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Assay of 6-mercaptopurine and its metabolites in patient plasma by high-performance liquid chromatography with diode-array detection

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Abstract

A reversed-phase high-performance liquid chromatography (HPLC) method was developed to determine 6-mercaptopurine (MP) and seven of its metabolites (6-thioguanine, 6-thioxanthine, 6-mercaptopurine riboside, 6-thioguanosine, 6-thioxanthine riboside, 6-methylmercaptopurine and 6-methylmercaptopurine riboside) simultaneously in human plasma. A volume of 100 μl of plasma was used. Protein was removed from the sample by a simple and easy ultrafiltration step and ultrafiltrate was directly injected onto the HPLC system. Analytes were detected and confirmed with a diode-array detector before quantitation at 295 and 330 nm. The limit of detection for the analytes ranged from 20 to 50 n*M*. For the majority of patients receiving a 1 g/m^2 MP intravenous infusion, MP and all metabolites except 6-thioguanine and 6-methylmercaptopurine riboside were present. This method serves as useful tool to characterize pharmacokinetics and pharmacodynamics of MP in oncology patients, and the small volume of plasma lends itself to pediatric studies. © 1999 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

6-Mercaptopurine (MP) constitutes an important part of the backbone for the treatment of childhood acute lymphoblastic leukemia (ALL). It is an inactive prodrug that is converted into cytotoxic and inactive metabolites (Fig. 1). MP is metabolized intracellularly into 6-thioinosine 5′-monophosphate (TIMP) by hypoxanthine phosphoribosyltransferase (HPRT), and subsequently into 6-thioxanthosine 5′-monophosphate (TXMP) and 6-thioguanosine nucleotides (mono-, di- and triphosphates, TGNs) [1]. TGNs are active metabolites of MP, which incorporate into DNA and RNA, causing cytotoxicity. Thioguanine (TG) base is an alternate substrate for HPRT; it is metabolized directly to 6-thioguanosine 5′-monophosphate (TGMP). Inactivation of MP and its metabolites is catalyzed by two primary enzymes: methylation by thiopurine *S*-methyltransferase (TPMT) and oxidation by xanthine oxidase. In vitro studies have shown that MP, TIMP and TGMP were methylated by TPMT to 6-methylmercaptopurine (MeMP), 6-methyl-thioinosine 5′-monophosphate (MeTIMP) and 6-methyl-thioguanosine 5′-mono-

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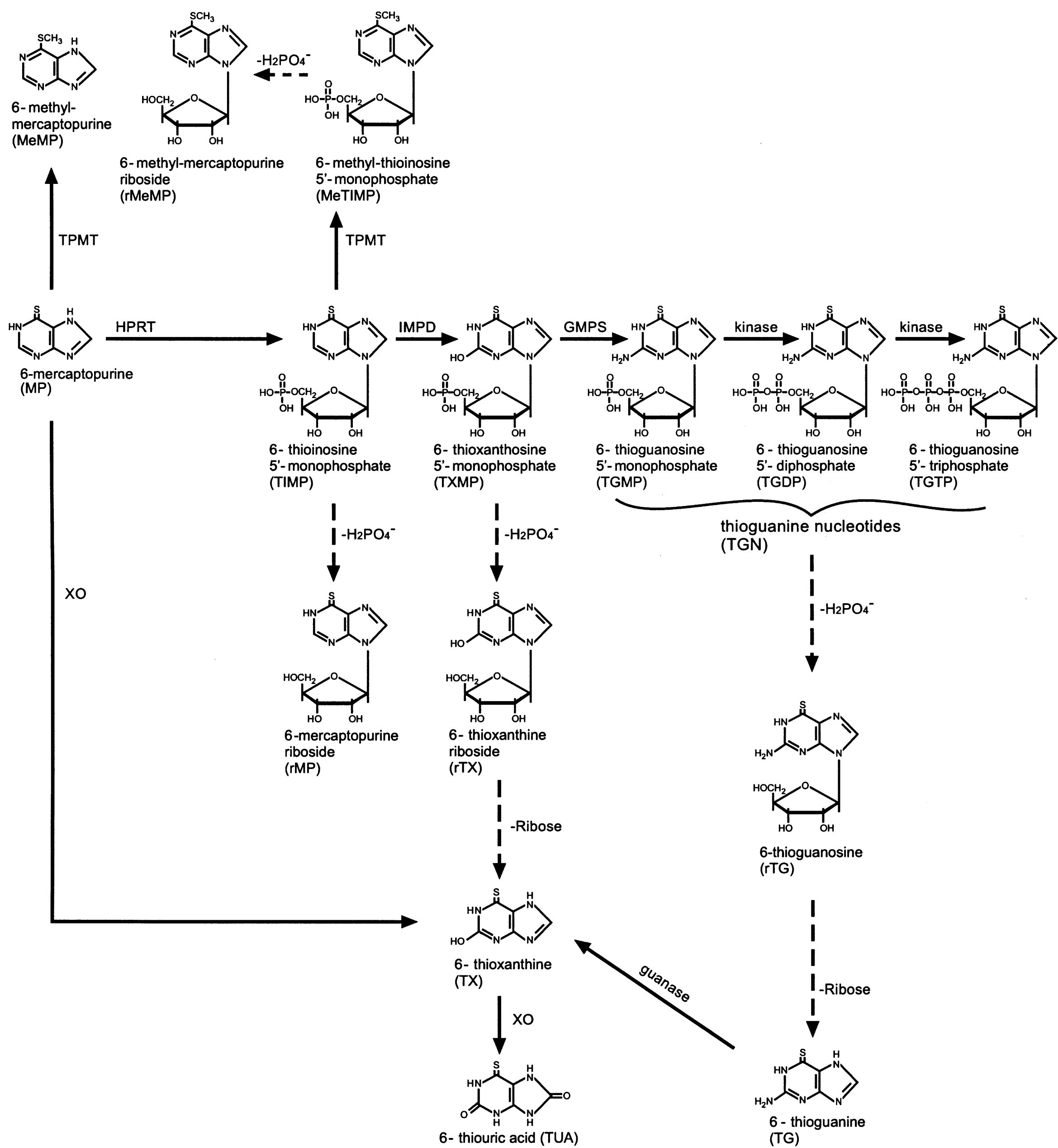

Fig. 1. 6-Mercaptopurine metabolic pathways are indicated by solid arrows: dashed arrows indicate putative products of dephosphorylation to nucleosides and further catabolism to nucleobases, process that have not yet been fully elucidated in plasma from patients. HPRT, hypoxanthine phosphoribosyltransferase; TPMT, thiopurine methyltransferase; XO, xanthine oxidase; IMPD, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthetase.

phosphate (MeTGMP) [1]. Xanthine oxidase, which is mainly active in liver, intestine and kidney [2], catabolizes MP to give inactive thiouric acid (TUA) via 6-mercapto-8-hydroxypurine and thioxanthine (TX) [3,4]. TX can also be formed from the metabolism of TG alternatively [5].

Published analytical methods for MP in biological fluids dated back to the 1950s [6] after its introduction by Elion et al. [7]. The initial drug analysis was performed in plasma in order to characterize the pharmacokinetics of MP. Recent studies have been focused on the determination of MP and its metabolites in erythrocytes [8–10] or DNA [11,12]; pharmacodynamic relationships have been established between red blood cell (RBC) TGNs with efficacy and toxicity of MP in childhood ALL [13,14]. The relationship between plasma pharmacokinetics and cellular levels of MP and its metabolites has not been well defined.

Various methodologies have been incorporated into high-performance liquid chromatography (HPLC) to detect MP in plasma [15–21], some of which have involved more complicated extraction or derivatization procedures. Attempts have also been made in recent studies to determine thiopurine nucleosides and bases in biological fluids. Most of the methods determined relatively few thiopurines [22–26], while Keuzenkamp-Jansen et al. analyzed nine thiopurine nucleosides and bases in human plasma and urine [5]. Other investigators concentrated on detection of specific MP metabolites such as 6-methylcaptopurine riboside (rMeMP) [27].

In the current study, we report a simple, quick and accurate method to determine plasma concentrations of MP, TG, TX, 6-mercaptopurine riboside (rMP), 6-thioguanosine (rTG), 6-thioxanthine riboside (rTX), MeMP and rMeMP simultaneously. Quantitation of thiopurine nucleosides and bases was achieved by using a gradient HPLC assay coupled with diode-array detection. Precision, accuracy and recovery were determined for all analytes in the study and application of the method for assay of plasma samples was demonstrated.

2. Experimental

2.1. Chemicals

6-Mercaptopurine (MP), 6-thioguanine (TG), 6-thioxanthine (TX), 6-mercaptopurine riboside (rMP), 6-thioguanosine (rTG), 6-methylmercaptopurine (MeMP) and 6-methylmercaptopurine riboside (rMeMP) were obtained from Sigma (St. Louis, MO, USA); 6-thioxanthine riboside (rTX) was a generous gift from the National Cancer Institute (Bethesda, MD, USA); TE buffer was from Promega (Madison, WI, USA); Microcon 10 ultrafiltration concentrators were from Amicon (Beverly, MA, USA); 0.2 μm nylon-66 filters used for mobile phase preparation was from Varian (Harbor City, CA, USA). All other chemicals were from Fisher (Fair Lawn, NJ, USA) and water used for all buffers was purified in a Milli-Q UV plus system (Millipore, Bedford, MA, USA).

2.2. Instruments

The liquid chromatograph consisted of an automated system with two Shimadzu (Columbia, MD, USA) LC-10AS liquid chromatograph pumps, a Shimadzu SCL-10A system controller, a Shimadzu SIL-10A autoinjector, a Shimadzu sample cooler kept at 4°C, and a Hewlett-Packard (Wilmington, DE, USA) 1100 diode-array detector. The data were acquired and analyzed using HP "Chemstations" software running on a Hewlett-Packard Vectra XA computer.

2.3. Chromatographic conditions

Separation of MP and its metabolites was performed on a Supelco (Bellefonte, PA, USA) reversed-phase analytical column (Supelcosil LC-18, 250×4.6 mm I.D., 5 μm particle size). A Supelco LC-18 guard column was used. There were two mobile phases. Mobile phase A consisted of 30 m*M* ammonium phosphate monobasic (pH 2.99)–methanol (98:2, v/v). Mobile phase B consisted of 30 m*M* ammonium phosphate monobasic (pH 2.99)–methanol (60:40, v/v). Flow-rate was maintained at 1 ml/min, with a pressure of less than 2500 p.s.i. throughout the run (1 p.s.i.=6894.76 Pa). The samples were isocratically eluted with 10% mobile phase B for the first 20 min, at which time a 15 min linear gradient to 100% mobile phase B was initiated and continued to the end of the run at 50 min. Thereafter the mobile phase was returned to 10% mobile phase B and 10 min were required for baseline stabilization. Autoinjection was made every 60 min with an injection volume of 50 μl. The autosampler was rinsed with 40% (v/v) methanol solution.

The diode-array detector was set for peak integration at 330 nm for the first 30 min, then changed to 295 nm until the end of the run, with UV scans from 200 to 400 nm for each marked peak.

2.4. Stock solutions, calibrators and control samples

The stock solutions of rMP, rTG, rTX and rMeMP were prepared using deionized water; MP, TG, TX and MeMP were dissolved in 0.5 *M* NaOH, and the pH of the solution was then adjusted to 4–5 with 0.5 *M* HCl. All stock solutions were stored at −70°C and periodically checked by HPLC.

Calibrators and control samples were prepared by spiking the stock solutions to pooled human plasma. The ranges of the calibration curves were different for the eight standards because of differences in anticipated concentration from patient plasma samples following an intravenous (i.v.) dose of MP at 1 g/m^2. The ranges were: MP, 1–64 μ*M*; rMP, 0.2–13 μ*M*; TG and rTG, 0.1–3 μ*M*; TX, rTX and MeMP, 0.2–6 μ*M*; and rMeMP, 0.02–1.3 μ*M*. Standard curves were fitted by linear regression using the peak area versus concentration curve.

2.5. Sample preparation

A 100-μl volume of plasma sample was pipetted into a 1.5-ml Eppendorf tube, and 25 μl of 1 *M* HCl was added. The sample was vortexed and kept on ice for 10 min. A 25-μl volume of TE buffer was added, and the sample was vortexed again and kept on ice for an additional 10 min. Protein was removed by ultrafiltration using a Microcon 10 concentrator at 4°C, 1700 *g* for 1 h. A 50-μl aliquot of ultrafiltrate was injected onto the HPLC system.

2.6. Precision, accuracy and recovery

Intra- and inter-day precision and accuracy of the assay were determined by analyzing plasma replicates at known low and high concentrations of thiopurines. Intra-day analysis was performed with 10 spiked replicates on the same day whereas inter-day evaluation was done on five different days. Accuracy was calculated as the percent error of the mean concentration from the spiked samples deviated from the target concentration (mean concentration/target concentration×100%) and precision was determined by the relative standard deviation (RSD). Precision and accuracy were also determined for three unknown plasma samples spiked with low, medium and high concentrations of thiopurines (each containing all eight analytes), with the analyst blinded to the sample preparation and concentrations.

Recoveries of analytes were determined by comparing the peak areas from spiked plasma samples with those from samples prepared with water at the same concentrations within the validated ranges. Seven replicates at low and high concentrations were analyzed.

2.7. Peak identification

Both retention time and UV spectra were used for peak identification. A UV spectra library was built for each of the standard compounds (MP, TG, TX, rMP, rTG, rTX, MeMP and rMeMP) by using control samples. The UV spectra of chromatographic peaks in patient samples with retention times corresponding to authentic standards were compared with the UV spectra of standards in the library to confirm peak identity.

2.8. Patients and sample collection

Blood was collected into heparinized tubes prior to and at 3, 6 and 20 h from the start of a 1 g/m^2 6 h i.v. MP infusion in children with ALL. After collection, the sample was centrifuged at 500 *g* for 2 min at room temperature. Plasma was aspirated into a screw cap vial and stored at −70°C until assay.

3. Results

3.1. Sample injections

A typical chromatogram of pooled human plasma spiked with the eight standards is presented in Fig. 2. Quantitation of analytes (TX, TG and rTG) in extracted samples was more reproducible when injected using a sample cooler than injection at room temperature. For example, for plasma spiked with rTG ranging from 0.2 to 3.2 μ*M*, the mean±standard

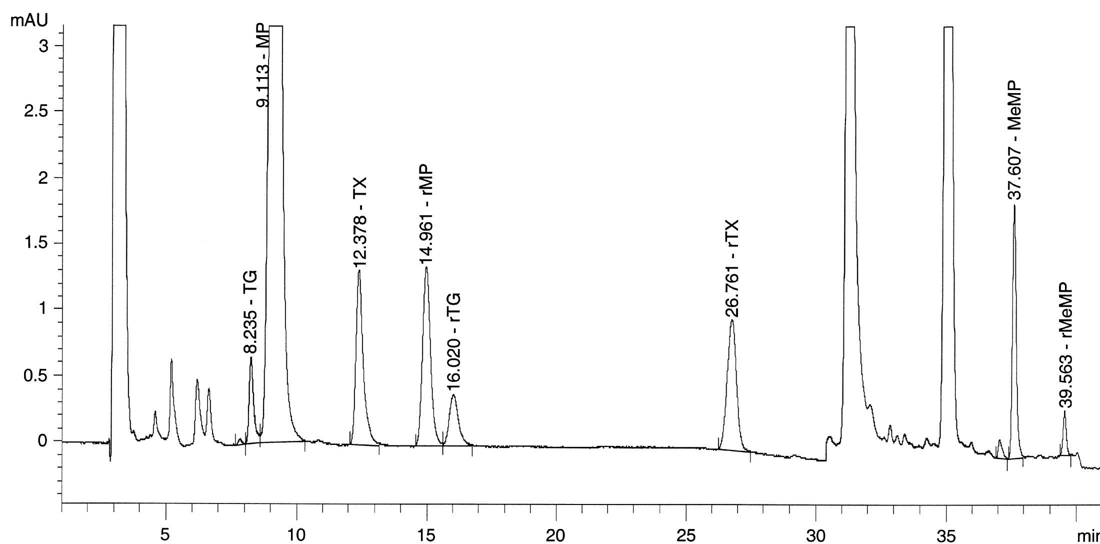

Fig. 2. Chromatogram of pooled human plasma spiked with thiopurine standards. Chromatographic conditions were as described in Experimental with numbers indicating retention time in minutes. The elution profile is as follows: TG, 6-thioguanine; MP, 6-mercaptopurine; TX, 6-thioxanthine; rMP, 6-mercaptopurine riboside; rTG, 6-thioguanosine; rTX, 6-thioxanthine riboside; MeMP, 6-methylmercaptopurine; rMeMP, 6-methylmercaptopurine riboside.

deviation (SD) difference in peak area among replicates was 28.4±13.1% at room temperature compared to 0.7±4.7% at 4°C. Because of that, an autosampler cooler was used for all subsequent assay procedures to decrease variability.

3.2. Calibration curves and detection limits

The standard curves demonstrated a linear relationship between peak areas and concentrations for all eight analytes (Table 1). The detection limit was about 20 n*M* for MP, TX, rMP, rTX, MeMP and rMeMP and 50 n*M* for TG and rTG.

3.3. Precision, accuracy and recovery

The intra-day ($n=10$) and inter-day ($n=5$) precision and accuracy of the assay are summarized in Table 2. RSD for intra- and inter-day analysis was <9% for all of the compounds and the accuracy ranged from 89.9 to 113% at known low and high concentrations of all eight analytes. Accuracy results

Table 1
Regression parameters for calibration curves of pooled human plasma spiked with thiopurine standards[a]

	Slope (area/μ*M*) (mean±SD)	*y*-Intercept (area) (mean±SD)	Correlation coefficient (mean±SD)
MP	32.2±0.97	−1.20±3.10	1.000±0.000768
TG	24.8±2.59	−1.19±0.75	0.999±0.000693
TX	28.9±1.39	0.40±1.16	0.999±0.000819
rMP	37.5±1.58	−1.57±1.12	0.999±0.000753
rTG	26.0±1.19	−0.82±0.31	0.999±0.000732
rTX	32.4±1.08	−1.47±1.09	0.999±0.000752
MeMP	23.3±1.20	−0.49±0.64	1.000±0.000612
rMeMP	29.3±1.51	0.40±0.13	0.999±0.000703

[a] Calibration curves were obtained from four consecutive runs performed over a more than half-year period.

Table 2
Precision and accuracy of standards in pooled human plasma spiked with known concentrations of thiopurines

Standard	Intra-day ($n=10$)			Inter-day ($n=5$)		
	Concentration (μ*M*) (mean±SD)	RSD (%)	Accuracy (%)	Concentration (μ*M*) (mean±SD)	RSD (%)	Accuracy (%)
MP	2.78±0.070	2.53	100.7	2.85±0.079	2.78	104.1
	58.6±0.550	0.94	101.6	57.8±1.08	1.88	100.3
TG	0.156±0.003	2.18	113.0	0.145±0.013	8.74	105.3
	2.92±0.024	0.81	101.2	2.91±0.061	2.09	101.0
TX	0.263±0.010	3.75	95.6	0.278±0.015	5.46	101.2
	5.90±0.063	1.07	102.5	6.09±0.465	7.63	105.8
rMP	0.531±0.015	2.76	96.2	0.566±0.015	2.72	102.6
	11.6±0.096	0.83	100.8	11.5±0.175	1.51	100.0
rTG	0.141±0.003	2.09	102.3	0.140±0.004	2.83	101.5
	2.87±0.026	0.89	99.7	2.85±0.071	2.50	99.1
rTX	0.266±0.006	2.15	96.6	0.276±0.017	6.21	100.1
	5.65±0.050	0.89	98.0	5.38±0.483	8.97	93.4
MeMP	0.272±0.012	4.26	98.6	0.268±0.010	3.77	97.0
	5.81±0.058	1.00	100.8	5.75±0.097	1.68	99.9
rMeMP	0.051±0.002	4.60	93.2	0.050±0.004	7.10	89.9
	1.16±0.017	1.47	99.9	1.17±0.027	2.28	100.9

determined by analysis of unknown plasma samples are shown in Table 3.

Recoveries from plasma were above 80% for all compounds at low concentrations and were above 85% for all analytes at high concentrations, with the exception of TX, which had somewhat lower recovery (Table 4).

3.4. Application to patient samples

The assay was suitable for assay of patient plasma. Chromatograms from a patient plasma sample before and 3 h from the start of a 6-h i.v. infusion of 1 g/m^2 MP are shown in Fig. 3a. The UV spectrum confirming identification for one of the peaks is also included in Fig. 3b.

4. Discussion

The current study reports the simultaneous analysis of MP and seven of its metabolites in a single chromatographic run. Our assay is similar to that reported by Keuzenkamp-Jansen et al. [5], although we also quantified rTX, which was present at significant concentrations with i.v. dosing of MP. Keuzenkamp-Jansen et al. used a protein precipitation step with perchloric acid in their sample preparation, and the eluted compounds were quantified using three different sets of wavelengths and chromatograms. We used hydrochloric acid for acidification to minimize possible oxidation of the thiopurines during preparation, and added an ultrafiltration step to remove protein. We found that perchloric acid destroyed rTX spiked to plasma samples. Our steps for protein removal and sample preparation were relatively quick and easy compared with other complicated extraction procedures, [15,19,20,25,28] and resulted in adequate recovery of all compounds, including rTX. The use of diode-array detection in the current method offered the advantage of confirming the identity of putative metabolites, particularly those in low abundance. Moreover, an intra-run wavelength change in our assay facilitated the quantitation of

Table 3
Precision and accuracy for three replicates of unknown plasma sample spiked with different concentrations of compounds, with the analyst blinded to the sample preparation and concentrations

Compound	Target concentration (μ*M*)	Mean observed concentration (μ*M*)	RSD (%)	Accuracy (%)
MP	1.79	1.68	0.95	93.7
	15.0	13.6	1.49	90.5
	31.9	27.7	1.06	86.8
TG	0.15	0.18	2.02	121.5
	1.24	1.32	1.27	106.0
	2.64	2.73	1.65	103.3
TX	0.28	0.27	2.12	93.5
	2.38	2.42	2.57	101.6
	5.06	5.09	1.57	100.6
rMP	0.59	0.58	1.19	98.1
	4.95	5.03	1.56	101.6
	10.5	10.4	1.23	99.2
rTG	0.16	0.15	1.82	96.7
	1.35	1.33	1.50	98.5
	2.86	2.78	2.61	97.3
rTX	0.30	0.28	2.57	92.6
	2.80	2.58	1.29	92.1
	5.95	5.47	3.23	91.9
MeMP	0.27	0.25	1.12	91.4
	2.31	2.33	1.72	100.6
	4.91	4.90	1.41	99.9
rMeMP	0.07	0.063	7.41	90.5
	0.59	0.66	0.20	111.1
	1.25	1.36	1.09	109.1

Table 4
Mean recoveries of thiopurines at low ($n=7$) and high ($n=7$) concentrations of analytes

	Compounds							
	MP	TG	TX	rMP	rTG	rTX	MeMP	rMeMP
Low concentration								
Concentration (μ*M*)	2.75	0.14	0.28	0.27	0.14	0.28	0.27	0.03
Mean recovery (%)	95.9	103.7	82.9	96.5	100.4	86.4	92.7	105.2
RSD (%)	3.6	10.8	3.2	2.1	4.4	4.0	4.8	8.6
High concentration								
Concentration (μ*M*)	57.5	2.88	5.78	5.70	2.86	5.76	5.71	0.60
Mean recovery (%)	92.4	95.5	74.7	91.0	94.4	98.6	93.6	88.7
RSD (%)	2.4	4.8	3.6	1.6	1.7	2.6	2.0	3.0

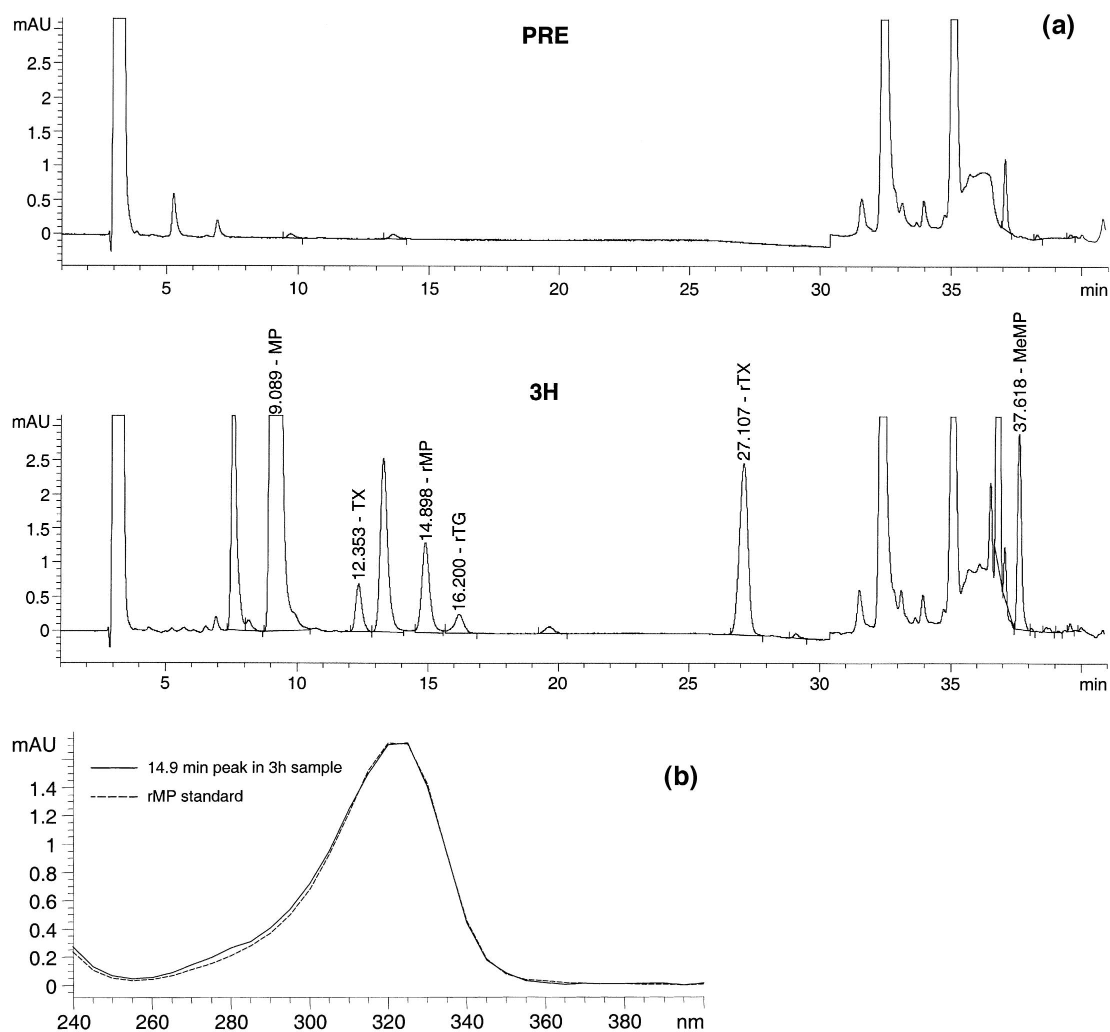

Fig. 3. (a) Chromatograms of patient plasma samples drawn immediately before and at 3 h after the start of a 6-h 6-mercaptopurine intravenous infusion (1 g/m^2). Analytes detected were: MP, 6-mercaptopurine; TX, 6-thioxanthine; rMP, 6-mercaptopurine riboside; rTG, 6-thioguanosine; rTX, 6-thioxanthine riboside; MeMP, 6-methylmercaptopurine. (b) UV spectrum confirming identification of peak corresponding to retention time of rMP in the same patient plasma sample obtained 3 h after MP infusion.

methylated and non-methylated MP bases and nucleosides in a single run, with the methylated compounds (having lower UV maxima) exhibiting later retention times (Fig. 2). Good separation of the eight analytes was observed using the current chromatographic conditions. Interfering peaks were excluded by the use of diode-array detection and sharp symmetric peaks were obtained for all standards (Fig. 2) spiked in human plasma.

Linearity for all thiopurine calibration curves was excellent over the anticipated concentration range with correlation coefficients of >0.999 for all the compounds (Table 1). Intra- and inter-day precision and accuracy were within 10% variation at low and high concentration of most analytes (Table 2) and for unknown plasma spiked with different concentrations of MP and its metabolites (Table 3). The recoveries for all compounds of the current assay were greater than 88% except TX and rTX. These high recoveries were comparable to methods re-

ported previously, [5,19–21,25,26] with relatively low variability. TX showed a recovery of only 75 to 80%, indicating that the current conditions may not be optimal for the determination of TX in plasma. However, TX is not thought to contribute to the cytotoxic effects of MP.

The limit of detection for MP, TX, rMP, rTX, MeMP and rMeMP in the present study was about 20 n*M* for each. This is equivalent to a range of about 150 to 300 pg on-column for the above compounds, with a detection limit of 150 pg for MP. Although the sensitivity of our assay for MP was less than that of Warren et al. (23 pg on-column) [21], Kato et al. (80 pg on-column) [20], and Narang et al. (75 pg on-column) [19], we used only 100 μl of plasma. Our detection limits of MP and its metabolites were comparable with those of other methods developed for multiple analytes analysis [5,24–26], but we used a lower plasma/blood sample volume. This makes our method suitable for use in pediatric pharmacokinetic studies in which blood volumes may be limiting.

Oral administration is the most commonly used dosing regimen for MP in maintenance therapy of childhood ALL. The usual MP concentration 5 h after an oral dose of 20–75 mg/m^2 MP in children with ALL on maintenance chemotherapy was higher than 66 n*M* and the maximum concentrations reported were from 0.066 to 4.3 μ*M* [29–32]. Although our method was calibrated to detect the higher MP concentrations during an i.v. infusion (1–64 μ*M*), the sensitivity was 20 n*M*, indicating that simply by recalibrating over a lower concentration range, the method would be suitable for use after oral dosing of MP in childhood ALL. Intravenous MP has been used in several recent treatment protocols [33–35], and thus studies evaluating drug interactions and pharmacodynamics of i.v. MP become more important. An assay that is suitable for measuring relatively high plasma concentration of MP and its metabolites after i.v. infusion will then be very useful.

Application of our assay was demonstrated by the analysis of plasma sample from a patient receiving 1 g/m^2 i.v. MP infusion; MP and five of its metabolites were detectable 3 h post infusion (Fig. 3a). There were two unidentified peaks (at 7.5 and 13.5 min, respectively) that did not correspond to the 6-methylmercapto-8-hydroxypurine (6MeM8OHP) metabolite described by Keuzenkamp-Jansen et al. [5], nor did their UV spectra match those of thiopurines. It is noted that TX, TG, rMP, rTX and rTG may be the breakdown products of TIMP, TXMP and TGNs of which TGNs are the active metabolites. MeMP is an inactive metabolite, whereas rMeMP has antitumor activity and can be activated to MeTIMP, which inhibits de novo purine synthesis [36]. It should also be acknowledged that the mechanisms by which some thiopurine nucleosides and bases make their ways from cells to plasma are incompletely characterized. Presumably nucleotides would have to undergo dephosphorylation to nucleosides prior to release from cells, some of which can be converted to the respective bases. Moreover, TX can be present as a product of xanthine oxidase acting directly on MP. TG and rMP have been found in plasma from children with ALL during consolidation and continuation therapy [25] whereas TX and rMP have been detected in urine obtained from patient receiving an i.v. infusion of MP [37]. The present method will be useful in determining MP metabolite concentrations in plasma, and will be used in conjunction with the assays for intracellular thiopurine concentrations to further define the metabolism and pharmacodynamics of MP in ALL.

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