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Short Communication

High-performance liquid chromatographic determination of thiopurine metabolites of azathioprine in biological fluids

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ABSTRACT

A selective and sensitive reversed-phase liquid chromatographic method for the analysis of thiopurine bases, nucleosides and nucleotides in biological samples was developed. A simple and rapid sample treatment procedure using perchloric acid deproteinization with dithiothreitol for the analysis of thiopurine bases and nucleosides is presented. The addition of dithiothreitol during sample collection and treatment improves recoveries. This procedure also allows the determination of thiopurine nucleotides by hydrolysis to their free bases after heating of the perchloric acid extract. The method was applied to the analysis of thiopurine metabolites in plasma and erythrocytes from lung-transplant patients under azathioprine therapy.

INTRODUCTION

Azathioprine is an effective immunosuppressive agent used with cyclosporine and corticosteroid in combination immunosuppressive protocols in organ transplantation [1]. However, dosage adjustment in azathioprine therapy are based on the occurrence of myelosuppressive toxicity. Azathioprine undergoes a complex metabolic pathway. It is rapidly converted into 6-mercaptopurine (6MP) which is either transformed into cytotoxic metabolites, such as nucleosides and nucleotides of 6-thioguanine (6TG) and 6 MP, or degraded to 6-thiouric acid (6TU) [2,3]. Thus it is extremely difficult to identify a biochemical marker that reliably predicts clinical outcome.

Determination of 6TU and 6MP plasma concentrations have been proposed for therapeutic drug monitoring [2,4], but this approach has not

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been considered to be predictive of azathioprine therapeutic efficacy or toxicity [5]. The immunosuppressive activity of azathioprine has been considered to depend on the formation of intracellular thiopurine nucleotides [6].

High-performance liquid chromatographic (HPLC) methods have been developed for the determination of nucleotides of 6TG [7] and/or nucleotides of 6MP [8–10]. Ion-exchange chromatography has been used to separate the thionucleoside mono-, di- and triphosphates [11]. Thionucleoside monophosphates of 6MP and 6TG have also been separated by ion-pair chromatography [9]. More recently, thionucleotides have been analysed after transformation to thiopurine bases by acid hydrolysis. The free base of each compound was then analysed by reversed-phase chromatography after laborious double-step solvent extraction [7,10].

In order to investigate more closely the relationship between thiopurine metabolites and the immunosuppressive activity of azathioprine, we have developed a selective and sensitive reversed-phase chromatographic method for the analysis of thiopurine bases and nucleosides. We established a simpler procedure for sample treatment, which consists of acid deproteinization of the biological samples and direct analysis of the extract for thiopurine base and nucleoside determination. This procedure also allows the determination of thiopurine nucleotides by conversion into their free bases after only a heating of the acid extract. We applied the method to the analysis of thiopurine metabolites in plasma and erythrocyte samples from lung-transplant patients under azathioprine therapy.

EXPERIMENTAL

Reagents

6-Mercaptopurine (6MP), 6-thioguanine (6TG), 6-thioxanthine (6TX), 6-thioguanosine (6TGuo), 6-thionosine (6TI), 6-thioinosinic acid (6TIMP) and DL-dithiothreitol (DTT) were purchased from Sigma (Isle d'Abeau, France). 6-Thiouric acid (6TU) and 6-thioguanosine monophosphate (6TGMP) were a gift from Burroughs Welcome (Research Triangle Park, NC, USA). Methanol, potassium dihydrogenphosphate and perchloric acid were obtained from Merck (Nogent-Sur-Marne, France). Owing to the limited availability of thiopurine standards, no suitable internal standard was found.

Apparatus and chromatographic conditions

The liquid chromatograph consisted of an automated system with two Model 510 pumps connected with a Model 715 sample processor, a Model 660 solvent programmer and a Model 481 variable-wavelength UV detector (all from Waters, St. Quentin Yvelines, France) and a D-2000 Chromatointegrator (Merck). The column (150 mm × 4.6 mm I.D.) was packed with Hypersil ODS 3 μm (Touzart et Matignon, Vitry, France). The analyses were performed in gradient elution mode using 0.02 *M* KH_2PO_4 (pH 3.5) and 0.02 *M* KH_2PO_4 (pH 3.5)–methanol (40:60, v/v) as eluents. The concentration of methanol in the elution solvent was varied from 0 to 4.8% over a period of 20 min using a convex gradient profile. The total run time (including equilibration time for the next run) was 40 min. The flow-rate was 1.2 ml/min, and the detection wavelength was set at 332 nm. The analyses were performed at ambient temperature.

Drug solutions

Stock solutions (100 μg/ml) of 6TG and 6TX were prepared by dissolving the drug in 0.1 *M* sodium hydroxide and making up to volume with 0.1 *M* hydrochloric acid containing 60 mg/l DTT. The others thiopurines were prepared in 0.1 *M* hydrochloric acid containing 60 mg/l DTT.

Sample collection and treatment

Blood samples of 10 ml were collected in heparinized tubes containing 1 mg of DTT and centrifuged without delay at low temperature (4°C). Plasma was decanted and stored at −20°C for the analysis of thiopurine bases and nucleosides. The leukocytes and the upper layer of the erythrocytes were removed. The erythrocytes were counted and drug concentrations were normal-

ized to $8 \cdot 10^8$ cells. A 1-ml volume of the remaining erythrocytes were transferred in a tube containing 10 mg of DTT, and rapidly deproteinized by 100 μl of perchloric acid (70%). Plasma was treated in the same way. The deproteinized samples were centrifuged at 2000 *g* for 10 min at 4°C. The supernatants were removed and 60-μl aliquots were injected into the column for thiopurine base and nucleoside analysis.

The remaining acid supernatant was heated for 45 min at 100°C. This procedure hydrolyses the thiopurine nucleotides and nucleosides to their free bases. The hydrolysate was cooled and then centrifuged, and 60 μl of the supernatant were injected into the column. All assays were run in duplicate.

RESULTS AND DISCUSSION

Chromatographic separation

Thiopurine bases and nucleosides have amino and hydroxyl groups. Thus their retention in chromatography can be influenced by the pH and the ionic strength of the mobile phase. The retention of thiopurine bases and nucleosides did not change significantly when the pH of the mobile phase was between 3.0 and 7.0. In this pH range, the compounds are in their neutral form according to their p*K* values, so they can be analysed by reversed-phase chromatography. The 6-thiopurine bases and nucleosides can be separated at pH 3.5. At this pH, the ionic strength of the buffer in the range 0.01–0.10 *M* did not significantly influence the retention of the compounds. In contrast, the retention behaviour of the bases and nucleosides was strongly influenced by the concentration of organic modifier (methanol). As expected, the retention decreased as the concentration of methanol increased, and this effect was used to establish the gradient elution system. From these data, the optimal mobile phase composition for the separation of thiopurine bases and nucleosides was determined. The chromatogram of a plasma spiked with the compounds of interest is shown in Fig. 1.

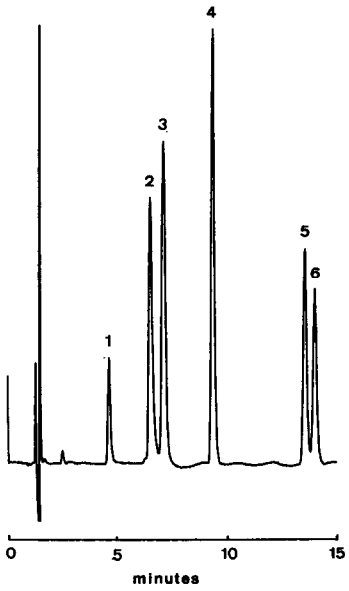

Fig. 1. Chromatogram of plasma spiked with 500 ng/ml of each compound. Peaks: 1 = 6TU; 2 = 6TG; 3 = 6MP; 4 = 6TX; 5 = 6TI; 6 = 6TGuo. Chromatographic conditions are described in Experimental.

Recovery

Analytical recoveries of thiopurine bases and nucleosides were determined by addition of known concentrations of compounds (50 and 500 ng/ml) to plasma or erythrocytes, and comparison of peak heights with those obtained by direct injection of aqueous standards. Recoveries (mean ± S.D.) were 89.0 ± 7.5% for 6TU, 81.0 ± 6.9% for 6TG, 81.0 ± 7.8% for 6MP, 88.0 ± 8.2% for 6TX, 88.0 ± 7.0% for 6TI and 88.0 ± 10.4% for 6TGuo. However, in erythrocytes, 6TU was not recovered. Zero recovery of 6TU from erythrocytes has been previously reported by Lennard [7] using a double-step solvent extraction with 0.3 ml of 10 m*M* DTT per 200 μl of packed cells.

The addition of DTT during sample collection and treatment protects the thiol group from oxidation. Furthermore, the addition of DTT results in analytical recoveries higher than 81% for each compound. When the biological samples were deproteinized without DTT, recoveries were *ca.* 50% lower and variable.

TABLE I

PRECISION AND ACCURACY IN ERYTHROCYTE SAMPLES

Compound	Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)
Intra-assay (n = 5)			
6TG	50	46	4.6
	500	494	2.9
6MP	50	49	4.8
	500	487	5.5
6TX	50	52	3.2
	500	485	2.2
6TI	50	48	0.0
	500	565	6.9
6TGuo	50	45	6.4
	500	546	6.3
Inter-assay (n = 5)			
6TG	50	43	7.8
	500	506	2.6
6MP	50	45	9.5
	500	510	5.8
6TX	50	58	9.8
	500	502	3.8
6TI	50	43	8.3
	500	498	6.1
6TGuo	50	44	8.1
	500	500	6.3

Linearity and precision

The linearity (peak height *versus* concentration) tested for each compound was excellent up to 7500 ng/ml with correlation coefficients greater than 0.998. The conversion of 6TGMP and 6TIMP into 6TG and 6MP, respectively, during the heating step under acidic conditions was 100%. The concentrations of 6TG and 6MP released from 6TGMP and 6TIMP were the same as those obtained from equimolar 6TG and 6MP added to control erythrocytes. The minimum amount detectable, at a signal-to-noise ratio of 4, was 1 ng for 6TU, 0.3 ng for 6TG, 0.2 ng for 6MP and 6TX, 0.4 ng for 6TI and 0.6 ng for 6TGuo.

The intra-assay and inter-assay precision and accuracy, determined by replicate analysis of erythrocyte samples spiked with the compounds of interest, are given in Table I. The Hypersil ODS column has long lifetime: *ca.* 600 samples were injected without any deterioration. There was no interference with the compounds of interest by related endogenous compounds, such as uric acid, hypoxanthine, xanthine, guanine and guanosine, or by other drugs, such as acyclovir, ganciclovir, cyclosporine, prednisone, allopurinol, oxipurinol, azathioprine, caffeine and theophylline.

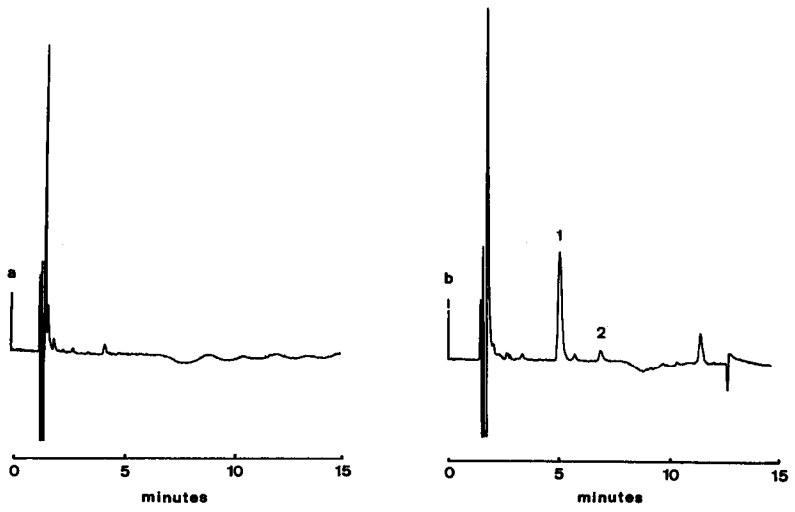

Fig. 2. Chromatograms of (a) blank plasma and (b) plasma sample from a patient under azathioprine therapy (2.5 mg/kg, daily). Blood samples were drawn 6 h after an oral dose of azathioprine. Peaks: 1 = 6TU (85 ng/ml); 2 = 6MP (5 ng/ml).

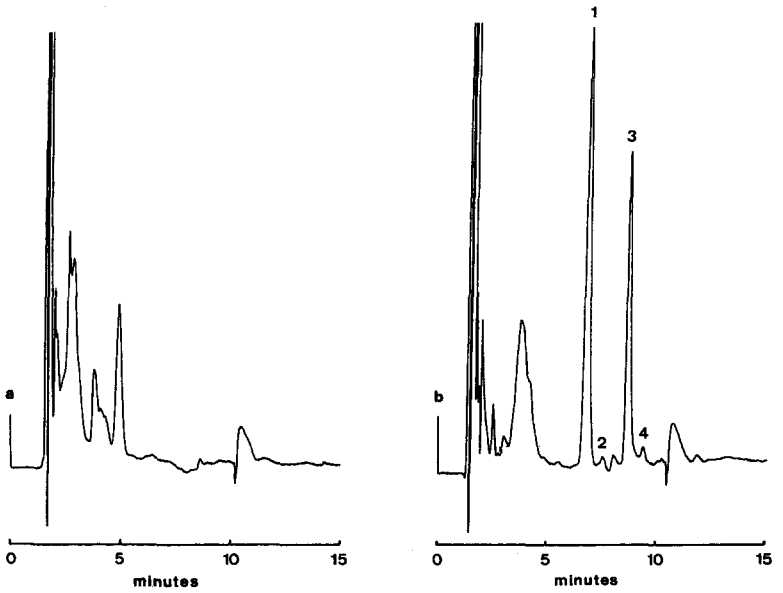

Fig. 3. Typical chromatograms of erythrocyte samples (a) before and (b) after heating of the PCA extract from a patient under azathioprine therapy. Peaks: 1 = 6TG (10 ng per $8 \cdot 10^8$ cells); 2 = 6MP (2 ng per $8 \cdot 10^8$ cells); 3 = unknown; 4 = 6TX (1 ng per $8 \cdot 10^8$ cells).

Analysis of patient samples

The method presented here was used to determine the thiopurine base, nucleoside and nucleotide contents in biological samples from lung-transplant patients under azathioprine therapy. Typical chromatograms of a blank plasma and a plasma sample from a patient who received azathioprine (2.5 mg/kg, daily) are shown in Fig. 2. Fig. 3 shows representative chromatograms of an erythrocyte sample before (a) and after (b) heating of the PCA extract.

CONCLUSION

The method described is selective and sensitive enough to determine thiopurine bases, nucleosides and nucleotides in biological samples obtained from patients under azathioprine therapy. The sample treatment procedure using PCA and DTT is simple and rapid compared with the tedious extraction methods reported in the literature. Furthermore, thiopurine nucleotides can be readily hydrolysed to thiopurine bases by heating the PCA extract.

The method is a suitable analytical tool for monitoring of thiopurine bases, nucleosides and nucleotides in patients under azathioprine therapy, and should be useful for gaining a better knowledge of the disposition and pharmacological activity of these compounds in organ-transplant patients.

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