Journal of Chromatography, 230 (1982) 373—380
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1265

QUANTITATION OF 6-MERCAPTOPURINE IN BIOLOGIC FLUIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: A SELECTIVE AND NOVEL PROCEDURE

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(First received November 23rd, 1981; revised manuscript received February 17th, 1982)

SUMMARY

A simple, selective, sensitive and rapid procedure is described for the quantitation of 6-mercaptopurine (6-MP) in biological fluids. A sensitivity of at least 5 ng/ml is easily achieved in plasma on a reversed-phase octadecylsilane (C_{18}) column using a high-performance liquid chromatography system following an initial protein precipitation and a clean-up step. Mean extractability of the drug from plasma following this procedure is greater than 98% and the overall coefficient of variation for the assay is below 6%. Plasma levels of 6-MP were measured in a rhesus monkey for 12 h following an intravenous administration of a single bolus dose (4 mg/kg) of 6-MP.

INTRODUCTION

Immunosuppressive agents are used to control the rejection reaction caused by antigenic differences that remain after tissue typing and donor—recipient matching. The antimetabolite azathioprine (AZA) is one of the most important immunosuppressive agents [1]. AZA is cleaved in the body to 6-mercaptopurine (6-MP) and methylnitroimidazole [2]. It is the biologically active forms of 6-MP that cause inhibition of protein and nucleic acid synthesis [3]. However, a significant amount of attention is being diverted lately to the use of 6-MP as a chemotherapeutic agent in the maintenance therapy of rapidly fatal forms of leukemia characterized by replacement of bone marrow by primitive or blast cells, e.g. acute lymphoblastic leukemia (ALL). Following standard therapy treatments and the resulting prolongation of life, an increasing incidence of leukemic infiltration of the central nervous system (CNS) is observed.

0378-4347/82/0000—0000/$02.75

In order to evaluate the use of 6-MP in meningeal leukemias, an exploratory study was conducted in monkeys to quantitate the partitioning of drug across the blood—brain barrier. Fast disappearance from plasma and slow partitioning of a polar drug, e.g. 6-MP, would tend to make its concentration in cerebrospinal fluid (CSF) and plasma very low. In order to define the disposition profile of 6-MP in biologic fluids a selective, sensitive and rapid assay procedure was desirable.

A review of the published literature revealed several procedures for quantitating 6-MP in serum, plasma and/or urine. Bailey et al. [4] quantitated 6-MP by gas chromatography of samples derivatized with tetramethylammonium hydroxide (TMAH) reagent while Finkel [5] measured fluorescence of purine-6-sulphonate, an oxidized product of 6-MP. Rosenfeld et al. [6] employed gas chromatography—mass spectrometry for determination of 6-MP following its derivatization. None of these methods had the desired sensitivity or any significant reproducibility data. Some procedures had analytic recoveries as low as 18% [6].

A procedure reported by Maddocks [7] in 1979 for simultaneous determination of 6-MP and AZA involved a lengthy extraction following the derivatization with phenylmercuric nitrate and subsequent measurement of fluorescence. Lin et al. [8] recently reported a procedure for the quantitation of 6-MP in plasma following AZA administration but gave no data on the specificity and sensitivity of the assay.

Ding and Benet [9] recently reported a high-performance liquid chromatographic (HPLC) procedure for quantitating both 6-MP and AZA with an extraction recovery of approximately 12% for 6 MP. Attempts to reproduce or achieve the desired and/or reported sensitivity proved unsuccessful using their methodology. Most of the HPLC analyses of thiopurines have been from tissue extracts [10, 11]. An ion-pair reagent heptanesulfonic acid was successfully employed by Day et al. [12] to determine 6-MP in plasma by reversed-phase ion-pair chromatography. The assay was not sufficiently sensitive to quantitate 6-MP levels in CSF following its intravenous administration or vice versa.

This paper presents a rapid, selective and sensitive method for quantitative determination of 6-MP in biological fluids by HPLC.

EXPERIMENTAL

Materials

6-MP was obtained from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.). 6-Thioguanine (TG) was procured from the National Cancer Institute (National Institutes of Health, Bethesda, MD, U.S.A.) and 6-thiouric acid (TU) was generously supplied by Dr. Elion of Burroughs Wellcome. Dithiothreitol (DTT, Cleland's Reagent) was obtained from Eastman-Kodak (Rochester, NY, U.S.A.) and stored at refrigeration temperature. Glacial acetic acid was obtained from Fisher Scientific (Silver Spring, MD, U.S.A.). Spectral grade dichloromethane (J.T. Baker, Phillipsburg, NJ, U.S.A.) was employed in the extraction. Both methanol and acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) used in the assay were HPLC grade. Water used was double distilled in a glass still. All other chemicals were reagent grade.

All HPLC solvents were passed through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) prior to use and degassed. Stock solutions of 6-MP, TG, TU were made in water and kept protected from light by aluminum foil. All solutions were stored at 4°C and showed insignificant degradation over a period of two months.

Methods

Plasma (1 ml) was placed in an 8-ml screw cap disposable glass vial (Fisher Scientific). To this were added 80 μl of a 10 μg/ml solution of TG as an internal standard and 10 μl of DTT (1 *M*). The glass vial was then vortexed for 10 sec and 2 ml of acetonitrile were added using a Repipet® dispenser (Lab-industries, Berkeley, CA, U.S.A.). Each vial was then vortexed for 30 sec and centrifuged for 5 min at 2000 *g*. The supernate was then decanted into another 8-ml disposable screw cap glass vial. Using a Repipet, 2 ml of dichloromethane were then added to each vial. The vials were then shaken for 5 min on an automatic reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) and centrifuged for 5 min at 2000 *g*. From the top aqueous layer, 750 μl were removed into a disposable 12 × 75 mm borosilicate culture tube and evaporated under a gentle stream of nitrogen at 37°C. The residue was reconstituted with 150 μl of distilled water and vortexed for 1 min. A 15-μl aliquot of the sample was injected into the HPLC system.

The chromatographic analysis of the samples was performed using a Spectra-Physics liquid chromatograph Model 3500B (Spectra-Physics, Santa Clara, CA, U.S.A.) equipped with a Schoeffel variable-wavelength UV Spectroflow monitor Model SF 770 (Schoeffel Instrument, Westwood, NJ, U.S.A.) for detection at 322 nm. The analysis was performed on a 25 cm × 4.6 mm I.D. Altex Ultrasphere Octadecylsilane (ODS) analytical column with a 5-μm particle size (Beckman Instruments, Berkeley, CA, U.S.A.). Preceding the analytical column was a 7 cm × 2.2 mm I.D. guard column packed with Co:Pell ODS (30–38 μm) particles (Whatman, Clifton, NJ, U.S.A.). A mobile phase of acetonitrile—acetic acid—water (3.5:0.2:96.3) at a flow-rate of 1.4 ml/min was used as the eluent. The back pressure ranged from 152 to 186 bars. The eluent was degassed prior to the HPLC run. Retention times for TU (chief metabolite of 6-MP), 6-MP, internal standard (TG), and DTT under these conditions were 3.8, 4.8, 6.5, and 13.8 min, respectively. A fresh standard curve for 6-MP in plasma was obtained with each batch of samples. Standard curves for 6-MP were almost identical when prepared in plasma or water. The concentration of 6-MP was estimated by means of the ratio of its peak height to the peak height of the internal standard as compared with a simultaneously run standard curve. Analytic recovery of 6-MP was determined by comparison of direct injection of standard aqueous solution with the injection of the same standards run through the assay procedure. No interfering peaks were observed in the chromatograms for the monkey plasma samples.

RESULTS AND DISCUSSION

Typical HPLC tracings of plasma samples with or without drug are shown in Fig. 1. Sharp symmetrical peaks are shown for TU, 6-MP, TG, and DTT. As is

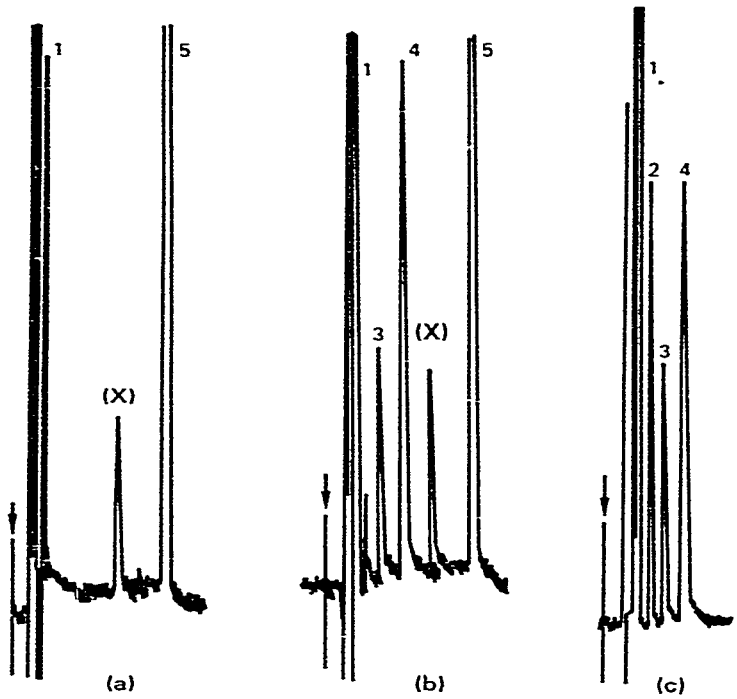

Fig. 1. Representative HPLC tracings of monkey plasma samples run through the assay procedure. Peaks: (retention times in the parentheses), (a) Blank plasma; (b) 8-h sample with 6-MP; (c) an aqueous mixture of TU, 6-MP and TG. 1, solvent front; 2, TU (3.8 min); 3, 6-MP (4.8 min); 4, TG (6.5 min); 5, DTT (13.8 min); X, peak from plasma.

often found with reversed-phase liquid chromatography, the compounds elute in order of decreasing polarity; DTT being the least polar, elutes last; whereas, the chief metabolite (TU) formed following oxidation of 6-MP by the enzyme xanthine oxidase, elutes first.

Fig. 2 shows a typical mean standard curve (n = 8) in plasma over a concentration range of 10—100 ng/ml parent drug (6-MP) over a 2-month period, where peak height ratio is plotted as a function of 6-MP concentration. Linearity in standard curves of 6-MP was established over an extended range of up to 10 μg/ml in human plasma. No attempt was made to quantitate the metabolite levels in plasma. However, as can be easily seen from Fig. 1c a clean separation was obtained between the parent compound 6-MP and its most predominant metabolite (TU) peak. Other polar metabolites of drug probably elute with the solvent front. Further studies are nor in progress to evaluate and describe the disposition of 6-MP in a subhuman primate following intravenous dosing.

The sensitivity of the assay for a 15-μl injection is at least 5 ng/ml for 6-MP. This was determined by observing the lowest concentration that maintained an arbitrary signal-to-noise ratio of 3. The assay sensitivity can be increased at least 2—8-fold by reducing the reconstitution volume to 100 μl and injecting up to 90 μl on the column. Table I shows the reproducibility of the assay at five concentrations. Each plotted value represents the mean of eight determinations

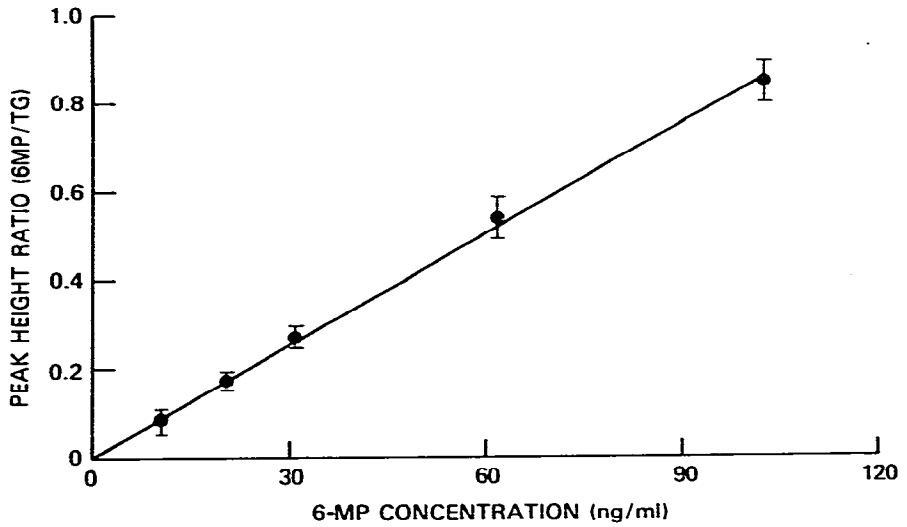

Fig. 2. Mean standard curve for 6-mercaptopurine in plasma over a 2-month period. Each point is a mean (± S.D.) of eight determinations.

TABLE I

ASSAY REPRODUCIBILITY OF EIGHT REPLICATES OVER A TWO-MONTH PERIOD FROM PLASMA

Actual concentration of standards (ng/ml)	Concentration of 6-MP		
	Mean	S.D.	C.V. (%)
10.4*	10.2	1.030	10.10
20.8	20.1	0.960	4.78
30.1	31.0	1.376	4.43
62.0	64.4	3.022	4.69
103.0	101.8	1.645	1.62
Mean			5.12

*n = 7 in this case.

over a 2-month period. Assay efficiency or recovery data are shown in Table II. The percent recovery of 6-MP was essentially complete and there was no statistically significant difference ($p>0.1$) in the recovery as a function of concentration.

Table I clearly shows that the mean coefficient of variation over the entire range is under 6%. One probable reason for the high variation observed in the low-concentration standard (Table I) could be that it was exposed to light at room temperature once for 1—2 h. Other evaluations of the variation associated with plasma standards containing 6-MP from 2 to 10 ng/ml, determined by injecting larger volumes, have shown the coefficients of variation to be about 6% for the 10 ng/ml and about 10% for 2 ng/ml sample.

The interday variability coefficients evaluated by comparing the peak height ratio of 6-MP to internal standard over a period of one week for two standard

TABLE II

ANALYTICAL RECOVERY DATA ON STANDARD SOLUTIONS OF 6-MP ASSAYED THREE TIMES

Concentration of standard (ng/ml)	Recovery (%)	
	Mean	S.D.
46.2	95.5	2.98
97.9	100.9	2.53
146.1	102.1	2.57
199.8	109.5	3.45
252.8	102.2	3.10
Mean*	102.0	
S.D.	4.99	

*n = 15.

plasma solutions, with concentrations representing each end of the standard curve, were 6.7% (n = 9) for 9.27 ng/ml and 4.0% (n = 12) for 97.9 ng/ml standard. Intraday coefficient of variation evaluated for one plasma standard (46.2 ng/ml) was less than 1.0% (n = 6).

In a study published earlier, Ding and Benet [9] reported no data on the reproducibility of their assay. With an overall extraction recovery of 12%, the coefficient of variation associated with the quantitation of 6-MP in biological fluids could be significantly high. However, as the data in Table I and Table II show, the assay procedure described here is sensitive and reproducible, the only step requiring some significant amount of time being the evaporation of the aqueous phase. Time required for the evaporation can be further shortened by using an Evapo-Mix (Buchler Instruments, Fort Lee, NJ, U.S.A.) if so desired.

The assay reported here has been successfully employed for the quantitation of 6-MP in plasma following an intravenous administration of a single bolus dose (4 mg/kg) to a monkey. Fig. 3 shows the plasma decay of 6-MP in a monkey which appears to follow a multicompartment open body model. The terminal half-life of the log-linear phase computed by linear regression analysis appears to be approximately 1.9 h.

HPLC analyses of the plasma samples revealed no interfering peaks in the chromatograms. Insignificant amount of metabolite (TU) was detected in the analysis of a few plasma samples. Thiouric acid, being even more polar than the parent compound, is eliminated even faster than 6-MP. Some other metabolites of 6-MP which are even more water soluble and are present in insignificant amounts in plasma probably elute with the solvent front and no attempt was made to separate them. Several drugs that might be administered concurrently with 6-MP were chromatographed to check for possible interference in the assay procedure. No interference was observed with methotrexate, AZA, caffeine, 5-fluorouracil, prednisone, theophylline, vinblastine and vincristine, and only cytarabine (ARA-C) showed a possible interference with the internal standard peak. However, the peak was rather small and broad at therapeutic drug concentration.

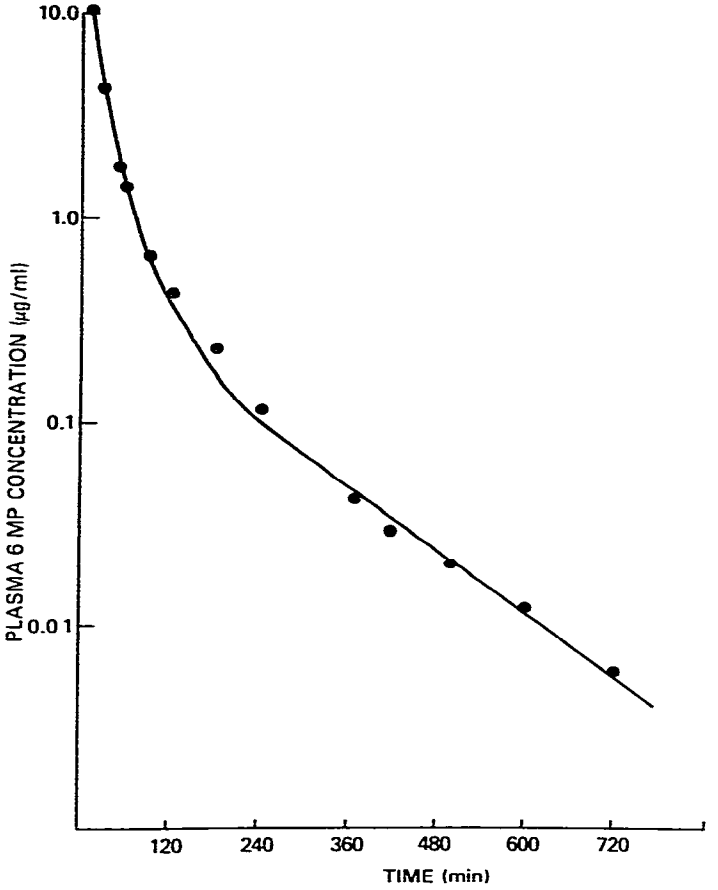

Fig. 3. Plasma concentrations of 6-MP observed in monkey 0-268 over a 12-h period following a single intravenous bolus dose (4 mg/kg).

As has been suggested in earlier reports [4, 9], it was deemed necessary to add sulfhydryl protecting reagent 1,4-dithiothreitol (DTT, Cleland's Reagent) immediately to all plasma standards, aqueous standards and monkey plasma samples to stabilize unsubstituted thiols. Ten microliters of DTT (1 *M* solution) were added per 1 ml of the sample resulting in a final concentration of 10 m*M* of DTT. Exclusion of DTT resulted in a lower peak height ratio (6-MP/TG) due to decomposition of drug during the extraction procedure. Bailey et al. [4] have also observed and reported such drug decomposition of 6-MP in serum.

We did not make any attempt to extract the drug into an organic phase, as earlier reports [6, 9] have shown the poor extractability of 6-MP. Excellent recovery and extractability can therefore be achieved by keeping the drug in the aqueous phase. Initial precipitation of plasma sample with acetonitrile removes most of the possible interferences from proteins and subsequent addition of dichloromethane selectively removes acetonitrile from the above solution leaving the drug in the desired aqueous phase.

This procedure basically takes advantage of the negligible solubility of 6-MP

in organic solvents, its small size and can therefore be suitably applied to other water soluble, low molecular weight compounds. It was also noted that longer column equilibration was required for TG when starting up the HPLC system. In order to avoid such delays, the mobile phase can be recirculated overnight through the system.

The assay reported here has been successfully employed for the quantitation of 6-MP in plasma following an intravenous administration of a single bolus dose (4 mg/kg) to a monkey. Fig. 3 shows the plasma decay of 6-MP in a monkey which appears to follow a multicompartment open body model. The terminal half-life of the log-linear phase computed by linear regression analysis appears to be approximately 1.9 h.

Further studies to elucidate the pharmacokinetics of 6-MP using this simple, precise and rapid assay procedure are presently underway in subhuman primates in our laboratories. Because of its simplicity and precision the assay lends itself to therapeutic drug monitoring as well. The assay has been successfully employed in quantitating drug levels in certain leukemic patients, following oral and/or intravenous 6-MP administration.

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