ELSEVIER

Journal of Chromatography B, 679 (1996) 147–154

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Simultaneous determination of azathioprine and 6-mercaptopurine by high-performance liquid chromatography

Erik C. Van Os[a,b,*], Jeffrey A. McKinney[b], Bradley J. Zins[a,b], Dennis C. Mays[b], Zachary H. Schriver[b], William J. Sandborn[a], James J. Lipsky[b]

[a]*Department of Medicine, Division of Gastroenterology, Mayo Clinic/Mayo Foundation, 200 First Street SW, Rochester, MN 59905, USA*

[b]*Department of Medicine, Clinical Pharmacology Unit, Mayo Clinic/Mayo Foundation, Rochester, MN 55905 , USA*

Received 1 August 1995; revised 13 December 1995; accepted 2 January 1996

Abstract

A specific, sensitive, single-step solid-phase extraction and reversed-phase high-performance liquid chromatographic method for the simultaneous determination of plasma 6-mercaptopurine and azathioprine concentrations is reported. Following solid-phase extraction, analytes are separated on a C_{18} column with mobile phase consisting of 0.8% acetonitrile in 1 m*M* triethylamine, pH 3.2, run on a gradient system. Quantitation limits were 5 ng/ml and 2 ng/ml for azathioprine and 6-mercaptopurine, respectively. Peak heights correlated linearly to known extracted standards for 6-mercaptopurine and azathioprine (r=0.999) over a range of 2–200 ng/ml. No chromatographic interferences were detected.

Keywords: Azathioprine; 6-Mercaptopurine

1. Introduction

Azathioprine (AZA) and its major metabolite 6-mercaptopurine (6MP) (Fig. 1) are immunosuppressive medications gaining increasing use in the treatment of a variety of conditions, including rheumatologic disorders, prevention of rejection following organ transplantation and inflammatory bowel disease. In the blood, AZA is rapidly converted to 6MP by non-enzymatic attack of sulfhydryl-containing compounds, such as glutathione, on the sulfide bond between the purine and the imidazole rings of AZA. 6MP is further metabolized via three pathways. Two pathways metabolize 6MP

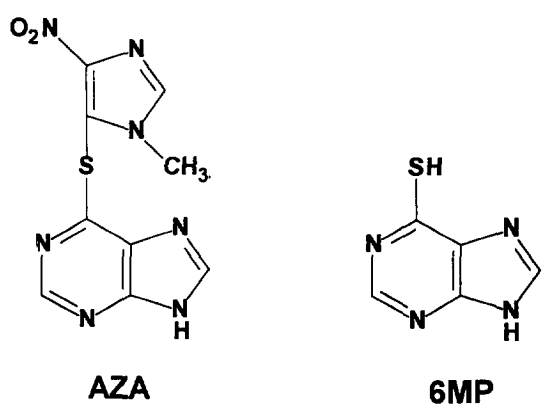


Fig. 1. Structure of AZA and 6MP.

*Corresponding author.

0378-4347/96/$15.00
PII S0378-4347(96)00007-2

to presumed inactive metabolites: 6-thiouric acid via xanthine oxidase and 6-methylmercaptopurine via thiopurine methyltransferase. 6MP is also metabolized to the drug's presumed active metabolites, the 6-thioguanine nucleotides, via hypoxanthine phosphoribosyltransferase and other enzymes [1].

Despite the fact that 6MP and AZA have had extensive use for over 25 years, few studies have evaluated the pharmacokinetics of both AZA and 6MP following AZA administration [2–5]. These studies have been limited by the instability of AZA in whole blood and the requirement of performing separate assays on plasma for 6MP and AZA. There is only one previous report of simultaneous determination of 6MP and AZA by HPLC [6]. This assay required converting 6MP to a N-ethylmaleimide derivative prior to chromatography, had a run time of 40 min and had a sensitivity of 10 ng/ml for each compound. The sulphydryl-protecting compound dithiothreitol (DTT) is often added to plasma to prevent breakdown of 6MP and is one factor limiting the ability to perform simultaneous assays for 6MP and AZA [1]. Addition of DTT to plasma containing AZA results in near complete conversion of AZA to 6MP [5]. For this reason plasma must be separated prior to DTT addition and separate assays for 6MP and AZA must then be performed.

An assay which allows simultaneous determination of plasma 6MP and AZA concentrations would simplify simultaneous pharmacokinetic studies of these compounds. We report a specific, sensitive, single-step solid-phase extraction method for the simultaneous determination of plasma 6MP and AZA concentrations by high-pressure liquid chromatography (HPLC).

2. Experimental

2.1. Chemicals

AZA, 6MP (Sigma, St. Louis, MO, USA); methanol, acetonitrile (Burdick and Jackson, Muskegon, WI, USA); disodium ethylenediamine tetraacetic acid (EDTA), triethylamine (TEA) (Aldrich, Milwaukee, WI, USA); glacial acetic acid, phosphoric acid (J.T. Baker, Phillipsburg, NJ, USA).

2.2. Solutions

Stock solution of 6MP is prepared by dissolving 4 mg 6MP in 2 ml of 0.1 *M* NaOH and adjusting the volume to 50 ml with water. Stock solution of AZA is prepared by dissolving 4 mg AZA in 50 ml methanol. These stock solutions are stable at 5°C for at least 2 months. The saturated solution of EDTA is prepared by placing 2.5 g EDTA in 25 ml water and vortexing aggressively for 5 min. The 0.2% acetic acid solution is prepared by diluting 2 ml of glacial acetic acid to 1 l with water.

2.3. Extraction

Blood is collected in 7 ml EDTA(K_3)-containing vacuum tubes (Sherwood Medical, St. Louis, MO, USA) and is immediately placed in an ice-water slurry. Within 30 min, the blood sample is centrifuged for 10 min at 1000 *g*, 4°C. The plasma is transferred to plastic cryotubes (Nunc, Naperville, IL, USA) and stored at −70°C. until analysis. Solid-phase extraction columns (C_{18} Sep-Pak, Waters, Millford, MA, USA) are sequentially pre-rinsed with 2.5 ml of methanol and 5 ml of 0.2% acetic acid. Plasma (1 ml) is then loaded onto the Sep-Pak following addition of 0.04 ml of saturated EDTA solution to the plasma. In addition, 0.1 ml of standard solution is added to blank plasma for the standard curve samples and 0.1 ml of water is added to plasma of the unknowns. The sulfhydryl-protecting agent dithiothreitol (DTT) is not included because AZA is immediately converted to 6MP in the presence of DTT [5]. The cartridges are rinsed with 2 ml of 0.2% acetic acid and then centrifuged at 2200 *g* for 5 min to remove excess water. The samples are eluted from the cartridges with 2 ml of methanol and evaporated to dryness under a stream of nitrogen at 37°C. Samples are then reconstituted in 0.2 ml of mobile phase, vortex-mixed for 30 s, transferred to 1.5 ml conical microfuge tubes and centrifuged in a microcentrifuge (Fischer Scientific, Pittsburg, PA, USA) at 11 000 *g* for 5 min to remove

particulate matter. A portion (0.175 ml) of supernatant is transferred to HPLC vials for analysis.

2.4. Chromatography

The pump used was LKB 2150 (Bromma, Sweden), with the LKB 2152 controller. Sample vials were loaded onto the LKB 2157-010 autosampler. The analytical column was a Hewlett-Packard (Rockville, MD, USA) octadecylsilane (ODS) Hypersil, 200×4.6 mm I.D., 5 μm particle size. It was protected by a Zorbax (Mac-mod Analytical, Chadds Ford, PA, USA) ODS 12.5×4 mm I.D. guard column and a 2.0-μm particulate filter. The mobile phase contained the following: A, 0.8% acetonitrile in 1 m*M* triethylamine, adjusted to pH 3.2 (within 0.03 pH units using standard laboratory techniques) with phosphoric acid; B, 20.0% acetonitrile in 1 m*M* triethylamine, adjusted to pH 3.2 with phosphoric acid. Analyses were performed on a gradient system: Time 0, 0% B; time 5 min, 0% B; time 7.5 min, 50% B; time 19 min, 50% B; time 21 min, 0% B. The column was reequilibrated for 9 min before the next injection. The flow-rate was 1.5 ml/min. Absorbance was monitored at 340 nm (UVIS 204, Linear Instruments, Reno, NV, USA) at a range of 10 mAU full scale. The injection volume was 0.08 ml.

2.5. Stability

The stability of 6MP and AZA at −70°C was determined by spiking a batch of blank plasma with two concentrations of 6MP (10 ng/ml and 50 ng/ml) and spiking a second batch of plasma with the same concentrations of AZA. Freshly spiked plasma along with a freshly prepared standard curve were analyzed at time zero. Spiked plasma was frozen and aliquots were thawed and analyzed at specified time intervals. With each batch of analytes a fresh standard curve was prepared. The stability of the drugs in plasma at room temperature was determined by comparing freshly spiked plasma with spiked samples left on a laboratory bench for 2 h. The stability of 6MP and AZA in extracted samples at room temperature was determined by comparing freshly extracted samples with the same samples following 30 h at room temperature in HPLC vials.

2.6. Accuracy

The accuracy of the assay was determined by running sets of spiked standards along with standard curves.

2.7. Recovery

The recoveries of 6MP and AZA were determined by comparing the response of aqueous standards to the response of the 100 ng/ml standard for each run.

2.8. Calculation of 6MP and AZA concentrations

Concentrations of 6MP and AZA are determined by comparing peak heights of unknown concentrations with peak heights of known standards. 6MP concentrations (20–200 ng/ml) were determined by using a standard curve created from standards 2, 5, 10, 20, 50, 100, 200 ng/ml and 6MP concentrations ≤20 ng/ml were determined using a truncated standard curve created from standards 2, 5, 10 and 20 ng/ml in order to improve the precision and accuracy at low concentration ranges [7]. AZA concentrations 20–200 ng/ml are determined by using a standard curve created from standards 5, 10, 20, 50, 100, 200 ng/ml and AZA concentrations ≤20 ng/ml are determined using a standard curve created from standards 5, 10 and 20 ng/ml.

3. Results

3.1. Sample stability

6MP was stable when stored at −70°C in plasma at 10 ng/ml and 50 ng/ml concentrations for at least 133 days (Fig. 2). AZA was stable when stored in plasma at −70°C in concentrations of 10 ng/ml and 50 ng/ml concentrations for at least 60 days (Fig. 3). This was determined by performing linear regression on the mean 6MP concentrations at specified time intervals for the 10 ng/ml and 50 ng/ml concentrations. The slopes were 0.0018 ng/ml per day and 0.0002 ng/ml per day, respectively, neither significantly different from zero (one-tailed *t*-test; $P=0.77$,

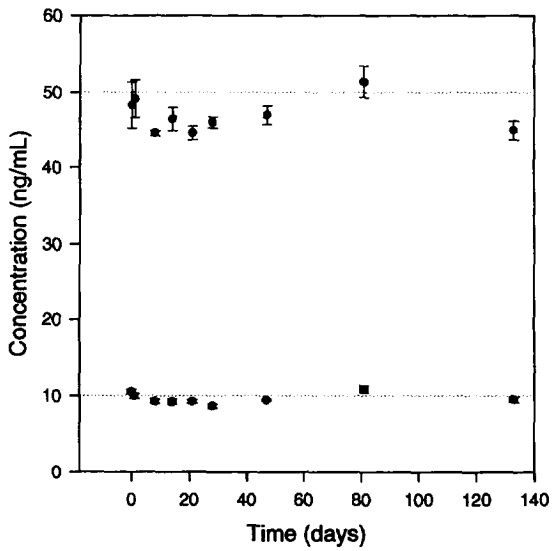


Fig. 2. Mean 6MP concentrations when frozen in plasma at two concentrations (50 ng/ml and 10 ng/ml) for 133 days. For each point $n=5$, except for the time zero and one day points of the 6MP 50 ng/ml study, where $n=17$.

0.99, respectively). A similar analysis was performed for AZA at the 10 ng/ml and 50 ng/ml concentrations. The slopes were 0.0587 ng/ml per day and −0.0241 ng/ml per day, respectively. The slope for the AZA 10 ng/ml per day is significantly different from zero ($P=0.04$), but is positive, suggesting no degradation. The slope of the AZA 50 ng/ml per day is not significantly different from zero ($P=0.76$). 6MP concentrations were stable in plasma at room temperature for at least 4 h, AZA concentrations were stable in plasma at room temperature for at least 2 h. No degradation of 6MP and AZA was detected in extracted samples while in the autosampler for 30 h at room temperature (data not shown).

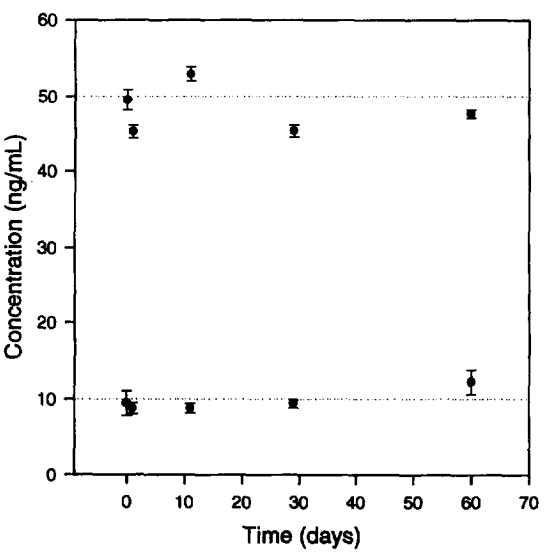


Fig. 3. Mean AZA concentrations when frozen in plasma at two concentrations (50 ng/ml and 10 ng/ml) for 60 days. For each point $n=5$.

Table 1
Inter-day variability assay for 6MP

Predicted concentration (ng/ml)	Measured concentration (mean±S.D.) (ng/ml)	C.V. (%)
2	2.06±0.26	12.7
5	4.74±0.30	6.4
10	10.3 ±0.26	2.5
20	19.9 ±0.12	0.6
50	47.5 ±1.22	2.6
100	97.1 ±1.60	1.7
200	202 ±0.91	0.5

3.2. Inter-day variability

Table 1 and Table 2 show the inter-day variability of the assay for 6MP and AZA, respectively, when five standard curves were prepared on five separate days. The standard curves for the inter-day vari-

Table 2
Inter-day variability assay for AZA

Peredicted concentration (ng/ml)	Measured concentration (mean±S.D.) (ng/ml)	C.V. (%)
5	4.98±0.83	16.8
10	10.0 ±1.25	12.5
20	20.0 ±0.42	2.1
50	48.9 ±1.95[a]	4.0
100	99.8 ±1.63	1.6
200	201 ±1.20	0.6

[a] $n=4$ for this concentration. One concentration was >8 standard deviations from the mean of the other four concentrations and was not included in the determination of the mean.

Table 3
Intra-day variability assay for 6MP

Predicted concentration (ng/ml)	Measured concentration (mean±S.D.) (ng/ml)	C.V. (%)
2	1.91±0.35	18.1
5	4.82±0.55	11.4
10	9.91±1.11	11.2
20	19.9 ±2.25	11.3
50	48.8 ±2.24	4.6
100	99.2 ±2.83	2.9
200	201 ±9.40	4.7

ability assays were linear, with a mean r value of 0.999.

3.3. Intra-day variability

Table 3 and Table 4 show the intra-day variability of the assay when five standard curves were performed on the same day. A reference standard curve was created from the mean peak heights for each predicted concentration. Back-calculated concentrations were then determined from this reference standard curve. The standard curves for the inter-day variability assays were linear, with a mean r value of 0.999.

3.4. Accuracy

For 6MP and AZA, sets of spiked plasma standards at the 10 ng/ml and 50 ng/ml concentrations were run along with fresh standard curves. The greatest deviation of the mean concentration for a set of standards from the known concentration of the standards was then determined for each compound at

Table 4
Intra-day variability assay for AZA

Predicted concentration (ng/ml)	Measured concentration (mean±S.D.) (ng/ml)	C.V. (%)
5	5.20±0.45	8.6
10	9.70±0.98	10.1
20	20.1 ±0.77	3.9
50	50.4 ±1.50	3.0
100	102 ±1.85	1.8
200	199 ±7.93	4.0

Table 5
Accuracy data

Compound	Concentration (ng/ml)	Sets run (n=5)	Greatest deviation of set mean (%)
6MP	10	2	5.3
	50	6[a]	11.8
AZA	10	2	11.9
	50	2	6.0

[a]Two sets n=5, four sets n=6

10 ng/ml and 50 ng/ml concentrations. Overall, our assay was accurate to within 11.9% (Table 5).

3.5. Recovery

The mean inter-day recoveries of 6MP and AZA for the 100 ng/ml standard were 77% and 91%, respectively. The mean intra-day recoveries of 6MP and AZA for the 100 ng/ml standard were 75% and 87%, respectively.

3.6. Chromatography

Fig. 4 shows the chromatograms of blank plasma prior to and after spiking with 50 ng/ml of 6MP and AZA. There were no interfering peaks in the blank plasma. Fig. 5 shows the chromatograms of a subject prior to and 30 min after an oral dose of 100 mg of AZA. There were no interfering peaks in the subject's pre-dose plasma. The following known and potential metabolites of AZA did not interfere with the assay: 6-thiouric acid, 6-methylthiouric acid, 6-methylmercaptopurine, 8-hydroxy-6-methylmercaptopurine, 6-thioxanthine, 6-thioguanine and 1-methyl-4-nitro-5-thioimidazole. Spectral analysis performed on the proposed 6MP and AZA peaks of aqueous standards, spiked plasma and patient samples were essentially identical in the region of 340 nm (Fig. 6). This demonstrates that there was no interference from metabolites of either compound.

3.7. Application of the assay

Fig. 7 is a time–plasma AZA and 6MP concentration curve for a human subject following 100 mg of oral AZA.

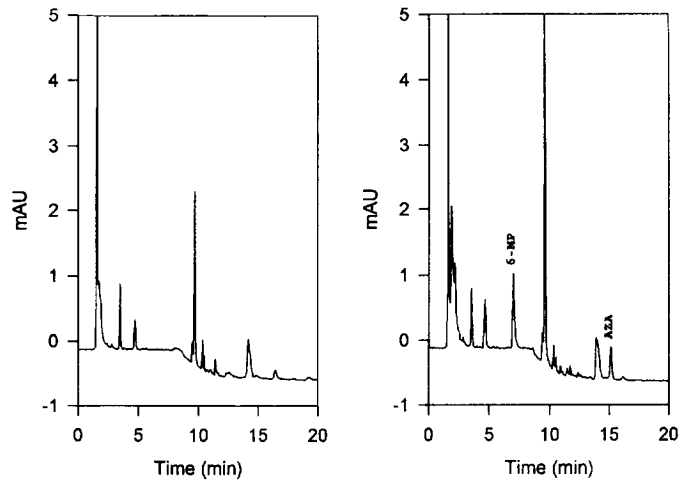


Fig. 4. Chromatograms of blank plasma prior to (left) and after (right) spiking with 50 ng/ml of 6MP and AZA. There were no interfering peaks in the blank plasma.

4. Discussion

We report a specific, sensitive HPLC assay for simultaneous determination of plasma AZA and 6MP concentrations. This assay is an improvement over the previously reported simultaneous assay [6]. Our assay does not require derivatization of 6MP, has a shorter run time and has greater sensitivity for both compounds.

Detailed data for the sensitivity and precision of our assay is provided. The quantitation limits for AZA and 6MP are 5 and 2 ng/ml, respectively. The most sensitive of five previous assays for AZA had a detection limit of 0.5 ng/ml [2,3,5,6,8]. AZA is detectable using our technique at concentrations lower than 5 ng/ml. Greater sensitivity for AZA could, therefore, be achieved by using higher injection volumes. The sensitivity of our assay for 6MP is well within the range of several more recent published HPLC assays for 6MP (1–10 ng/ml) [5,9–16].

Pharmacokinetic studies reporting both 6MP and

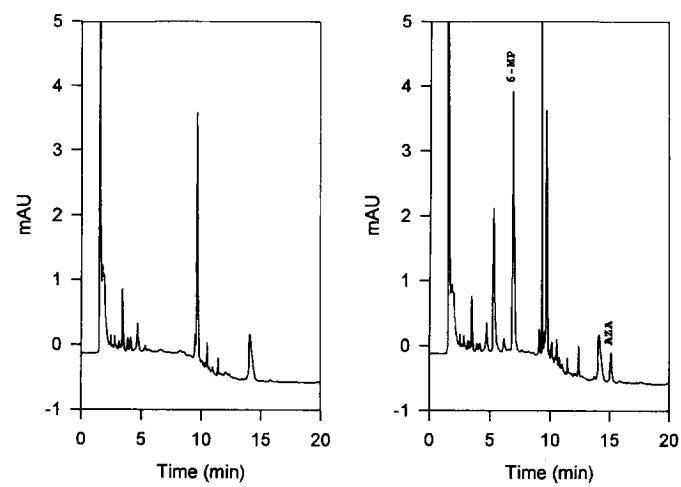
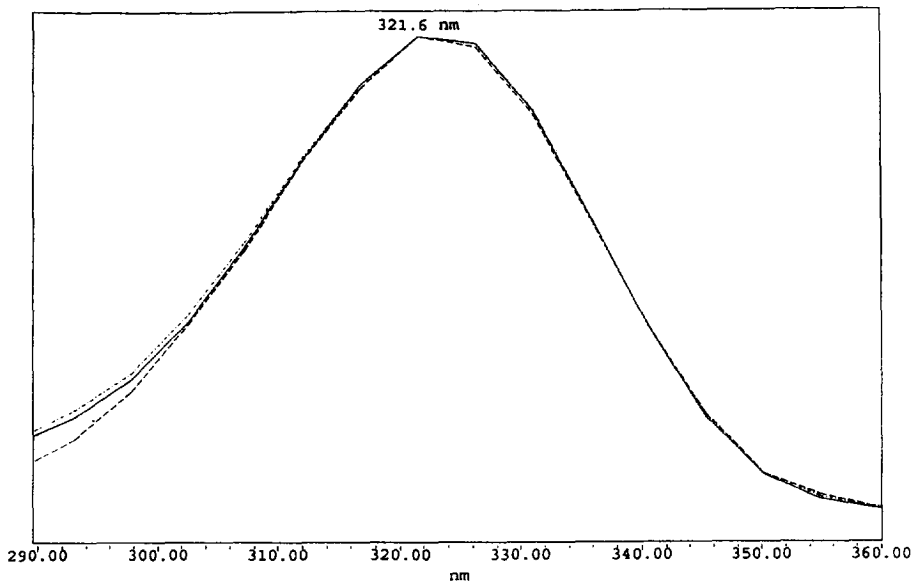
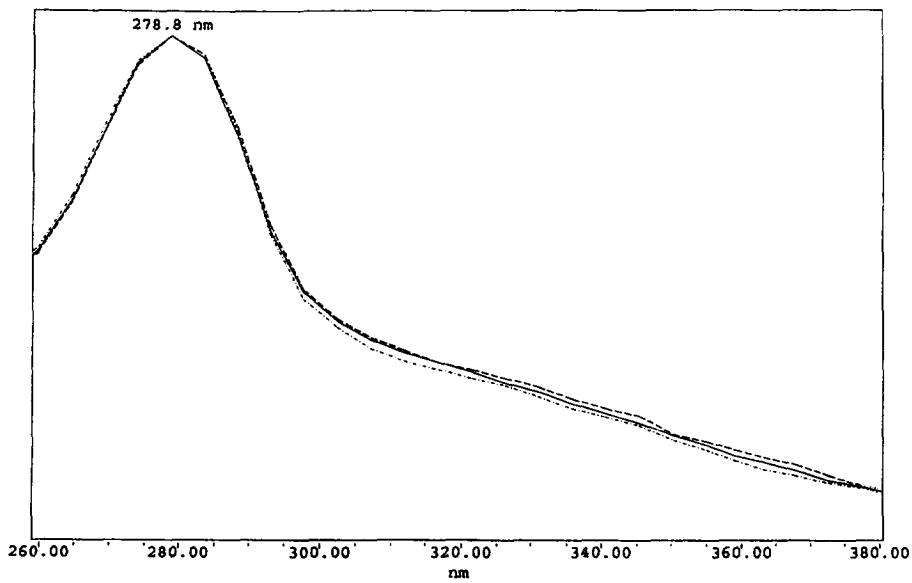


Fig. 5. Chromatograms of subject plasma prior to (left) and 30 min after (right) an oral dose of 100 mg of AZA. There were no interfering peaks in the subject's pre-dose plasma.



---- 6-Mercaptopurine spiked into human plasma
—·—· 6-Mercaptopurine in plasma of human subject 1.25 hours after 100mg p.o. dose AZA
—— 6-Mercaptopurine in H_2O



---- Azathioprine spiked into human plasma
—·—· Azathioprine in plasma of human subject 1.25 hours after 100mg p.o. dose AZA
—— Azathioprine in MeOH

Fig. 6. Spectral analysis of of the chromatographic peaks corresponding to (top) 6MP and (bottom) AZA for aqueous standard, spiked human blank plasma and human plasma 1.25 h post-oral AZA dose.

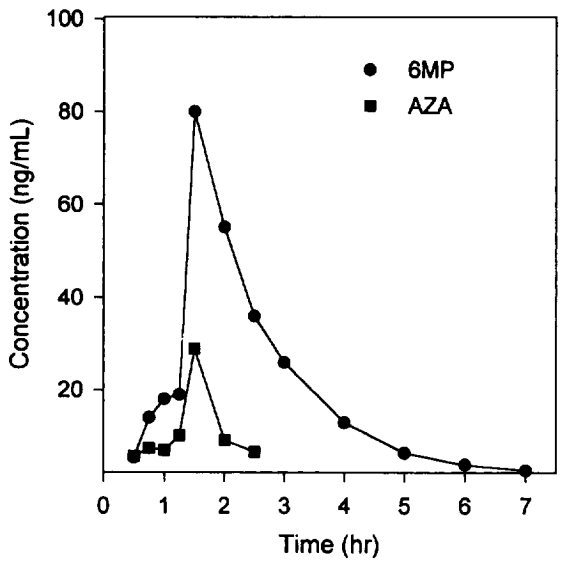


Fig. 7. Concentration–time curve for 6MP and AZA following 100 mg of AZA administered orally to a medication-free, non-smoking 30-year-old caucasian female weighing 69.3 kg.

AZA concentrations have previously required separation of samples with individual chromatographic determinations of 6MP and AZA [2–5]. Our assay simplifies pharmacokinetic studies of this type. We have demonstrated application of the assay in one subject following 100 mg of oral AZA (Fig. 7). The levels of 6MP are considerably higher than those of AZA, suggesting rapid conversion of AZA to 6MP. Similar studies in larger numbers of subjects, following a variety of delivery methods, would provide important insight into the absorption and metabolism of these compounds, facilitating development of improved delivery methods. The simplicity, sensitivity and rapidity of this assay allows it to be easily adapted for clinical use.

Acknowledgments

This work was supported by grant FD-T-000-886-03 from the Department of Health and Human Services. The authors would like to thank Burroughs-Wellcome and Dr. Gertrude Elion for generously supplying AZA metabolites used in this study.

References

[1] L. Lennard, Eur. J. Clin. Pharmacol., 43 (1992) 329.
[2] T.L. Ding and L.Z. Benet, J. Chromatogr., 163 (1979) 281.
[3] S.N. Lin, K. Jessup, M. Floyd, T.-P.F. Wang, C.T. Van Buren, R.M. Caprioli and B.D. Kahan, Transplantation, 29 (1980) 290.
[4] B. Odlind, P. Hartvig, B. Lindström, G.L. Lönnerholm, G. Tufveson and N. Grefberg, Int. J. Immunopharmacol., 8 (1986) 1.
[5] J. Liliemark, B. Pettersson, P. Lafolie, T. Zweig and C. Peterson, Ther. Drug Monit., 12 (1990) 339.
[6] K. Tsutsumi, Y. Otsuki and T. Kinoshita, J. Chromatogr., 231 (1982) 393.
[7] Y.-S. Uang, F.-L. Kang and K.-Y. Hsu, J. Chromatogr. B, 673 (1995) 43.
[8] A. El-Yazigi and F.A. Wahab, Ther. Drug Monit., 14 (1992) 312.
[9] D.J. Warren and L. Slørdal, Ther. Drug Monit., 15 (1993) 25.
[10] P. LaFolie, S. Hayder, O. Björk, L. Åhström and J. Liliemark, Acta Pædiatr. Scand., 75 (1986) 797.
[11] J.L. Rudy, J.C. Argyle, N. Winick and P. Van Dreal, Ann. Clin. Biochem., 25 (1988) 504.
[12] I. Bruunshuus and K. Schmiegelow, Scand. J. Clin. Lab. Invest., 49 (1989) 779.
[13] G.L.C. Chan, G.R. Erdmann, S.A. Gruber, P. Stock, S. Chen, N.L. Ascher and D.M. Canafax, Eur. J. Clin. Pharmacol., 36 (1989) 265.
[14] L. Endresen, S.O. Lie, I. Storm-Mathisen, H.E. Rugstad and O. Stokke, Ther. Drug Monit., 12 (1990) 227.
[15] K.T. Lin, F. Varin, G.E. Rivard and J.-M. LeClerc, J. Chromatogr., 536 (1991) 349.
[16] Y. Kato, T. Matsushita, T. Yokoyama and K. Mohri, Ther. Drug Monit., 13 (1991) 220.