaggregant action of the tested compounds with adenosine diphosphate as the aggregant. Under these conditions, all of the compounds presented a higher capacity of aggregation inhibition. Compound IV at 5×10^{-6} *M* totally inhibited aggregation; *i.e.*, approximately 2 mg of the compound can produce complete platelet aggregation inhibition in humans.

REFERENCES

(1) A. Gaarder, J. Jonsen, S. Laland, A. Hellem, and P. A. Owren, *Nature (London)*, **192**, 531 (1961).

(2) G. W. Schnetzer, *Am. Heart J.*, **83**, 552 (1973).

(3) J. F. Mustard and M. A. Packham, *Pharmacol. Rev.*, **22**, 97 (1970).

(4) P. R. Emmons, M. J. Harrison, A. J. Honour, and J. G. R. Mitchell, *Nature (London)*, **208**, 255 (1965).

(5) A. A. Hassanein, A. G. G. Turpie, G. P. MacNicol, and A. S. Dorglas, *Br. Med. J.*, **5**, 83 (1970).

(6) M. Nakanishi, H. Imamura, and K. Goto, *Yakugaku Zasshi*, **90**, 564 (1970).

(7) J. J. Sixma and A. M. C. Treesnichnigg, *Scand. J. Haematol.*, **8**, 417 (1971).

(8) E. F. Elslager, N. F. Haley, J. R. MacLean, S. C. Perricone, D. Potoczak, H. Veloso, D. Worth, and R. H. Wheelock, *J. Med. Chem.*, **14**, 397 (1971).

(9) H. Ott and G. M. Smith, *Br. J. Pharmacol.*, **43**, 460 (1971).

(10) R. D. Mackenzie, T. R. Blohm, E. M. Auxier, J. M. G. Henderson, and J. M. Steinbach, *Proc. Soc. Exp. Biol. Med.*, **137**, 662 (1971).

(11) G. P. Claxton, J. M. Grisar, E. M. Roberts, and R. W. Fleming, *J. Med. Chem.*, **15**, 500 (1972).

(12) E. Deutsh, K. Lachner, K. Moser, and L. Stockinger, *Thromb. Diath. Hemorrh.*, **26**, 145 (1971).

(13) K. Kikugawa and M. Ichino, *Chem. Pharm. Bull.*, **21**, 1151 (1973).

(14) W. N. Beverung and R. A. Partyka, *J. Med. Chem.*, **18**, 224 (1975).

(15) K. Izuka and K. Kikugawa, *Chem. Pharm. Bull.*, **20**, 614 (1972).

(16) M. H. Maguire, F. Penglis-Caredes, and G. R. Gough, *Experientia*, **30**, 922 (1974).

(17) J. Lasierra Cirujeda *et al.*, *Anal. Real Acad. Farm.*, **2**, 347 (1974).

(18) E. Szarvasi *et al.*, *Eur. J. Med. Chem.*, **11**, 115 (1976).

(19) A. Monge, J. A. Palop, M. T. Martínez, and E. F. Alvarez, *An. Quim.*, **73**, 278 (1977).

(20) G. U. R. Born and M. J. Cross, *J. Physiol.*, **168**, 178 (1963).

ACKNOWLEDGMENTS

Supported by the Subdireccion General de Promocion Científica del Ministerio de Educación y Ciencia.

The authors thank Dr. P. Angulo of Clinica Universitaria, Pamplona, Spain, and Prof. Dr. E. Fernández-Alvarez of Centro Nacional de Química Orgánica, Madrid, Spain, for their helpful discussions and suggestions.

Assay of Mercaptopurine in Plasma Using Paired-Ion High-Performance Liquid Chromatography

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Received May 26, 1977, from the *School of Pharmacy, Florida Agricultural and Mechanical University, Tallahassee, FL 32307.* Accepted for publication September 21, 1977.

Abstract □ A sensitive, quantitative, and specific high-performance liquid chromatographic method for mercaptopurine in plasma is described. The analysis, in which mercaptopurine and the internal standard, 6-methylthio-2-hydroxypurine, are chromatographed as ion-pairs with heptane–sulfonic acid, employs a simple and rapid sample preparation based on deproteination using 60% trichloroacetic acid. Quantitation of plasma samples to 0.2 μg of mercaptopurine/ml is reported. The retention times of the major metabolites do not interfere.

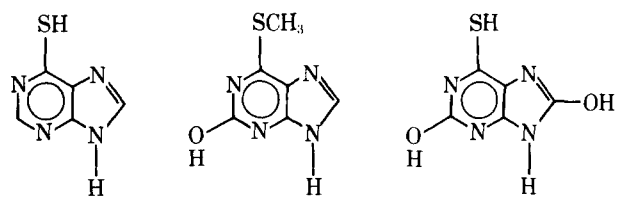
Keyphrases □ Mercaptopurine—high-performance liquid chromatographic analysis in plasma □ High-performance liquid chromatography—analysis, mercaptopurine in plasma □ Antineoplastic agents—mercaptopurine, high-performance liquid chromatographic analysis in plasma

To investigate plasma mercaptopurine (I) levels for pharmacokinetic studies, a sensitive and accurate assay was needed that would distinguish I from its metabolites. Several methods have been reported for I in biological fluids, but each has some disadvantages in specificity and sensitivity (1–3).

Recently, a GLC method was described that separates I from its metabolites (4). However, to achieve a sensitivity of 0.5 μg/ml, I must be extracted from a biological sample and then methylated to achieve a sample suitable for assay. The use of cation-exchange high-performance liquid chromatography (HPLC) for the detection, but not the quantification, of I in biological samples was reported (5, 6). The described method is specific and convenient and has sufficient sensitivity to measure accurately concentrations of mercaptopurine normally encountered at therapeutic levels.

EXPERIMENTAL

Reagents—All compounds used in the assay were used as received.



I II III

0022-3549/78/0700/1027$01.00/0

Table I—Statistics of Calibration Curve and Reproducibility

Concentration, μg/ml	Mean PHR[a]/ Micrograms of I	*SD*	*CV*, %
0.2	1.355	0.065	4.8
0.5	1.375	0.045	3.3
0.75	1.386	0.021	1.5
1.0	1.380	0.013	0.95

[a] PHR = peak height ratio of I to II. Each value represents the average of five determinations.

The solvents employed were reagent grade and were used without further purification.

Instrumentation—The liquid chromatograph[1] was equipped with a double-beam UV detector and a wavelength kit for monitoring at 313 nm. The stationary phase was a 300 × 4-mm prepacked stainless steel column containing a monomolecular layer of octadecyltrichlorosilane (C_{18}) chemically bonded to a high efficiency porous silica surface[2].

The mobile phase was a paired-ion solution containing 0.005 *M* 1-heptanesulfonic acid sodium salt[3], 1% acetic acid, and 10% methanol in distilled water. It was filtered[4] and then deaerated prior to use by the brief application of a vacuum. The temperature was ambient, and the solvent flow rate was 1.0 ml/min. The amount of sample injected was 10 μl for concentrations of I in the 0.2–1.0-μg/ml range. The detector was set at a sensitivity of 0.005 absorbance unit full scale, and the chart speed was 30.5 cm (12 in.)/hr.

Preparation of Standard Solutions—Mercaptopurine[5], 10.0 mg, was weighed accurately into a 100-ml volumetric flask and dissolved by adding 5 ml of distilled water and 200 μl of 1.0 *N* NaOH and then diluted to volume with Sorensen buffer (7). The final concentration was 100 μg/ml.

The blank was prepared as the standard but without I.

The internal standard, 6-methylthio-2-hydroxypurine[5] (II), 3.0 mg, was weighed accurately into a 100-ml volumetric flask and dissolved in and diluted to volume with methanol. The final concentration was 30 μg/ml.

Procedure—Concentrations of I ranging from 2.0 to 100 μg/ml were prepared by diluting the standard solution with the blank. To 0.5 ml of plasma (heparinized) were added 50 μl of the appropriate dilution of I and 50 μl of the internal standard (II). The solution was mixed[6] for 5 sec and then allowed to equilibrate for 10 min. The solution was then deproteinized by the addition of 100 μl of 60% (w/v) trichloroacetic acid. After being mixed[6] for 15 sec and standing for 2 min, the mixture was centrifuged at 2500 rpm for 10 min.

To 200 μl of the clear supernate was added 10 μl of a 50% (w/v) NaOH solution. After 5 sec of mixing[6], a 10-μl sample was used for analysis. The peak height ratio of I to II was plotted on the ordinate and the amount of I added was plotted on the abscissa to obtain the standard curve; this curve proved to be linear over the range evaluated. The reproducibility and accuracy of the assay method are given in Table I.

RESULTS AND DISCUSSION

Under the chromatographic conditions described, the retention volumes of I and II were 4.6 and 8.4 ml, respectively. Figure 1A shows the chromatogram of rat plasma as a control. Figure 1B shows the chromatogram of plasma containing 0.2 μg of I/ml together with 1.5 μg of II/ml. The chromatogram of 1.0 μg of I/ml in plasma is given in Fig. 1C.

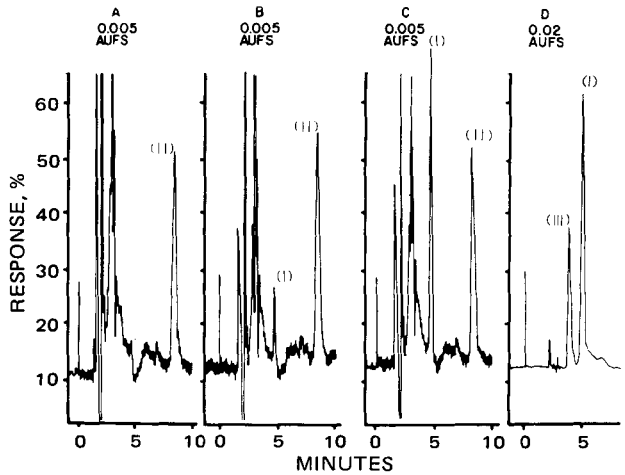


Figure 1—*A: Chromatogram of control plasma. B: Chromatogram of plasma containing 0.2 μg of I/ml and II. C: Chromatogram of plasma containing 1.0 μg of I/ml and II. D: Chromatogram of I in the presence of 6-thiouric acid (III).*

The two major metabolites of I that could interfere in the assay are 6-thiouric acid (III) and 6-methylmercaptopurine. Figure 1D shows that, although 6-thiouric acid comes off before I, it does not interfere. 6-Methylmercaptopurine comes off at a much later time than II.

The data in Table I show a zero coefficient of variation at 1.13 μg/ml when extrapolated by linear regression. The coefficient of variation of the linear regression is −0.985, indicating that the coefficient of variation of the assay is linear with respect to concentration and approaching zero at this point. The sensitivity and reproducibility of this method are better than those previously reported for the determination of I in plasma (1, 2). The lower range of the plasma concentration curve represents over a twofold improvement from that reported using a GLC method (4). The procedure described is currently being employed to obtain concentration–time data for I in animal species.

REFERENCES

(1) T. L. Loo, J. K. Luce, M. P. Sullivan, and E. Frei, *Clin. Pharmacol. Ther.*, **9**, 180 (1968).

(2) J. M. Finkel, *Anal. Biochem.*, **21**, 362 (1967).

(3) J. J. Coffey, C. A. White, A. B. Lesk, W. I. Rogers, and A. A. Serpick, *Cancer Res.*, **32**, 1283 (1972).

(4) D. G. Bailey, T. W. Wilson, and G. E. Johnson, *J. Chromatogr.*, **111**, 305 (1975).

(5) D. J. Nelson, C. J. L. Bugge, H. C. Krasny, and T. P. Zimmerman, *ibid.*, **77**, 181 (1973).

(6) H. J. Breter, *Z. Klin. Chem. Klin. Biochem.*, **12**, 223 (1974).

(7) "Remington's Pharmaceutical Sciences," 14th ed., Mack Publishing Co., Easton, Pa., 1970, p. 1554.

ACKNOWLEDGMENTS

Supported in part by Research Grant RR-08111 from the National Institutes of Health, Minority Biomedical Support Program, and in part by the National Cancer Institute, Bethesda, Md.

The authors thank Mr. Paul Grenier of the Florida A & M University School of Pharmacy for technical assistance.

[1] Model ALC/GPC 244 equipped with a model U6K injector and a model 52110 dual-pen 10-mv Houston recorder, Waters Associates, Milford, Mass.

[2] μBondapak C_{18}, Waters Associates, Milford, Mass.

[3] Eastman Kodak Co., Rochester, N.Y.

[4] Filter apparatus, 47 mm (5.0-μm pore size), Millipore Corp., Bedford, Mass.

[5] Sigma, St. Louis, Mo.

[6] Vortex-Genie, Scientific Industries, Springfield, Mass.