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Isolation of 6-mercaptopurine in human plasma by aluminum ion complexation for high-performance liquid chromatographic analysis

KUN T. LIN*, FRANCE VARIN, GEORGES E. RIVARD and JEAN-MARIE LECLERC
Centre de Recherche, Hopital Ste-Justine, Montreal, Quebec H3T 1C5 (Canada)

ABSTRACT

A sample preparation technique and a high-performance liquid chromatographic method for 6-mercaptopurine (6-MP) that is simple, sensitive and without interference from its metabolites is described. 6-Thioguanine (6-TG) is added as an internal standard to the plasma sample, which is then treated with an aqueous solution of aluminum perchlorate to denature the plasma proteins and form complexes with 6-TG, 6-MP and its major metabolite, 6-thiouric acid (6-TUA). These complexes coprecipitate with proteins on centrifugation. 6-MP and its analogues are then extracted from the precipitate with perchloric acid containing sodium hydrosulfite and the extract is chromatographed on an Ultrasphere ODS column eluted with 0.1 *M* phosphoric acid and 0.001 *M* dithiothreitol in deionized water. The eluate is monitored at 340 nm. No interefering peak was encountered in over 300 clinical plasma samples. 6-TUA was separated from 6-MP and was found to be present in much higher concentration than 6-MP itself throughout the sampling time (6 h) following oral administration of the drug.

INTRODUCTION

6-Mercaptopurine (6-MP, purine-6-thiol, 6-purinethol, NSC-755, CAS 50-44-2; Fig. 1) is one of the major antineoplastic agents used in the maintenance therapy for acute lymphoblastic leukemia (ALL). As a rule, the drug is taken orally. It has been reported that there is no correlation between the prescribed dose of 6-MP and the clinical response in children with ALL [1]. Some of these unpredictable responses may be due to large individual variations in the pharmacokinetics of 6-MP [2–5]. The problem is further complicated by the fact that many pharmacokinetic data have been obtained by different analytical methods and some of these published methods might measure plasma levels of 6-MP in addition to its metabolites [6].

In this paper we describe a simple sample preparation method for isolating 6-MP and its major metabolite, 6-thiouric acid (6-TUA; Fig. 1) from either plasma or serum from patients. A high-performance liquid chromatographic (HPLC) system is used to separate 6-MP from 6-TUA. This method is shown to be reliable.

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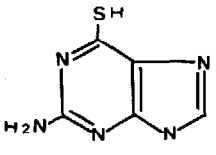
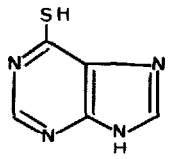


6-THIOGUANINE



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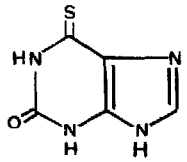
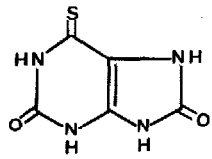


6-THIOXANTHINE



6-THIOURIC ACID

Fig. 1. Structures of 6-thioguanine, 6-mercaptopurine, 6-thioxanthine and 6-thiouric acid.

EXPERIMENTAL

Materials

6-MP, 6-thioguanine (6-TG; Fig. 1) and 6-thioxanthine (6-TX; Fig. 1) were purchased from Sigma (St. Louis, MO, U.S.A.). 6-TUA was synthesized enzymatically from 6-TX, as described previously [7]. Aluminum perchlorate was obtained from Aldrich (Milwaukee, WI, U.S.A.), dithiothreitol from Boehringer Mannheim Canada (Dorval, Quebec, Canada), sodium hydrosulfite from Fisher Scientific (Fair Lawn, NJ, U.S.A.), phosphoric acid (85.9%, analytical-reagent grade) from J. T. Baker (Phillipsburg, NJ, U.S.A.) and citrated normal human plasma from American Hospital Supply (Miami, FL, U.S.A.).

Collection of plasma sample

Venous blood (1 ml) was withdrawn with a polypropylene syringe and transferred to a polypropylene test-tube (100 × 17 mm) containing 20 units of heparin (B.P.), and chilled immediately in ice–water. For pharmacokinetic studies a blank blood sample was taken immediately before the oral administration of 6-MP (50 mg/m^2 body surface). Thereafter, samples were taken at 0.25, 0.5, 1.0, 1.5, 2.0, 4.0 and 6.0 h. The blood samples were centrifuged and the plasma samples were stored at −20°C in a polypropylene test-tube until analyzed.

Sample preparation

To 250 μl of plasma in a 1.5-ml Eppendorf polypropylene centrifuge tube were added 5 μl of 6-TG (2.5 μg/ml) as an internal standard and 25 μl of 1 *M* aluminum perchlorate (aqueous solution). The mixture was allowed to stand at room tempera-

ture for 15 min, chilled in ice–water for 15 min and then centrifuged at 15 600 *g* for 15 min. The supernatant was discarded and the precipitate was resuspended in 500 μl of a 50 m*M* aqueous solution of aluminum perchlorate by stirring to break up the precipitate. It was vortex mixed for 20 s, followed by a 15-min centrifugation (as above), after which the supernatant was thoroughly removed. The precipitate was then resuspended in 150 μl of 0.4 *M* perchloric acid, and then 5 μl of freshly prepared 0.2 *M* sodium hydrosulfite solution (in deionized water) were added. The contents of the tube were mixed and the mixture was allowed to stand at room temperature for 30 min, chilled in ice–water and centrifuged for 15 min (as above). The supernatant was then pipeted into a 1.1-ml STV glass vial (Chromacol, London, U.K.) and placed in the autosampler for injection.

HPLC system for the assay of 6-MP and 6-TUA

The Beckman System Gold for HPLC (Beckman, Berkeley, CA, U.S.A.) was used for the assay. The system consisted of an ODS (40 μm) guard column (45 × 2.0 mm I.D.), an Ultrasphere ODS (5 μm) analytical column (150 × 4.3 mm I.D.), a solvent Module 126, a Module 166 UV–VIS detector, a Model 506 autosampler, an IBM PS/2 Model 50 computer running on a Gold software Version 3.10 and an Epson FX-86e printer. The effluent was monitored at 340 nm. The sample injection volume was 50 μl and the flow-rate of the mobile phase was 1.0 ml/min. The mobile phase was prepared by mixing 6.8 ml of phosphoric acid (85.9%), 993.2 ml of deionized water and 154.3 mg of dithiothreitol and filtering through a 0.45-μm HA-type filter (Millipore, Bedford, MA, U.S.A.). The final composition of the mobile phase was 0.1 *M* phosphoric acid with 0.001 *M* dithiothreitol. It was used without further degassing. The entire HPLC operation was carried out at room temperature (21–23°C).

Quantification

The minimum quantifiable concentration of 6-MP by the detector was 3 ng/ml for a 50-μl injection volume at a peak-area ratio of sample to blank (0.4 *M* perchloric acid) of 5, and that of 6-TUA was 5 ng/ml at a peak-area ratio of 4. Normal human plasma, spiked with both 6-MP and 6-TUA at concentrations of 0, 12.5, 50, 200, 400 and 800 ng/ml, was assayed in parallel with the sample. Calibration graphs of the peak-area ratio of 6-MP to 6-TG *versus* 6-MP concentration and that of 6-TUA to 6-TG *versus* 6-TUA concentration were constructed automatically by the Gold system. The graphs were linear over the range 0–800 ng/ml for both 6-MP and 6-TUA with y (for 6-MP) = $90.2091x + 3.1073$ and y (for 6-TUA) = $340.0171x - 3.9877$. The coefficient of determination (r^2) was 0.9997 for 6-MP and 0.9997 for 6-TUA.

The percentage recovery was calculated by comparing the peak areas obtained for normal human plasma, spiked with various amounts of 6-TG, 6-MP and 6-TUA, with those observed following injection of pure compounds made up in 0.4 *M* perchloric acid containing 0.066 *M* of sodium hydrosulfite. The standard solutions were 1.613 times more concentrated than the concentrations listed in Table II, taking into account the fact that 250 μl of initial plasma sample resulted in a final volume of 155 μl of extract. The results of the recovery studies are summarized in Table I. A higher recovery for 6-MP could be obtained if the final extraction of the drug from protein with perchloric acid was performed on two successive aliquots of 0.1 ml, but for practical purpose this extra work is not justifiable.

TABLE I

RECOVERY OF 6-MERCAPTOPURINE, 6-THIOURIC ACID AND 6-THIOGUANINE FROM HUMAN PLASMA

Compound	*n*	Amount added (ng/ml)	Mean recovery (%)
6-MP	10	25	61.0
	10	50	60.3
	10	100	63.2
6-TUA	10	50	33.3
	10	200	33.3
	10	800	35.2
6-TG	30	50	51.3

Precision studies were carried out on the same group of samples by calculating the peak-area ratio of 6-MP to that of 6-TG. The relative standard deviations (R.S.D.) for 6-MP at concentrations of 25, 50 and 100 ng/ml were 3.6, 3.7 and 2.5%, respectively. The R.S.D.s for 6-TUA at 50, 200 and 800 ng/ml were 8.5, 5.1 and 5.8%, respectively.

Pharmacokinetic analysis

Standard non-compartmental pharmacokinetic analysis was carried out on the 6-MP plasma concentration–time profile according to published methods [8]. The area under the drug concentration *versus* time curve (AUC) and that under the moment curve (AUMC) were obtained by the trapezoidal method. Other parameters were derived as follows: elimination half-life (h) = $0.693/K_{el}$ (elimination rate constant), clearance ($l/h \cdot m^2$) = dose/AUC and distribution volume (l/m^2) = dose·AUMC/AUC^2.

RESULTS AND DISCUSSION

Thiols form metallic derivatives easily and rapidly in neutral solution [9]. In the sample preparation procedure described, aluminum ion was used to form a complex with 6-MP in the plasma [10]. The complex was then coprecipitated with the protein, as aluminum perchlorate is also an excellent protein precipitant. The supernatant was discarded and the precipitate was extracted with perchloric acid containing sodium hydrosulfite to obtain 6-MP and its analogues. Fig. 2 shows a typical chromatogram of a plasma sample from a leukemic patient who had taken 6-MP orally. Fig. 2A was obtained from a blood sample taken just before the administration of 6-MP. The only peak observed is that of 6-TG (t_R = 4.73 min), which was added to the sample as an internal standard. In a total of 60 blank blood samples taken from ten different patients, no peak was ever observed that might interfere with the detection of 6-TG, 6-MP or 6-TUA. In fact, peaks were virtually never detected in blank samples. Fig. 2B represents the chromatogram from the sample taken 2 h after 6-MP had been administered orally. The peaks for both 6-MP (t_R = 6.08 min) and 6-TUA (t_R = 7.57 min) are clearly visible. The presence of 6-TUA in the plasma is not surprising, as there is xanthine oxidase activity in both intestinal mucosa and liver to catalyze the oxidation of 6-MP to 6-TUA via 6-thioxanthine (6-TX; Fig. 1). 6-TX, which has

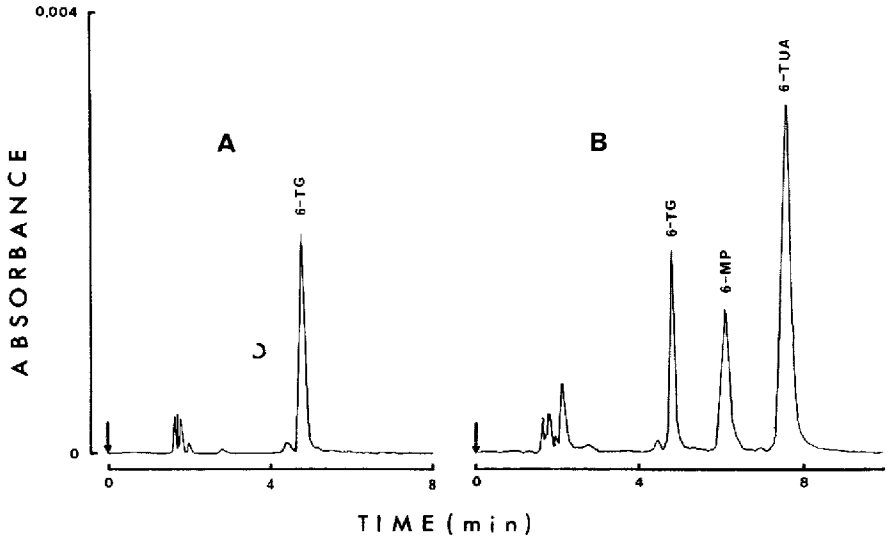


Fig. 2. Representative chromatograms obtained from plasma samples taken (A) immediately before drug administration and (B) 2 h after 6-mercaptopurine (50 mg/m^2) had been administered orally.

a longer retention time in our system (11.63 min), was virtually undetectable or showed up only as a very minor peak. 6-TX is a more effective substrate for xanthine oxidase than 6-MP and will not accumulate in the plasma [7].

Fig. 3 shows representative results of pharmacokinetic studies for 6-MP on a patient. The appearance of 6-MP in the plasma after drug administration is always accompanied by a much higher concentration of 6-TUA. This holds true for all the patients that we studied.

Table II summarizes the pharmacokinetic studies with 6-MP. The elimination half-life observed is compatible with the recently reported values [9]. However, the peak concentrations were far below those reported by others. As many workers did not discuss 6-TUA in their reports, this problem remains to be solved.

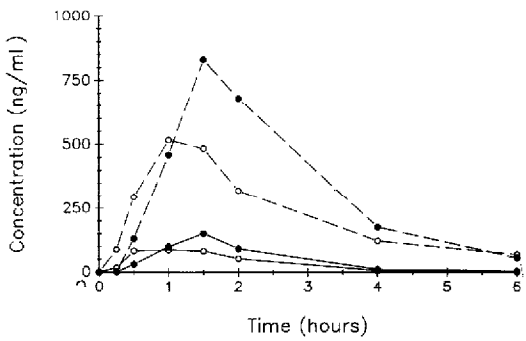


Fig. 3. Typical plasma concentration *versus* time curves for 6-mercaptopurine (solid lines) and 6-thiouric acid (broken lines) in a patient who had received 6-mercaptopurine (50 mg/m^2) orally at (●) 6 a.m. and (○) 6 p.m.

TABLE II

PHARMACOKINETICS OF 6-MERCAPTOPURINE IN EIGHT PATIENTS

The results were obtained from a total of 23 studies involving eight patients.

Parameter	Value[a]
Elimination half-life (h)	1.2 ± 0.1 (27%)
Clearance (l/h · m^2)	341 ± 36 (30%)
Distribution volume (l/m^2)	831 ± 110 (37%)
Peak concentration (ng/ml)	72 ± 9 (36%)
Peak time (h)	1.3 ± 0.1 (20%)

[a] The values are expressed as means ± standard error of the mean with the relative standard deviation in parentheses.

It has long been known that sulfhydryl compounds bind avidly to acid-denatured plasma proteins. The binding of 6-MP and 6-TG to the protein, however, is incomplete and variable. With normal plasma, freshly spiked with 6-MP and 6-TG, we found that only about 10–15% of the added 6-thiopurines bound to protein precipitate, and the binding increased with time of sample storage. As may be expected, it is also greatly affected by the hemolytic state of the blood sample, as 6-MP and 6-TG are known to bind to acid-denatured erythrocyte protein [11]. This direct binding of 6-thiopurines to the protein can be prevented by dithiothreitol and other reducing agents. By using aluminum perchlorate as a protein precipitant, all 6-thiopurines coprecipitate with the protein and thus remain in a single fraction. Although Al^{3+} is known to form only weak complexes with sulfhydryl ligands [10], with the largely excessive concentration used in our method it is safe to assume that only a very small amount of 6-thiopurines can exist in the free sulfhydryl form. It has been reported that 6-MP, 6-TG and their derivatives can undergo considerable distortion to accommodate the metal ion to form a five-membered chelate ring in which metallic ion interacts with the mercapto group at the 6-position on the purine molecule and also accepts the donation of an unshared pair of electrons on the nitrogen at the 7-position [12]. The coordination number of Al^{3+} can be 4, 5 or 6, and it is therefore possible for Al^{3+} to form complexes with 6-thiopurines, and also to bind to the protein precipitate by directing its unoccupied coordination position toward the protein. Chelate compounds are generally more hydrophobic than the parent ligands [13], thus also favoring coprecipitation with protein through hydrophobic interactions. The precipitate is then treated with perchloric acid and sodium hydrosulfite to regenerate free 6-thiopurines, as the chelates are broken down owing to protonation of the sulfur and the nitrogen atoms, and the disulfide bridges, through which 6-thiopurines may bind directly to protein, are reduced to sulfhydryl groups by the reducing agent. Sodium hydrosulfite was used in place of dithiothreitol because the latter absorbs at 340 nm and interferes with 6-TUA detection in our HPLC system.

One of the most widely used techniques for the isolation of 6-MP was described by Maddocks [14], in which phenylmercury(II) acetate was allowed to react with 6-MP in alkalinized plasma to form a complex, 6-phenylmercury(II) mercaptopurine. The complex was isolated from plasma with an organic solvent and the solvent was extracted with dilute hydrochloric acid to release free 6-MP into the aqueous medium.

Whalen *et al.* [6] used this technique for sample preparation prior to HPLC analysis of 6-MP. They noted some drawbacks of their method in that the ethyl acetate used for the extraction of 6-phenylmercury(II) mercaptopurine was carried over to the final acid extract and became an interfering peak in the chromatogram. They had to connect two analytical ODS columns from two different manufacturers in order to separate the three peaks, namely those of 6-TG, 6-MP and ethyl acetate. Further, they also reported that the 6-MP peak heights decreased after several injections owing to the interaction between the binding sites on the column and phenylmercury(II) acetate, which was also carried over from the extraction [6]. In our system, no organic solvent was used, and a single analytical column separated 6-TG, 6-MP and 6-TUA. In addition, no appreciable difference in the chromatogram was observed even after overnight automatic injection of over 60 plasma samples.

The same peak identification table, which allows the Gold systems to identify a compound by its retention time in the column, was used for the entire unattended overnight operation. Our procedure for isolating 6-MP and its metabolites from plasma was far simpler than any methods described so far. We used only one polypropylene Eppendorf centrifugation tube for each sample for the entire extraction process. No transfer of liquid was needed during the process until the last step, when the final extract must be transferred to the automatic sampler for injection.

The mobile phase for our HPLC system was also easy to prepare: it is simply dilute phosphoric acid containing dithiothreitol; no adjustment of pH is required. No undue deterioration of the analytical column has been noted, although the pH of the solution was observed to be 1.5. This low pH was necessary to obtain an optimum separation of 6-TG, 6-MP and 6-TUA. At pH values above 2, 6-MP and 6-TUA were not separated.

We are currently studing the chronopharmacokinetics of 6-MP by this method. Over 300 plasma samples have been analysed and no major technical problem has been encountered thus far.

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