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DETERMINATION OF 6-MERCAPTOPURINE AND AZATHIOPRINE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Using 1-ml plasma samples, levels of 6-mercaptopurine (6MP) as low as 5 ng/ml and azathioprine (AZA) as low as 40 ng/ml can be detected using a high-performance liquid chromatography reversed-phase column procedure following extraction. Both compounds were stable in frozen plasma for seven weeks. AZA stability in blood was temperature dependent; the half-lives of AZA breakdown to 6MP at 37° were 28 and 46 min in blood drawn from two rhesus monkeys. Plasma levels of 6MP were measured in a rhesus monkey following 6MP (1.47 mg/kg) and AZA (3 mg/kg) intravenous administration. 6MP levels were also measured in three renal transplant patients on daily 50- and 100-mg AZA doses. Peak levels (45—75 ng/ml) were reached within an hour and 6MP levels were detected for up to 7 h.

INTRODUCTION

The immunosuppressive agent azathioprine (AZA), 6-(1-methyl-4-nitro-5-imidazolyl) thiopurine, is primarily used as an adjunct for preventing rejection of organ transplantation. It is metabolized in vivo to 6-mercaptopurine (6MP), which is anabolized to the biologically active thioinosinic acid, methylthioinosinic acid and thioguanylic acid [1].

In our attempt to determine the bioavailability and pharmacokinetics of AZA, we endeavored to measure plasma levels of the parent drug and 6MP. A limited number of analytical methods have been developed to measure levels of AZA or 6MP in plasma and urine. Methods employing radioisotopes such as [^{35}S]AZA [2—4] or [^{14}C]AZA [5] with the label in the imidazole moiety, give non-specific information on the absorption, distribution and elimination characteristics of the drug. The fluorescence method of Finkel [6] for measuring serum levels of 6MP was not sensitive enough to measure the low levels found in animals or man after AZA or 6MP administration. The spectro-

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photometric method by Chalmers [7] for measuring urinary AZA and 6MP is also not sufficiently sensitive nor specific for our purpose. Other methods of determining 6MP include gas chromatography (GC) [8] and mass spectrometry (MS) [9]; both methods involve derivatization of 6MP prior to analysis. The gas—liquid chromatographic (GLC) method [8] is not sensitive enough to measure 6MP serum levels following the usual oral doses of AZA given to patients. The GC—MS method [9] reports a limit of detection of 20 ng/ml and yields 18% recovery for the combined extraction, derivatization, drying and measurement procedures.

Most high-performance liquid chromatographic (HPLC) analyses of thiopurines have been limited to determination in tissue extracts [10—14]. DeMiranda et al [15] however, did separate AZA and its metabolites in rat plasma and urine using LC techniques. Day et al. [16] used paired ion HPLC to determine 6MP in plasma. A sensitivity of 0.2 μg of 6MP per ml is reported. In the present work, a sensitive and specific assay for 6MP and AZA is described using HPLC. Both 6MP and AZA can be assayed from a one-ml plasma sample.

EXPERIMENTAL

Materials

6MP monohydrate, AZA and 8-OHMP hemihydrate were generously supplied by Burroughs Wellcome (Research Triangle Park, N.C., U.S.A.). 9-Methylmercaptopurine (9MMP) and 6-methylmercaptopurine (6MMP) were purchased from Heterocyclic Chemical Co. (Harrisonville, Mo., U.S.A.). Dithioerythritol (DTE) was obtained from Sigma (St. Louis, Mo., U.S.A.) and stored at 4°. HPLC grade ethyl acetate was from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Acetonitrile was of nanograde purity (Mallinckrodt, St. Louis, Mo., U.S.A.). Stock solutions of 6MP, 9MMP, 6MMP, and AZA were dissolved in methanol and stored at 4°.

Procedure

One ml of plasma was placed in a 15 mm × 150 mm test tube; 120 ng 9MMP and 500 ng 6MMP were added as internal standards for 6MP and AZA, respectively; 200 μl of 2 *N* hydrochloric acid and 5 ml ethyl acetate were added. The tube was capped and shaken for 10 min using a tube rotator (BBL, Cockeysville, Md., U.S.A.). The tube was centrifuged for 10 min and the organic layer was transferred to another test tube for analysis of AZA. The extraction was repeated with another 5 ml of ethyl acetate. The organic portions were combined together in one tube and 1 ml of 1 *M* sodium acetate buffer, pH 5.1 was added. The sample was shaken for 10 min and centrifuged for 10 min. The ethyl acetate layer was transferred to a nipple tube and evaporated to dryness under nitrogen. The residue was reconstituted with 50 μl of acetonitrile, vortexed for 1 min and centrifuged for 5 min; 15 μl of the sample were injected into the HPLC apparatus for AZA analysis using a μBondapak C_{18} column.

To the plasma left after the two ethyl acetate extractions, 10 μl of a 1% solution of DTE in distilled water were added; 1 ml of 1 *M* sodium acetate

buffer, pH 5.1 and 10 ml ethyl acetate were added. The sample was shaken and centrifuged, each process for 10 min. The organic layer was transferred to a nipple tube and evaporated to dryness under nitrogen. The sample was reconstituted with 50 μl of HPLC buffer used for 6MP analysis (see below), 50 μl of 0.2 *N* sulfuric acid and 100 μl of ethyl acetate. The tube was vortexed for 1 min, centrifuged for 5 min and about 90 μl of the aqueous phase was injected into the HPLC apparatus, using a LiChrosorb column for analysis.

HPLC analysis was performed using a Perkin-Elmer liquid chromatograph Series 2, equipped with a LC-55 Perkin-Elmer spectrophotometer for detection at 325 nm for 6MP and 280 nm for AZA. 6MP was assayed with a LiChrosorb RP-18 column, 10 μm particle size, 25 cm × 4.6 mm I.D. (E. Merck, Darmstadt, G.F.R.) and a Spectrum 921 filter. The eluent is comprised of 1% methanol, 0.5% acetonitrile and 60 mg DTE per liter 0.005 *M* potassium phosphate buffer at pH 4.0; flow-rate 2 ml/min. AZA was analyzed with a μBondapak C_{18} column, 10 μm particle size, 30 cm × 3.9 mm I.D. (Waters Assoc., Milford, Mass., U.S.A.); the eluent is 11% acetonitrile in 0.01 *M* sodium acetate buffer pH 4.0: flow-rate 2 ml/min.

Stability study of 6MP and AZA in plasma

Plasma samples spiked with 45 ng 6MP per ml were frozen and assayed at 0, 2, 8, and 52 days. A similar stability study was carried out for AZA spiked at 0.41 μg/ml plasma.

Stability of AZA in blood

The stability of AZA in blood drawn from two rhesus monkeys was determined at 37°, room temperature and in ice. Blood was spiked with AZA and incubated at the respective temperatures. Aliquots of blood were removed at 0, 30, 60, 120, 180 min and immediately centrifuged. An additional aliquot was removed at 15 min for blood incubated at 37°. The plasma was then assayed for AZA.

Animal studies

Intravenous preparations of AZA at 3 mg/kg and 6MP at 1.47 mg/kg were administered to a rhesus monkey in separate studies. The solution of AZA was prepared by injecting 10 ml water into a 100-mg vial of the drug (pH of final solution = 9.7). The solution of 6MP was prepared by dissolving the powder in sodium hydroxide solution and diluting with saline (pH of final solution = 10). Blood was sampled at 0, 5, 10, 15, 20, 30, 40, 60, 90, 120, 180, 240 min and kept in ice before it was centrifuged. The plasma was frozen and assayed for 6 MP and AZA.

Patient studies

Informed consent was obtained from three renal transplant patients, hospitalized at the Moffitt Hospital, University of California, San Francisco. Two of these patients were receiving daily oral AZA doses of 50 mg while the third was receiving 100-mg daily doses. Eight-ml blood samples were taken 10 times over a 12-h period. The blood samples were immediately placed in

an ice bath until the plasma was separated by centrifugation. Plasma samples were split for analysis of oral steroids in another study and for the AZA and 6MP measurements described here.

RESULTS AND DISCUSSION

It was found necessary to add DTE during the final extraction step and in the HPLC solvent system for 6MP analysis. Following these additions, improved peak heights of 6MP and 9MMP were observed at 325 nm. Consistent peak heights for duplicate injections of the same amount of thiopurine were observed when DTE was used. This suggests that DTE may have a stabilizing effect on the unsubstituted thiols. Bailey et al. [8] have also used DTE as a sulfhydryl-protecting reagent in their extraction procedures for GC analysis of 6MP.

The addition of DTE, however, would convert AZA to 6MP if the unchanged drug was present in the plasma sample. For this reason AZA was separated from 6MP in the initial extraction steps. AZA was then analyzed separately from 6MP. If the amount of AZA in the plasma was less than 100 ng/ml, very little of the drug would be left in the plasma following extraction to be converted to 6MP upon adding DTE.

Sample HPLC chromatograms are depicted in Fig. 1 for 6MP and 8-hydroxymercaptopurine (8-OHMP) (left portion of Fig. 1) and AZA (right portion of Fig. 1). Blank plasma samples are designated as I while chromatograms for plasma with added drug and internal standard are designated II.

Thiouric acid, the chief metabolite formed following oxidation of 6MP by the enzyme xanthine oxidase is very poorly extracted from plasma. It

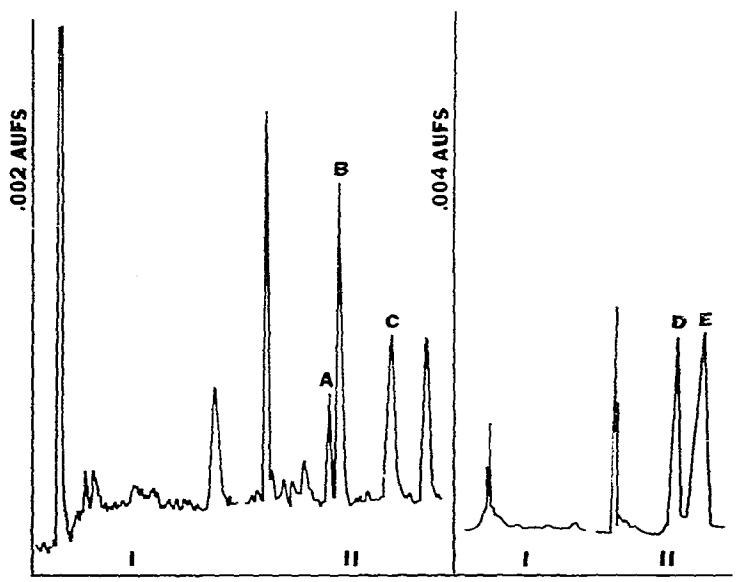

Fig. 1. HPLC chromatograms of plasma samples without (I) and with (II) added drug and internal standard. Numbers in parentheses indicate retention times in min. A = 8-OHMP (5 min); B = 6MP (5½ min); C = 9MMP (8 min); D = 6MMP (6 min); E = AZA (8 min).

elutes at an earlier retention time than 6MP and 8-OHMP after passing through the column.

Over the 6MP concentration range of 10—100 ng/ml, standard curves of peak height ratio (6MP/9MMP) versus 6MP concentration were constructed (slope = 0.0138, intercept = 0.0509, r^2 = 0.9970). The limit of sensitivity for accurate measurement of 6MP is 5 ng/ml. Standard curves for AZA were constructed over a 0.05—0.80 μg/ml plasma concentration range using peak height ratios of AZA to 6MMP (slope = 1.2156, intercept = —0.0094, r^2 = 0.9976). 6MMP was used as the internal standard since it was not detected in the plasma of monkeys dosed with AZA. AZA was recovered to the extent of 68% from spiked plasma samples. The overall recovery of 6MP, however, was considerably less (12%) approximating the recovery previously reported for the CG—MS method [9]. The major loss of drug occurs in the initial extraction steps.

The stability study of 6MP and AZA in plasma showed that samples could be kept frozen for at least 7 weeks without decomposition. As indicated in Table I, an average value of 44 ± 4 ng/ml was measured for samples spiked with 45 ng/ml of 6MP; an average of 0.39 ± 0.02 μg/ml was obtained for AZA samples spiked at 0.41 μg/ml.

Chalmers et al. [17] have shown that AZA is rapidly converted to 6MP at pH 7.35 and 37° in the presence of 1 m*M* glutathione. We attempted to determine whether the breakdown by glutathione in whole blood was significant after the blood was drawn from the animal and before it was spun down. The stability study in blood (see Fig. 2) showed that blood samples kept in ice were relatively more stable compared to those left at room temperature, and even more so than those incubated at 37°. The half-lives of AZA decline at 37° were 28 min and 46 min for blood drawn from two monkeys. This compares well with the half-life of 47 min determined for the conversion of AZA to 6MP by the addition of glutathione [17].

Plasma levels of 6MP following a 1.47 mg/kg dose of 6MP to a 6.8-kg rhesus monkey are depicted in the upper curve in Fig. 3. The concentration time curve appears to follow a 2-compartment body model. This same monkey also received a 3 mg/kg intravenous dose of AZA (equivalent to 1.65 mg/kg 6MP if AZA is quantitatively converted to 6MP). AZA concentrations fell

TABLE I

STABILITY STUDY OF 6MP AND AZA IN PLASMA

Day	Concentration	
	6MP (ng/ml)	AZA (μg/ml)
0	44	0.37
	47	
2	41	0.40
	41	0.41
8	50	0.35
	46	0.41
52	44	0.38
	39	0.39
Mean ± S.D.	44 ± 4	0.39 ± 0.02

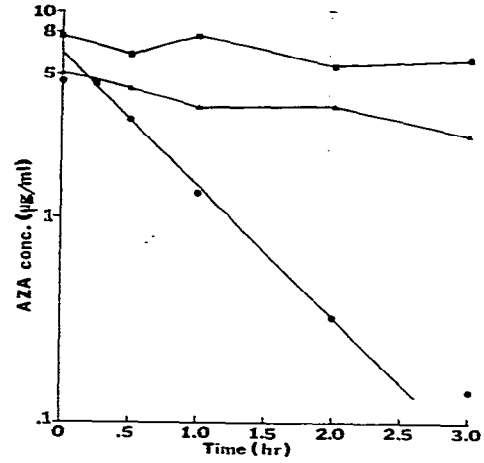

Fig. 2. Stability study of AZA in blood from a rhesus monkey, □, In ice; 7, at room temperature; •, at 37°.

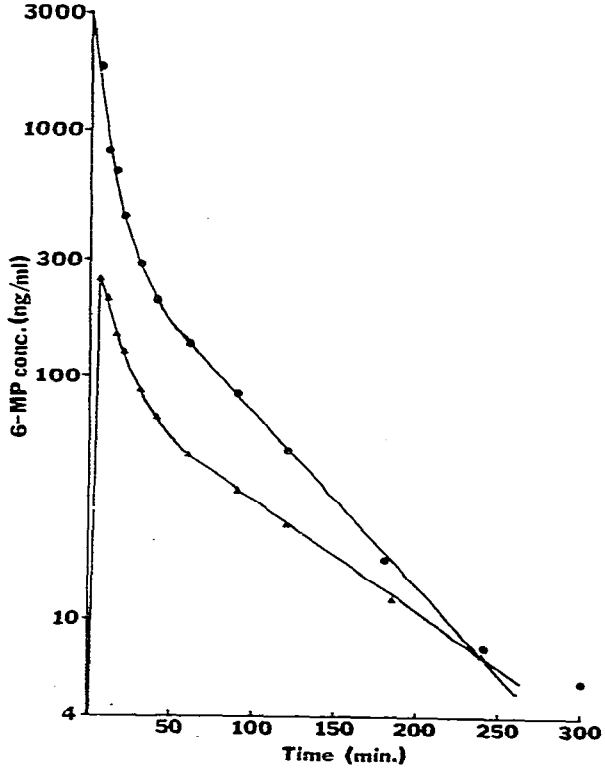

Fig. 3. Plasma levels of 6MP in a 6.8-kg rhesus monkey after an intravenous bolus dose of 6MP (●) at 1.47 mg/kg and AZA (▲) at 3 mg/kg.

rapidly to levels below assay sensitivity. However, 6MP concentrations following this AZA dose are depicted in the bottom curve of Fig. 3. Note that peak 6MP levels are achieved at the first 5 min sampling point after AZA dosing. Plasma levels of 8-OHMP were found following a 9 mg/kg oral dose of AZA to one rhesus monkey. 8-OHMP is derived from the oxidation of AZA to 8-OHAZA in the animal by the enzyme aldehyde oxidase [18]. 8-OHAZA is subsequently cleaved to 8-OHMP and the imidazole moiety.

Randomized blood samples were collected from three renal transplant patients on daily oral doses of AZA. Plasma was analyzed for 6MP as shown in the semilogarithmic plot of the data in Fig. 4. Absorption of the drug appears to be rapid and levels of 6MP can be detected for up to 7 h. Further studies to define the kinetics of AZA and 6MP in rhesus monkeys and renal transplant patients are presently ongoing in our laboratory using the assay procedures described in this paper.

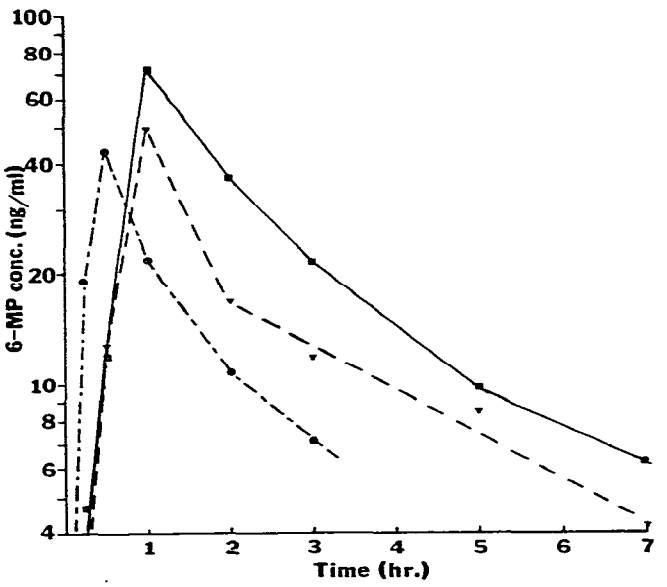

Fig. 4. Plasma levels of 6MP in 3 renal transplant patients, each receiving an oral dose of AZA daily. Key: ●, D.H., 74.3 kg, 100 mg AZA; ▲, A.R., 65.3 kg, 50 mg AZA; ■, A.V., 54.4 kg, 50 mg AZA.

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