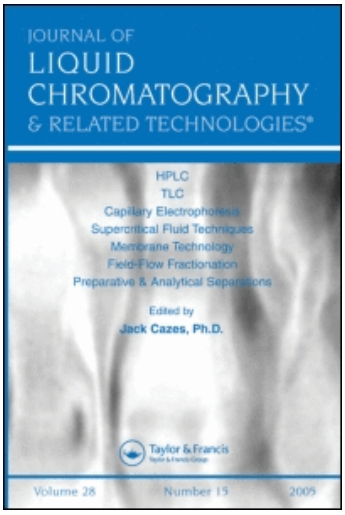

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HPLC Determination of 6-Thiouric Acid and 6-Mercaptopurine in Organ Transplant Patient Serum

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HPLC DETERMINATION OF 6-THIOURIC AND 6-MERCAPTOURINE IN ORGAN TRANSPLANT PATIENT SERUM

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ABSTRACT

A sensitive high performance liquid chromatographic method for the simultaneous determination of 6-thiouric acid and 6-mercaptopurine in serum is described. Our intent was to develop a procedure that could be used for pharmacokinetic studies and therapeutic drug monitoring in organ transplant patients taking azathioprine. Serum samples were precipitated with acetonitrile containing 6-n-propyl-2-thiouracil as the internal standard. The chromatographic separation was performed with an octadecylsilane column and gradient solvent system consisting of acetonitrile and 0.01 M sodium dihydrogen phosphate, pH 6.1. An initial acetonitrile concentration of 1% was used to elute 6-thiouric acid but was increased to 16% to recover the 6-mercaptopurine and internal standard. The flow rate was increased from 1.3 ml/min to 1.5 ml/min during the analysis. The column effluent was monitored at 353 nm and 323 nm for detection of 6-thiouric acid and 6-mercaptopurine, respectively. Statistical analysis of standard curve data showed good intra- and inter-day accuracy, precision and reproducibility throughout a concentration range of 10 - 2500 ng for 6-thiouric acid and 10 - 500 ng for 6-mercaptopurine/ml of serum. The method has been applied to the quantification of 6-thiouric acid and 6-mercaptopurine in serum from two kidney allograft recipients.

INTRODUCTION

Azathioprine (AZA) has been used for immunosuppression in organ transplantation and autoimmune diseases for over 25 years. Information on azathioprine disposition remains limited resulting from the lack of specific and sensitive assays for AZA (1-5), and the contention that plasma AZA concentrations do not correlate with pharmacological activity (6).

Intracellular thiopurine ribonucleotides have been identified as the active metabolites of AZA (7), unfortunately, the assay procedures required to measure these compounds have proven difficult (8-10). It has been suggested that AZA therapy might be monitored by determining plasma concentrations of 6-thiouric acid (6TU), an end-product of AZA metabolism (11). The first analytic attempts were spectrophotometric assays of 6TU that showed poor sensitivity (12-14), with detection limits of 10 ug/ml in plasma and 1 ug/ml to 50 ug/ml in urine. VanScoik et al (15) developed a high performance liquid chromatographic (HPLC) assay with a detection limit of 12.5 ng of 6TU/ml of plasma. The method was used in single-dose kinetics in rats which were free of other drug substances. This suggested to us that the need existed to develop an accurate, specific, and reliable method for the quantification of 6TU and 6-mercaptopurine (6MP) in human serum. This paper describes an analytic HPLC approach which demonstrated sufficient sensitivity to be used in detailed pharmacokinetic studies of transplant patients taking azathioprine.

MATERIALS AND METHODS

Instrumentation

The chromatographic analysis was performed with a Hewlett Packard 1090 liquid chromatograph equipped with a diode array detector and Data Processing Unit (DPU) software. The column was an octadecylsilane, 250 x 4.6 mm packed with 5 um spherical material

(Zorbax ODS, DuPont, Biomedical Products, Wilmington, DE). A 20 x 3.2 mm ODS precolumn (Brownlee Labs, Inc., Santa Clara, CA) was also used. The effluent was monitored at 353 nm for the detection of 6-TU and 323 nm for 6-MP and the internal standard. The column compartment was maintained at 30°C.

Reagents

The 6-MP, 6-n-propyl-2-thiouracil (PTU), and dithiothreitol (DTT) were obtained from Sigma Chemical Company (St. Louis, MO). Sodium dihydrogen phosphate (reagent grade), methanol (HPLC grade), and water (HPLC grade) were from Mallinckrodt Inc. (Paris, KY). The sodium hydroxide (reagent grade) and acetonitrile (HPLC grade) were obtained from Fischer Scientific (Fair Lawn, NJ). 6-Thiouric acid was the gift of Burroughs-Wellcome Co. (Research Triangle Park, NC).

Drug Solutions

A 200 ug/ml solution of 6-TU was prepared in water. A 200 ug/ml stock solution of 6-MP was prepared by dissolving 10 mg in 0.1 N NaOH. The solution was gradually acidified to contain 0.1 N HCl at final volume. Both solutions were covered with aluminum foil and stored at 4°C.

Internal Standard (IS) and Precipitation Solution

A 1 mg/ml stock solution of PTU was prepared in methanol. The precipitation solution consisted of diluting an aliquot of the internal standard stock solution in acetonitrile to a final concentration of 8 ug/ml.

Mobile Phase

Sodium dihydrogen phosphate solution (0.01 M) was prepared in water, and the pH was adjusted to 6.1 with 2 N NaOH. The mobile phase for the chromatography consisted of 99% buffer and 1% acetonitrile (v/v) to elute the 6-TU. Between 2 min and 10 min the

percentage of acetonitrile was increased to 16% to elute the 6-MP and IS. At 13.5 min, 60% acetonitrile was maintained for 4 min to flush the column. The gradient was then reversed and the column allowed to equilibrate at 1% acetonitrile for 4 min prior to the next sample injection.

Preparation of Serum Standards

To a volume of drug free serum, 10 ul of DTT and an aliquot of each drug solution was added to prepare standards containing 10 - 500 ng 6-MP and 10 - 2500 ng 6-TU/ml serum. After mixing, a 300 ul aliquot was then placed into a 10 x 75 mm pyrex tube, and 1 ml of the precipitation solution containing IS was added. The tubes were vortexed and centrifuged for 10 min at 1400 x g. The supernatant was transferred to a glass tube and evaporated to dryness under nitrogen at 40°C. The residue was dissolved in 100 ul of mobile phase, centrifuged, and the supernatant transferred to glass HPLC microvials. A 25 ul aliquot was injected onto the column.

Quantification

Standard curves for serum were constructed utilizing three replicates at each concentration. The peak height ratios of 6-TU and 6-MP to internal standard were plotted against concentration (ng/ml serum).

Recovery

Spiked samples containing known concentrations of 6-MP and 6-TU (10 - 500 and 10 - 2500 ng/ml, respectively) in serum and water were carried through the analysis procedure in triplicate. The peak height ratios obtained from the serum samples (mean observed conc) were compared to the ratios obtained from the equivalent water samples (mean recovery conc) to estimate percent recovery after serum protein precipitation.

Patient Samples

Serum samples were collected from two kidney allograft recipients at 0, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 24 hours after receiving 200 mg AZA orally. The serum was stored at -20°C immediately after collection and analyzed within 36 hours. The patients were also receiving prednisone, cotrimoxazole, clotrimazole, folic acid, theophylline, diltiazem, furosemide, clonidine, ranitidine, and metolazone. Standard curves for determining patient drug levels were prepared daily.

Quality Control (QC) Samples

Drug-free serum was spiked with DTT and known concentrations of 6-MP and 6-TU. Three QC levels (10, 200, 500 ng/ml for 6-MP, 100, 500, 2500 ng/ml for 6-TU) were prepared, aliquoted, and stored at -20°C. Prior to analysis, the QC samples were brought to room temperature and carried through the serum assay. The amount of drug found in the QC samples was calculated by comparison to the standard curve.

RESULTS AND DISCUSSION

The sample precipitation technique used in this study quantitatively detected 10 ng of 6-TU and 6-MP/ml of serum. No interference from normal serum constituents was observed after precipitation and chromatography (Fig. 1A).

Statistical analysis of standard curve data by linear regression indicated excellent linearity and reproducibility throughout the concentration range of 6-TU and 6-MP as shown in Table 1. Data collected from three standard curves on three separate days (Table 2) showed the inter-day precision of 6-TU was 6.5% at 10 ng/ml and 0.1% at 2500 ng/ml (correlation coefficient of 0.9998, a slope of 0.9998, and an intercept of 0.3060). Similar analysis of 6-MP data showed inter-day precision of 9.1% and 0.6% at the 10

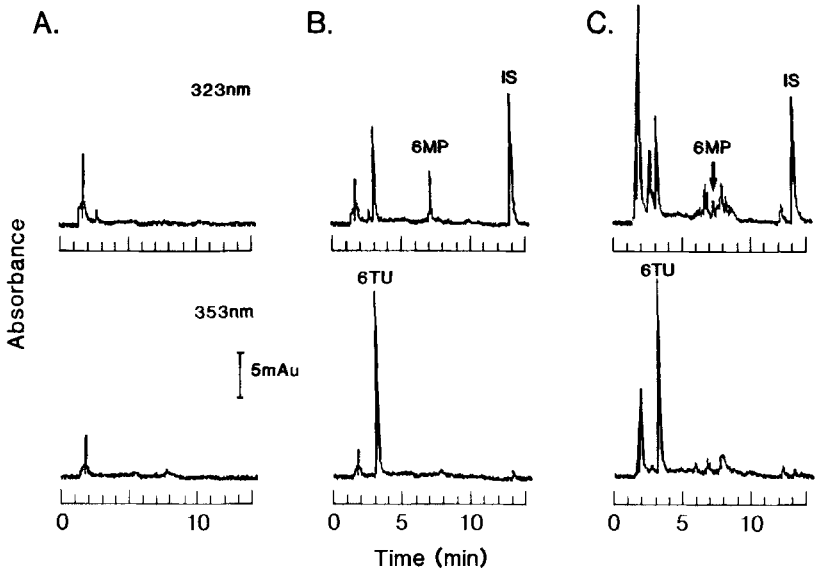

Figure 1. HPLC Chromatogram of 6TU and 6MP
Key: A, blank serum
B, 500 ng/ml 6TU, 200 ng/ml 6MP serum
C, kidney allograft recipient # 2, 2 hrs after azathioprine dose

and 500 ng/ml concentrations, respectively (correlation coefficient of 0.9998, slope of 1.0000, intercept of 0.0093).

The reproducibility of 6-TU and 6-MP while stored at -20°C in the presence of DTT was determined by analyzing QC samples over a 1 month period (Table 3). The data showed good precision and reproducibility for both drugs at all three levels suggesting negligible decomposition under these conditions. The overall percent recovery of 6-TU was 97% while recovery of 6-MP was 99% (Table 4).

Simultaneous dual-wavelength monitoring which has been made easy with computerized diode array technology has eliminated the problem of quantitating 2 or more analytes possessing different UV spectral characteristics. 6-Thiouric acid showed peak UV absorb-

TABLE 1.
Linearity and Precision of 6MP, 6TU Standard Curve in Serum

Drug	Theoretical conc (ng/ml)	Observed conc (ng/ml)*	C.V. (%)
6MP	10	12.7 ± 0.1	0.8
	25	26.0 ± 0.6	2.3
	50	52.4 ± 2.5	4.8
	100	99.0 ± 0.4	0.4
	200	192.8 ± 1.0	0.5
	400	396.9 ± 5.8	1.5
	500	505.2 ± 4.5	0.9
		correlation coefficient = 0.9997	
		intercept = -0.0033	
		slope = 0.0018	
6TU	10	14.4 ± 2.0	14.1
	25	27.4 ± 2.5	9.1
	50	52.1 ± 1.9	3.7
	100	114.4 ± 1.7	1.5
	500	500.8 ± 17.5	3.5
	1000	961.1 ± 6.3	0.7
	2500	2514.7 ± 22.5	0.9
		correlation coefficient = 0.9998	
		intercept = 0.0024	
		slope = 0.0009	

n = 3 replicates at each concentration.
* mean ± standard deviation

ance of 353 nm while 6-MP showed maximal absorbance at 323 nm. The difficulty in quantitating 6-TU and 6-MP simultaneously was not in the detection, but in the chromatography. The water solubility of 6-TU precluded its resolution from the solvent front at acetonitrile percentages exceeding 2% while flowing at 1.3 ml/min. The 6-MP (and internal standard), however, are less polar and required an increased acetonitrile concentration to elute from the column.

TABLE 2.
Inter-Day Precision of 6MP and 6TU

Drug	Theoretical conc (ng/ml)	Mean Observed conc (ng/ml)*	C.V. (%)
6MP	10	12.7 ± 1.2	9.1
	25	26.5 ± 0.5	1.9
	50	50.0 ± 2.5	5.0
	100	99.5 ± 2.0	2.0
	200	195.1 ± 3.3	1.7
	400	396.6 ± 5.3	1.3
	500	504.7 ± 2.9	0.6
6TU	10	13.8 ± 0.9	6.5
	25	26.6 ± 3.9	14.5
	50	48.0 ± 5.2	10.9
	100	111.5 ± 5.2	4.7
	500	510.9 ± 11.9	2.3
	1000	963.8 ± 4.7	0.5
	2500	2511.9 ± 2.5	0.1

n = 9 replicates at each concentration
* mean ± standard deviation

TABLE 3.
Reproducibility of 6MP, 6TU Quality Controls

Drug	Theoretical conc (ng/ml)	Mean Observed conc (ng/ml)*	C.V. (%)
6TU	10	12.7 ± 1.7	13.7
	500	494.9 ± 11.7	2.4
	2500	2624.6 ± 40.0	1.5
6MP	10	12.8 ± 1.0	8.1
	200	206.5 ± 12.9	6.3
	500	537.4 ± 27.5	5.1

n = 6 replicates at each concentration
* mean ± standard deviation

TABLE 4.
Percent Recovery of 6MP and 6TU from Serum

Drug	Theoretical conc (ng/ml)	Mean Observed conc (ng/ml)*	Mean Recovery conc (ng/ml)*	% Recovery
6MP	10	13.8	15.1	92
	25	26.5	25.0	106
	50	50.2	50.6	99
	100	97.8	98.9	99
	200	193.5	191.5	101
	400	401.7	402.1	100
	500	501.5	501.8	100
		overall recovery = 99%		
6TU	10	14.3	14.2	100
	25	22.4	27.0	83
	50	39.1	49.7	79
	100	114.7	98.6	116
	500	524.2	502.4	104
	1000	961.0	989.1	97
	2500	2510.65	2503.9	100
		overall recovery = 97%		

n = 3 replicates at each concentration
* = mean ± standard deviation

A slight increase in flow rate was used to hasten the chromatography and retain good peak shape. The column was maintained slightly above ambient temperature to obtain reproducible retention times. A change of two or three degrees had a significant effect on the elution of 6-TU. The column flushing cycle was necessary when analyzing several serum samples to extend the life of the column.

A kinetic profile of 6-MP and 6-TU serum concentrations in kidney allograft recipient #1 receiving an oral regimen of 200 mg AZA daily is presented in Figure 2. Peak serum 6-TU and 6-MP concentrations were observed at 6 hours and 2 - 4 hours, respectively. Despite poor renal performance in kidney allograft recipient #2 (Fig. 1C), a 25 ng/ml level of 6MP was still resolved.

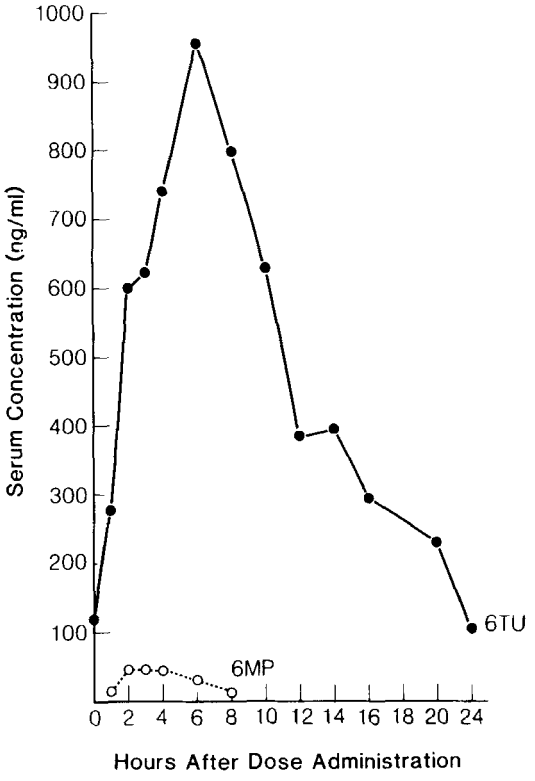

Figure 2. Serum concentrations of 6TU and 6MP in kidney allograft recipient # 1

CONCLUSION

An HPLC method for the simultaneous analysis of 6-MP and 6-TU in serum has been described. The procedure is simple, involving precipitation of the serum proteins with acetonitrile, and employs gradient analysis with dual-wavelength monitoring. The method has been used to monitor 6-MP and 6-TU levels in a kidney allograft recipient receiving numerous other medications. The study is currently being expanded to include other transplant recipients.

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