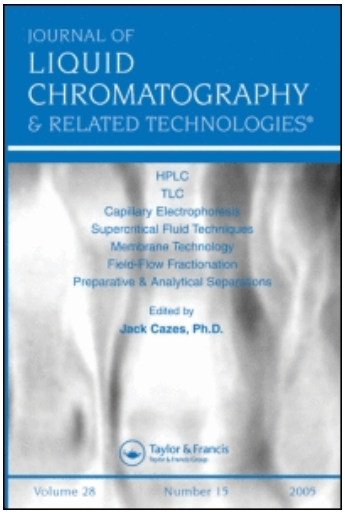

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Analysis of Azathioprine and 6-Mercaptopurine in Plasma in Renal Transplant Recipients After Administration with Oral Azathioprine

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ANALYSIS OF AZATHIOPRINE AND 6-MERCAPTOPURINE IN PLASMA IN RENAL TRANSPLANT RECIPIENTS AFTER ADMINISTRATION WITH ORAL AZATHIOPRINE

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ABSTRACT

Studies on the pharmacokinetics of azathioprine (AZA) and its main metabolite, 6-mercaptopurine (6-MP) in patients treated with oral AZA, such as renal transplant recipients, require an analytical method with a high sensitivity. Since the substances differ substantially in their physicochemical properties, it is hardly feasible to develop an extraction procedure and select chromatographic conditions for the simultaneous determination of both substances. Therefore, we have developed two specific chromatographic assays for determination of AZA and 6-MP in plasma. A solid-phase extraction (C_8 Isolute) procedure was used for AZA with guaneran as internal standard (IS), while 6-MP was purified on mercurial cellulose and 6-mercaptopurine arabinoside was used as IS. Chromatographic analyses were achieved using C_8 and C_{18} reversed phase columns for AZA and 6-MP, respectively. The methods were reproducible with intra- and inter-assay coefficients of variations below 6 %. The average recoveries of AZA, 6-MP and their respective IS were higher than 79%. The limit of quantitation of AZA and 6-MP in plasma is 0.2 ng.

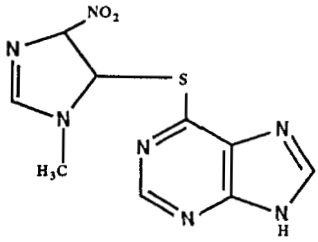
INTRODUCTION

Azathioprine (AZA), a pro-drug of 6-mercaptopurine (6-MP) (Fig. 1), has been widely used either alone or in combination with other immunosuppressants such as prednisolone and cyclosporin, to prevent the rejection of transplanted organs (1, 2). It has also been used in the treatment of autoimmune and inflammatory diseases (3-5). There is a marked intra- and inter-patient variability in the pharmacokinetics of AZA and its main metabolite, 6-MP (6-10). In clinical use, AZA is generally administered on a mg per kg body weight basis and dose adjustments are based on the occurrence of myelosuppression with leukopenia (11).

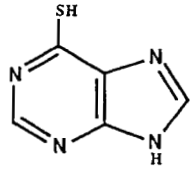
AZA is rapidly metabolized *in vivo* to 6-MP, the immediate metabolite; 6-thiouric acid (6-TU), the final end-product; and 6-thioguanine nucleotides (TGN), the active moiety intracellularly (12-14). Determination of AZA and 6-MP plasma concentrations have been suggested for therapeutic drug monitoring (TDM) (9, 15). Little is known about the relationship between the pharmacokinetics of the AZA and 6-MP and clinical effects, due to lack of assay suitable for large TDM studies.

Several methods for the quantitative determination of AZA and 6-MP in biological fluids have been proposed. These include liquid chromatography (LC) with UV (15-20) or fluorescence detection (21, 22) and gas chromatography-mass spectrometry (23). Some of these methods do not use an internal standard (IS) (18) or use 6-thioguanine as IS (15, 17, 21), which has been detected in plasma, urine (24) and erythrocytes (25) as a minor metabolite and it is therefore less suitable as an IS.

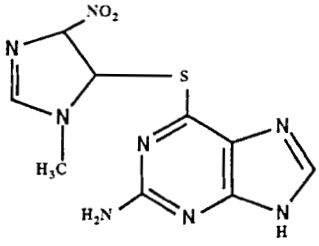
The extraction procedure, the chromatographic condition, and choice of IS in this study were modified from previous methods in order to obtain a better specificity and sensitivity with low intra- and inter-assay variability. With this optimized method in our hands we now have the opportunity to once more address the issues of correlation between AZA and 6-MP pharmacokinetics and clinical outcome.



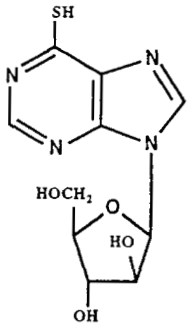
Azathioprine
Mw 277.29



6-Mercaptopurine
Mw 152.19



Guaneran
Mw 302.18



6-Mercaptopurine arabinoside
Mw 284.30

1. Structural formulae of AZA, 6-MP and the used IS.

EXPERIMENTAL

Chemicals

Guaneran (6-[(1-Methyl 4-nitro-5-imidazolyl)thio)-2-aminopurine], was a generous gift from Dr. Gertrude Elion (Wellcome Foundation, Research Triangle Park, N.C.). Azathioprine, 6-mercaptopurine and 6-mercaptopurine arabinoside were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Mercurial cellulose was prepared according to described procedure (26). Methanol and acetonitrile were HPLC grade (JT Baker, Deventer, Holland). Sodium dihydrogen

phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$ p.a.) was from Merck (Merck, Darmstadt, Germany).

Equipments

Reversed phase LC was performed at room temperature using a CM4000 multiple solvent delivery system pump (Milton Roy Co., Rochester, NY, USA), a CMA-240 autosampler (Carnegie Medicine, Inc., Stockholm, Sweden) and a variable wavelength UV detector (Milton Roy Co., Rochester, NY, USA). For the solid-phase extraction, a VacMaster (International Sorbent Technology, Mid-Glamorgan, UK) was used.

Determination of plasma AZA

Solid-phase extraction cartridges C8 (Isolute 100 mg, part Nr 290-0010-A, International Sorbent Technology, Mid-Glamorgan, UK) were conditioned before use with 2.5 ml of methanol and 3.5 ml of 10 mM phosphate buffer pH 7.0. Fifty µl of guaneran solution (1.5 µg/ml) was added as IS to 1 ml plasma and mixed with 2 ml 10 mM phosphate buffer pH 7.0. The mixture was applied on the cartridge with an approximate flow rate of 2.5 ml/min. The cartridge was then washed sequentially with 3 ml of 1% acetonitrile in phosphate buffer pH 7.0. The cartridge was then dried by aspirating air for 2 minutes. The compound was eluted with 2 ml of 5% methanol in ethyl acetate. The eluate was collected in a glass test-tube and evaporated to dryness under a stream of nitrogen in a water bath (40°C). The residue was finally dissolved in 50 µl of mobile phase and 20 µl was injected directly on an HSpecosphere 3CR C8 column, (80 x 4.6 mm, 3 µm, Perkin-Elmer, Norwalk, CT, USA). The mobile phase consisted of 9 % acetonitrile in 10 mM sodium phosphate buffer, pH 6.2 at a flow rate of 1.2 ml/min. AZA and guaneran were detected at 280 nm.

Determination of plasma 6-MP

To 1 ml plasma, 50 µl of 6-mercaptopurine arabinoside (IS) (2.8 µg/ml) solution and 2-3 mg mercurial cellulose were added. The mixture was vortexed for 30 seconds and centrifuged (550 g, 5 min.). The pellet

was resuspended in 2 ml phosphate buffered saline (PBS) and centrifuged. This was repeated twice. Finally, the pellet was resuspended in 250 µl of freshly prepared 20 mM 2-mercaptoethanol, centrifuged and 100 µl injected directly on an ODS column (250 x 4.6 mm, 5 µm, Beckman, Fullerton, CA, USA). The mobile phase consisted of 10 mM sodium phosphate buffer with 2% acetonitrile, pH 3.0 at flow rate of 1.2 ml/min. 6-MP was detected at 323 nm.

Assay validation

The intra- and inter-assay variability of AZA and 6-MP assays were examined at two concentration levels and in a pooled plasma from patients treated with AZA. Standard samples were prepared by spiking blank plasma with known amounts of AZA and 6-MP and used for calibration curves. The capacity factor of each compound was calculated as $(t_r-t_0)/t_0$, where t_r is the retention time of the compound and t_0 is the retention time of the first distortion of baseline.

Assay specificity

As AZA is frequently coadministered with other immunosupressive drugs and antiinflammatory drugs, we examined possible chromatographic interferences with AZA and 6-MP. The following substances were injected without a sample preparation onto the column: cyclosporin, prednisolone, chloroquine, acetylsalicylic acid, diltiazem and nifedipine.

Analysis of data

The chromatographic data was collected and processed on a Macintosh Classic computer (Apple Inc., Chicago, IL, USA) equipped with Chromac 3.1. software (Drew Ltd, London, UK). AZA and 6-MP concentrations in patient samples were determined by comparing the response factor (i.e. ratios of their respective peak areas to that of the IS) of samples with those of standard curve determined from at least seven data points.

RESULTS AND DISCUSSION

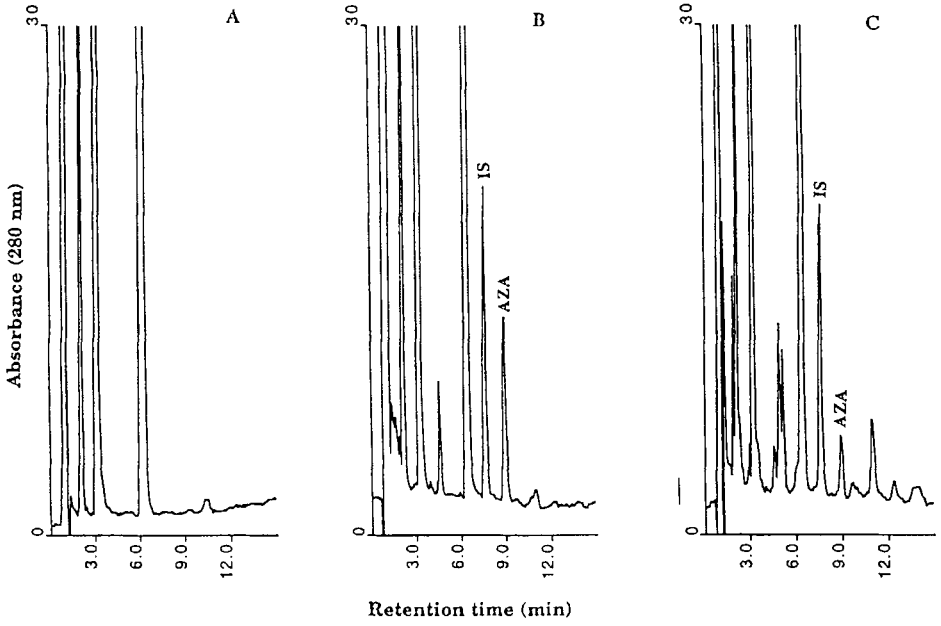
Specificity. Table 1 shows summary of the chromatographic conditions used in the present study. Figures 2A-C and 3A-C show chromatograms obtained from blank plasma, plasma spiked with AZA and 6-MP and their respective internal standards and plasma samples obtained from a patient receiving a single oral dose of AZA (150 mg). A satisfactory separation of substances and their respective IS from endogenous compounds was achieved in less than 20 minutes. Capacity factors (k') of AZA and IS were 10.3, and 8.5, respectively. The corresponding k' of 6-MP and IS were 2.9 and 5.8, respectively. Endogenous plasma components did not interfere with AZA, 6-MP and/or the used IS. Only a very small peak interfering with the IS of 6-MP was observed in plasma. However, the size of this peak was less than 3% of that IS and did not have any significant impact on the calculation of the drug concentration. None of the substances mentioned in Experimental was found to interfere with AZA and 6-MP and/or internal standards (not shown). It must be noted that coffeine elutes before guaneran (IS) and it may interfere with the measurement of IS at high concentrations. In such a case, a different mobile phase for the chromatographic column should be used (18 % methanol in 10 mM sodium phosphate buffer, pH 6.2).

Recovery. The drug recovery was determined by comparing spiked plasma samples with aqueous solutions of AZA, 6-MP and IS. Mean overall recoveries of AZA and 6-MP in the range 2-100 ng/ml were 79.0 ± 8.3% and 82 ± 6.9%, respectively. The IS guaneran and 6-mercaptopurine arabinoside had the recoveries of 92.0 ± 7.9% and 80.0 ± 7.4%, respectively.

Precision of the method. Table 2 shows the results of a precision study, both intra- and inter-assay variability for two levels of plasma AZA and 6-MP. The results show good precision (CV < 6 %) for both inter- and intra-assays. At lower concentrations of AZA and 6-MP (<2 ng/ml), the intra-assay variability was 15.8 and 7.5 %, respectively.

Table 1. Summary of conditions for HPLC analyses of azathioprine (AZA) and 6-mercaptopurine (6-MP).

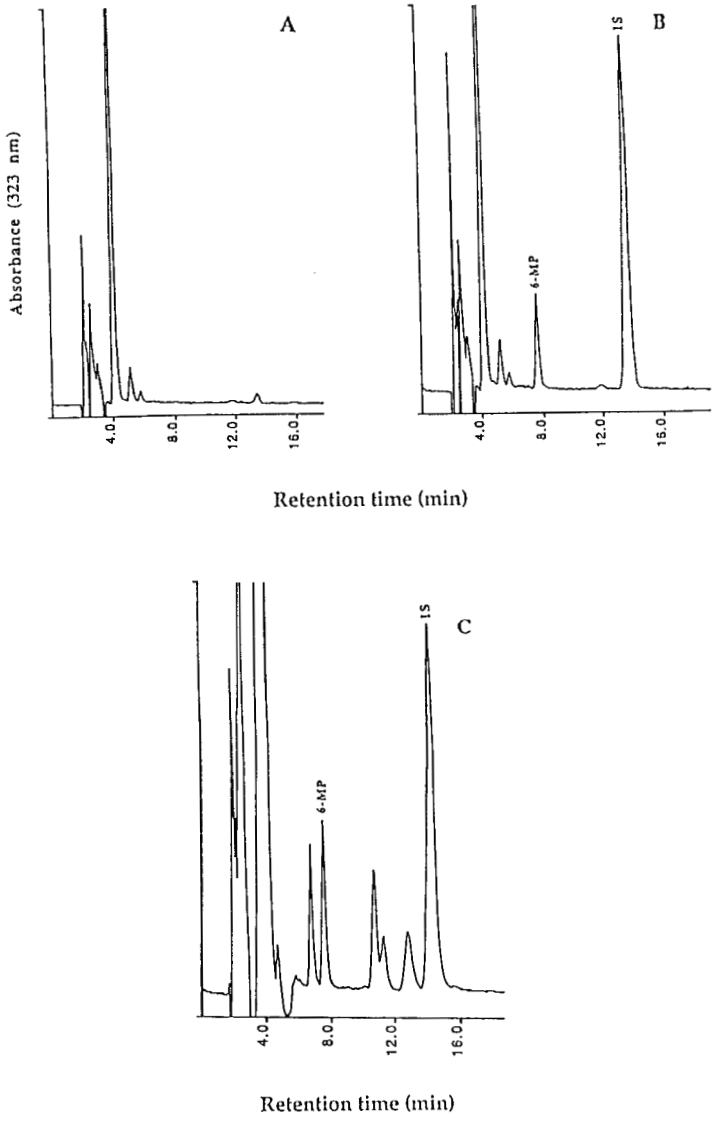
Parameter	Assay conditions for AZA	Assay conditions for 6-MP
Column	HSpecosphere 3CR C8 (80 x 4.6 mm, 3 μm)	ODS (250 x 4.6 mm, 5 μm)
Mobile phase	Sodium phosphate buffer (10 mM, pH 6.2), 9 % acetonitrile	Sodium phosphate buffer (10 mM, pH 3.0), 2 % acetonitrile
Temperature	Ambient	Ambient
Range (AUFS)	0.001	0.001
Flow rate	1.2 ml/min	1.2 ml/min
Wavelength	280 nm	323 nm
Internal standard	Guaneran	6-mercaptopurine arabinoside
Retention time	7.7 min for IS, 8.9 min for AZA	7.6 min for 6-MP, 13.6 min for IS



2. Representative chromatograms of (A) a blank plasma sample, (B) a blank plasma supplemented with 13.8 ng/ml of azathioprine (AZA) and 75 ng/ml of the internal standard (IS), and (C) a patient's plasma sample (3.9 ng/ml) collected 2 hours after a single oral dose of AZA to which 75 ng/ml of IS was added.

Limit of detection and quantitation. The smallest detectable quantity of AZA and 6-MP, defined as at least three times the baseline noise signal was about 0.2 ng for both substances injected onto the column. This amount corresponds to a concentration of 0.5 ng/ml of plasma. Lower concentrations could be quantitated by injecting larger sample volumes.

Linearity. The linearity of these methods was studied in spiked plasma solutions in the concentration range of 0.5-55 ng/ml for AZA and 1-200 ng/ml for 6-MP. The results from linear regression analysis were slope = 0.094, Y intercept = 0.015, and r = 0.999 for AZA (Figure 4A)



3. Representative chromatograms of (A) a blank plasma sample, (B) a blank plasma supplemented with 15.2 ng/ml of 6-mercaptopurine (6-MP) and 140 ng/ml of the internal standard (IS), and (C) a patient's plasma sample (26.5 ng/ml) collected 2 hours after a single oral dose of azathioprine (AZA) to which 140 ng/ml of IS was added.

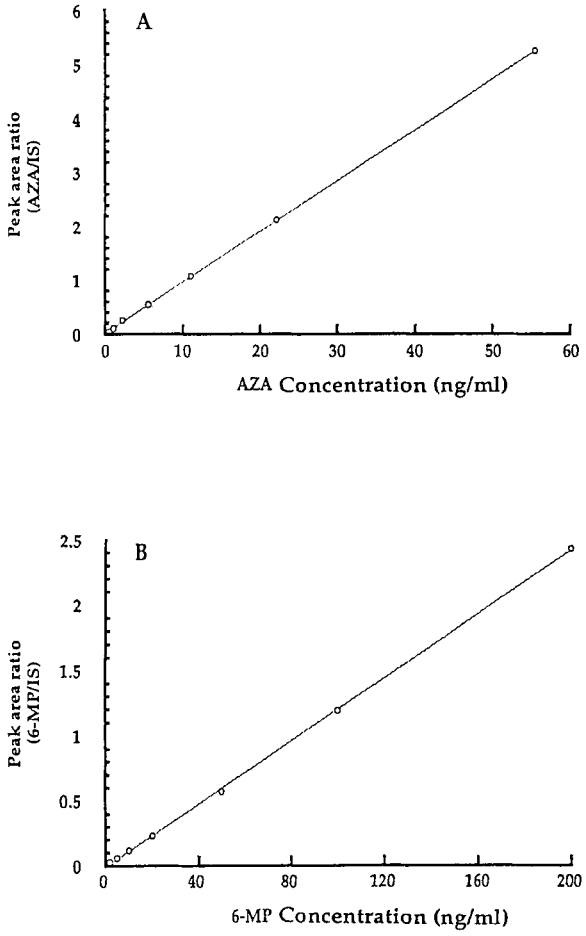
Table 2. Intra- and inter-assay variabilities of Azathioprine (AZA) and 6-mercaptopurine (6-MP) methods in blank human plasma and in plasma from patients treated with oral AZA.

	Spiked level ng/ml	Measured mean (ng/ml)	CV %	n
Intra-assay				
AZA	5.5	5.7	5.6	8
	55.0	58.9	2.7	8
	-	7.0*	5.8	10
6-MP	10.0	10.4	1.6	6
	100.0	109.5	2.3	6
	-	143.5*	3.6	8
Inter-assay				
AZA	5.5	5.6	1.9	5
	55.0	55.8	4.3	5
6-MP	10.0	10.21	5.0	5
	100.0	105.9	4.3	5

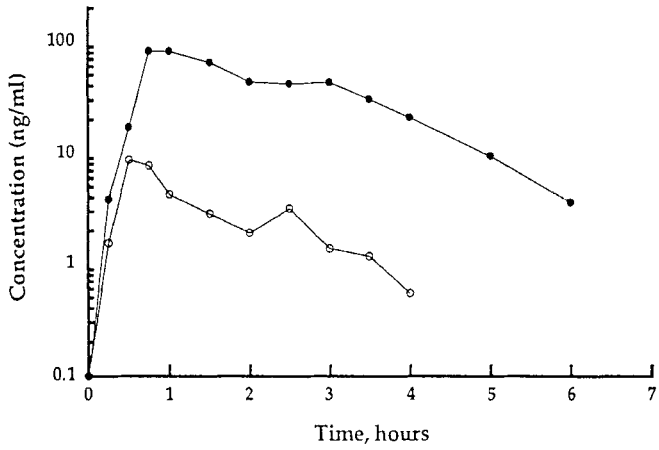
* pooled plasma from patients treated with AZA

and slope = 0.012, Y intercept = -0.010, and r = 0.999 for 6-MP (Figure 4B).

Clinical application of the method. An example of the disappearance of AZA and 6-MP in plasma studied over a period of 6 hours following an oral administration of 150 mg of AZA is shown in Figure 5. Plasma concentration versus time curve was fitted to a two compartment model using an iterative non-linear least-squares fitting program with a weighting factor 1. The absorption half-life of AZA was found to be 0.4 h. The elimination half-life and area under the plasma concentration versus time curve were 0.9 h and 16.8 $ng/ml^{-1}/h$, respectively. The corresponding values for 6-MP were 0.9 h and 218.9 $ng/ml^{-1}/h$, respectively.



4. Calibration curve for the assays of AZA (A) and 6-MP (B) in human plasma.



5. Semilogarithmic plot of plasma AZA (○) and 6-MP (●) concentration-time curve obtained in one patient with renal transplant after a 150 mg oral dose of AZA.

CONCLUSION

The plasma AZA and 6-MP extraction procedures used in the present study are simple and reproducible with low intra- and inter-assay variabilities. The choice of 6-mercaptopurine arabinoside as IS is superior to 6-thioguanine, since the latter has been found in plasma, urine and in erythrocytes as a minor metabolite. The limit of quantitation (0.2 ng) is sufficient for studying the pharmacokinetics and TDM of AZA in plasma during a period of 6 hours and that of 6-MP during an even longer period following the oral administration of a dose of AZA. Furthermore we suggest that this method may be evaluated for TDM in the clinical setting in a large scale.

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