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Short communication

Determination of thiopurine nucleotides in human lung tissue by high-performance liquid chromatography

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Abstract

A method for the analysis of thiopurine nucleotides in human transbronchial lung biopsy was developed. The sample treatment procedure is based on perchloric acid homogenisation and deproteinisation with dithiothreitol and hydrolysis of thiopurine nucleotides into their free bases by heating of the acid extract. Then, the free bases were analyzed in the gradient elution mode on a Hypersil ODS, 3-μm column using dihydrogenphosphate buffer–methanol as eluent. Mean analytical recoveries for 6-thioguanosine monophosphate and 6-thioinosinic acid from lung tissue were 97.0 ± 2.0 and $98.0 \pm 1.8\%$ at a concentration of 3.0 nmol/ml and the minimum detectable amounts were 3.5 and 2 pmol, respectively. The procedure described is simple and represents a suitable method for the investigation of thiopurine nucleotides in tissues.

1. Introduction

Azathioprine, a thiopurine compound that exhibits immunosuppressive activity, is currently used with cyclosporine and corticosteroid in a combined immunosuppressive protocol in organ transplantation [1]. The immunosuppressive effect is related to its rapid metabolism to 6-mercaptopurine and subsequent intracellular conversion to nucleotides of 6-thioguanine and 6-mercaptopurine. These nucleotides act as metabolite analogs and the pharmacodynamic activity mainly resulted from the inhibition of de-novo purine synthesis and/or incorporation into DNA and RNA [2,3]. Although graft as well as patient survival have been improved during the last years, the frequency of rejection episodes in lung transplant patients is higher than in other organ transplantations. In order to elucidate the relation between thiopurine metabolites and the immunosuppressive and/or myelotoxic activity of azathioprine, we investigated the distribution of thiopurine nucleotides in human. Despite the extensive use of azathioprine in lung transplantations, data on the diffusion of thiopurine nucleotides into the lung are not available. Therefore, we have developed a method for the determination of thiopurine nucleotides in transbronchi-

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al lung biopsy performed during graft rejection monitoring in lung transplant patients.

2. Experimental

2.1. Reagents

6-Thioguanine (6TG), 6-mercaptopurine (6MP), 6-thioxanthine (6TX), 6-thioinosinic acid (6TIMP) and DL-dithiothreitol (DTT) were obtained from Sigma (Isle d'Abeau, France). 6-Thiouric acid and 6-thioguanosine monophosphate (6TGMP) were a gift from Burroughs Welcome (Research Triangle Park, NC, USA). Methanol, potassium dihydrogenphosphate and perchloric acid were from Merck (Nogent-sur-Marne, France).

2.2. Chromatographic conditions

Thiopurine nucleotides were analysed using a high-performance liquid chromatographic method described previously [4]. The column (150 × 4.6 mm I.D.) was packed with Hypersil ODS 3 μm and the analyses were performed in the gradient elution mode using 0.02 *M* KH_2PO_4 (pH 3.50) and 0.02 *M* KH_2PO_4 (pH 3.50)–methanol (40:60, v/v) as eluents. The flow-rate was 1.2 ml/min and the detection was performed at 332 nm.

2.3. Sample collection and storage

Transbronchial lung biopsies were performed during the monitoring of lung allograft rejection [5]. Tissue samples were immediately frozen in liquid nitrogen to preserve the nucleotide pool and stored at −80°C until analysis. Simultaneously, 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 −80°C for the analyses of thiopurine bases. The leukocytes and the upper layer of the erythrocytes were discarded. The erythrocytes were counted and stored at −80°C for the analysis of thiopurine nucleotides.

2.4. Sample treatment procedure

Thawed lung tissue was transferred to a tube containing 10 mg of DTT and precooled 0.6 *M* perchloric acid (500 μl/10 mg of tissue, v/w) and the samples were homogenized for 2 min at 6000 rpm in a Stirpack homogenizer (Bioblock Scientific, France). After centrifugation at 2000 *g* for 10 min at 4°C, the supernatant was removed and heated for 45 min at 100°C. This resulted in a conversion of the thiopurine nucleotides into their free bases by acid hydrolysis. Then, the hydrolysate was cooled and centrifuged, and 70 μl of the supernatant were injected onto the column.

Plasma and erythrocyte samples (1 ml) were deproteinized by 100 μl of perchloric acid (70%) with 10 mg of DTT. The supernatants were analyzed according to the previously published method [4].

3. Results and discussion

A chromatogram of a plasma sample containing 6-thiouric acid, 6-thioguanine, 6-mercaptopurine and 6-thioxanthine is shown in Fig. 1. Chromatograms of a blank lung biopsy (a) and lung tissue sample (b) from a lung transplant patient under azathioprine therapy are shown in Fig. 2.

Due to the lack of a suitable standard for most of the active nucleotide metabolites, 6TGMP and 6TIMP were used to evaluate the sample treatment procedure. The degree of conversion of 6TGMP and 6TIMP into 6TG and 6MP respectively during the heating step under acidic conditions was 100%.

The mean analytical recoveries of 6TGMP and 6TIMP from lung tissue determined by adding known concentrations of the compounds in perchloric acid prior to homogenisation were 97.0 ± 2 and 98.0 ± 1.8% (mean ± S.D.) for 6TGMP and 6TIMP respectively, at a concentration of

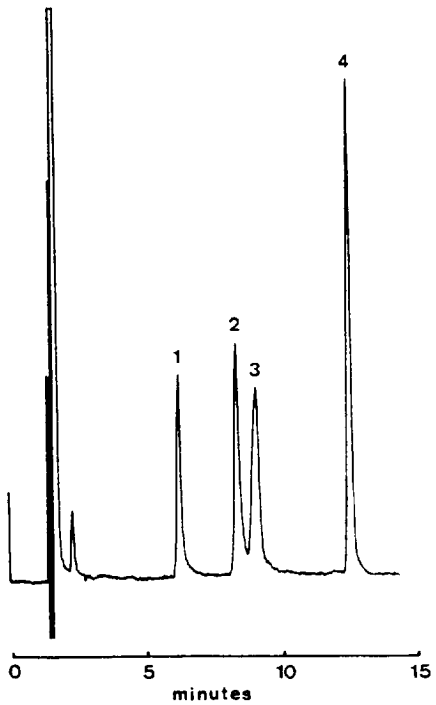

Fig. 1. Chromatogram of a plasma sample containing 0.60 nmol/ml of each compound. Peaks: 1 = 6TU, 2 = 6TG, 3 = 6MP, 4 = 6TX.

3.0 nmol/ml. The linearity (peak height versus concentration) was excellent up to 20 nmol/ml with correlation coefficients greater than 0.998. The regression equations were $y = 0.29x + 0.08$ for 6TGMP and $y = 0.39x + 0.02$ for 6TIMP. The minimum detectable amount defined as a signal-to-noise ratio of 4 was 3.5 pmol for 6TGMP and 2.0 pmol for 6TIMP, which corresponds to 2.5 nmol/g of tissue and 1.4 nmol/g of tissue for 6TGMP and 6TIMP, respectively. The intra-assay and inter-assay precision and accuracy in lung biopsy are given in Table 1.

Thiopurine nucleotide concentrations in trans-bronchial lung biopsy were determined in a lung transplant patient treated with azathioprine at a dosage of 2.5 mg/kg daily in combination with cyclosporine and methylprednisolone. Lung biopsy and blood samples were collected 6 h after oral administration. In lung tissue, 6-thio-guanine nucleotides (6TGN) and 6-mercapto-purine nucleotides (6MPN) were recovered at concentrations of 19 nmol/g of tissue and 2 nmol/g of tissue, respectively. Nucleotides of 6-thioxanthine were not detectable. In erythro-

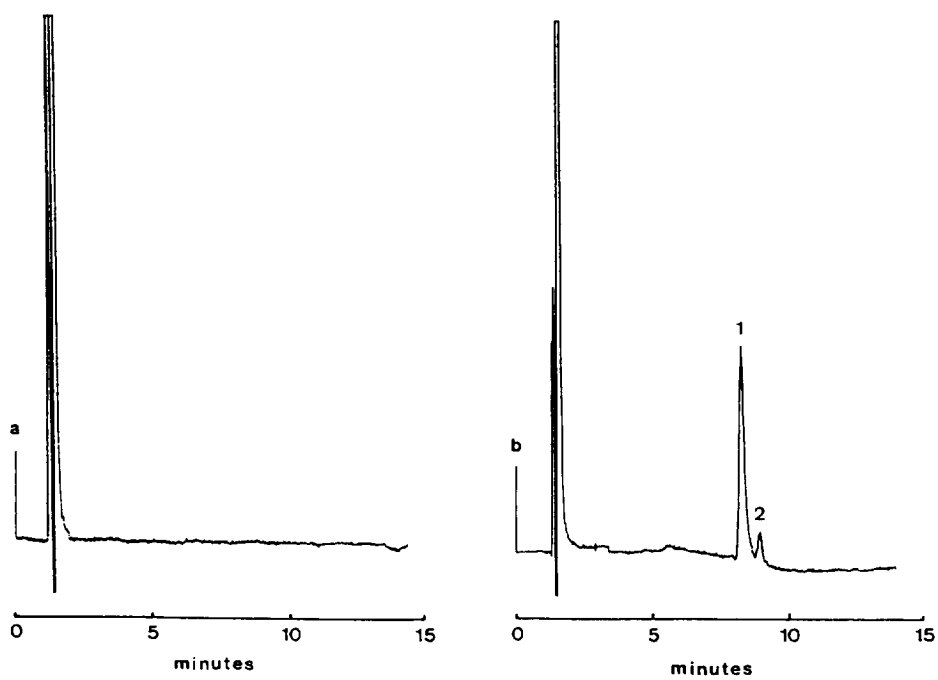

Fig. 2. Chromatogram of (a) blank lung biopsy and (b) lung biopsy from a lung transplant patient under azathioprine therapy (2.5 mg/kg, daily). Peaks: 1 = 6TGN, 2 = 6MPN.

Table 1
Precision and accuracy

Compound	Concentration added (nmol/ml)	Concentration found (nmol/ml)	C.V. (%)
Intra-assay (n = 5)			
6TGMP	0.60	0.61	2.6
	3.00	2.90	1.2
6TIMP	0.60	0.57	1.5
	3.00	2.90	2.0
Inter-assay (n = 5)			
6TGMP	0.60	0.58	8.6
	3.00	2.68	6.0
6TIMP	0.60	0.61	9.6
	3.00	3.14	7.9

cytes, only 6TGN was found at a concentration of 42 pmol/10^8 cells. In the plasma sample, 6-thioguanine was recovered at a concentration of 0.09 nmol/ml.

These results show that thiopurine nucleotides of 6-thioguanine and 6-mercaptopurine that are considered as active metabolites, can diffuse into the lung at significant levels. Further investigations are needed to define the role of these metabolites in the immunosuppressive and/or myelotoxicity effect of azathioprine in organ transplant patients.

In conclusion, the sample treatment procedure described for the analysis of thiopurine nucleotides in human lung biopsy is simple and represents a suitable method for the investigation of thiopurine nucleotides in tissues.

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