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Short communication

Sample preparation for the determination of purine nucleotide analogues in tissues

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Abstract

A sample treatment procedure for the determination of thiopurine and ganciclovir nucleotides in human tissues was developed. Owing to the lack of suitable standards for most of the active nucleotide analogues, the procedure was based on two steps: (1) perchloric acid homogenization and deproteinization of the tissue specimen and (2) conversion of purine nucleotides into parent drug or free bases by enzymatic or acid hydrolysis. The parent drug or purine bases formed were then analysed on a Hypersil ODS column using isocratic elution with dihydrogenphosphate buffer for ganciclovir nucleotides or the gradient elution mode with dihydrogenphosphate buffer–methanol for thiopurine nucleotides. The sample treatment procedure was evaluated using guanosine triphosphate (GTP), 6-thioinosinic acid (6TIMP) and 6-thioguanosine monophosphate (6TGMP) as standards. Mean analytical recoveries determined by adding known concentrations of standards to the tissue specimen before sample processing were higher than 97%. The sample preparation described is simple and represents a suitable method for the investigation of active nucleotide pool in tissues.

Keywords: Sample preparation; Nucleotides; Purine nucleotides; Thiopurine nucleotides; Ganciclovir nucleotides; 6-Thioinosinic acid; 6-Thioguanosine monophosphate

1. Introduction

Many drugs used as antiviral, immunosuppressive and antimetabolite agents are purine analogues [1,2]. The pharmacodynamic activity of these compounds is related to their intracellular conversion to nucleotides. The phosphorylated forms act as metabolite analogues and their activity mainly results from the inhibition of de novo purine synthesis and/or incorporation into DNA and RNA [3,4]. Analytical methods are essential for investigating the purine nucleotide analogue pool in tissues. However, data on tissue preparation for the analysis of purine analogues are scarce. Moreover, the lack of available standards of active nucleotides represents a major drawback in the development of analytical methods.

Sample preparation being an essential step prior to HPLC analysis of biological materials, we have developed a sample treatment procedure for the determination of purine nucleotide analogues in human tissues. The method was applied to the investigation of thiopurine and ganciclovir nucleotides in lung and myocardial tissues from azathioprine-treated patients and heart-transplant recipients under ganciclovir therapy.

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2. Experimental

2.1. Reagents

6-Thioguanine (6TG), 6-mercaptopurine (6MP), 6-thioxanthine (6TX), 6-thioinosinic acid (6TIMP), guanosine triphosphate (GTP), DL-dithiothreitol (DTT) and alkaline phosphatase, type VII-NT (10 000 glycine units/mg protein) were purchased from Sigma (St. Quentin Fallavier, France). 6-Thioguanosine monophosphate (6TGMP) was a gift from Burroughs Welcome (Research Triangle Park, NC, USA). Potassium dihydrogenphosphate, perchloric acid and methanol were obtained from Merck (Nogent-sur-Marne, France).

2.2. Chromatographic analysis

Ganciclovir nucleotides and thiopurine nucleotides were analysed using the high performance liquid chromatographic methods described previously [5,6]. The column (150 × 4.6 mm I.D.) was packed with Hypersil ODS (3 μm).

For ganciclovir nucleotide analysis, potassium dihydrogenphosphate solution (0.02 mol/l) was used as the eluent. The flow-rate was 1.5 ml/min and detection was set at 254 nm. The determination of thiopurine nucleotides was performed in the gradient elution mode using 0.02 mol/l KH_2PO_4 (pH 3.50) and 0.02 mol/l KH_2PO_4 (pH 3.50)–methanol (40:60, v/v) as mobile phase. 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 flow-rate was 1.2 ml/min and the detection wavelength was 332 nm.

2.3. Sample collection and storage

Myocardial and transbronchial lung biopsies were performed during the monitoring of allograft rejection [7]. Tissue samples were immediately frozen in liquid nitrogen to preserve the nucleotide pool [8–10] and stored at −80°C until analysis.

2.4. Sample treatment procedure

The sample treatment procedure is based on two steps: (1) homogenization and deproteinization of tissue specimens using perchloric acid and (2) conversion of purine nucleotide analogues into parent drug or free bases by enzymatic or acid hydrolysis. The main steps of the procedure are the following. Precooled 0.6 *M* perchloric acid was added to lung or heart biopsy (500–700 μl per 10 mg of tissue). DTT (10 mg) was added to tissue specimen from azathioprine-treated patients to protect thiol group from oxidation and to improve analytical recoveries [11]. The samples were then homogenized for 2 min at 6000 rpm in a stirpack homogenizer (Bioblock Scientific, France) and centrifuged without delay at 2000 *g* for 10 min at 4°C. The supernatants were then treated in two different ways depending on the compounds analysed.

Mono-, di- and triphosphates of ganciclovir were converted into ganciclovir by enzymatic hydrolysis. The supernatant was neutralized to pH 7–8 with sodium hydroxide. A 200-μl volume of the supernatant was incubated with 10 μl of alkaline phosphatase for 30 min at 37°C. Enzymatic reaction was stopped by heating for 2 min at 100°C. The supernatant was then evaporated to dryness at room temperature with an RC 1022 centrifugal evaporator (Jouan, St. Nazaire, France). The residue was reconstituted in 30 μl of mobile phase and 20-μl aliquots were injected into the column.

Thiopurine nucleotides were converted into their free bases by acid hydrolysis. The acid supernatant was heated for 45 min at 100°C. The hydrolysate was cooled and centrifuged and 70-μl aliquots were injected on to the column.

3. Results

Representative chromatograms of (a) blank lung biopsy and (b) a lung tissue sample from an azathioprine-treated patient are shown in Fig. 1. Fig. 2 shows typical chromatograms of (a) blank myocardial tissue and (b) a myocardial extract

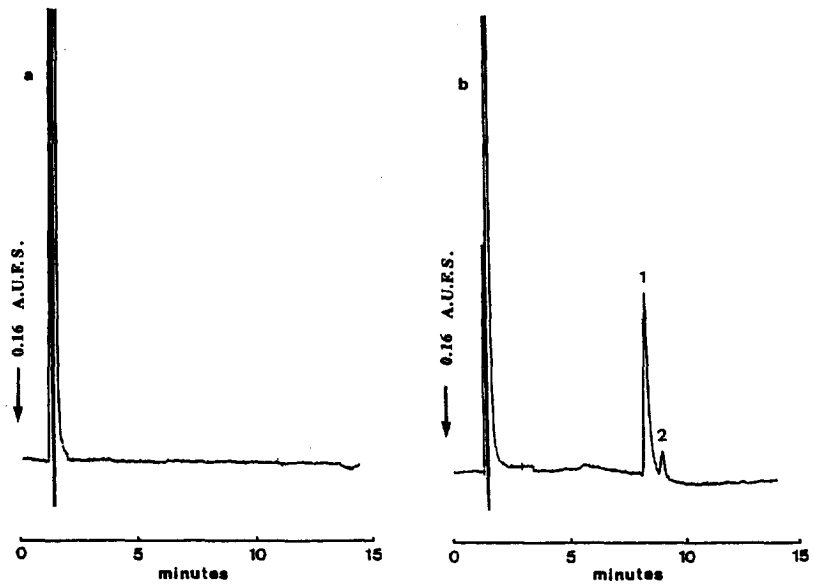

Fig. 1. Chromatograms of (a) blank transbronchial lung biopsy and (b) transbronchial lung biopsy from a lung-transplant patient under azathioprine therapy (2.5 mg/kg daily). For chromatographic conditions, see Experimental. Peaks: 1 = 6-thioguanine nucleotides; 2 = 6-mercaptopurine nucleotides.

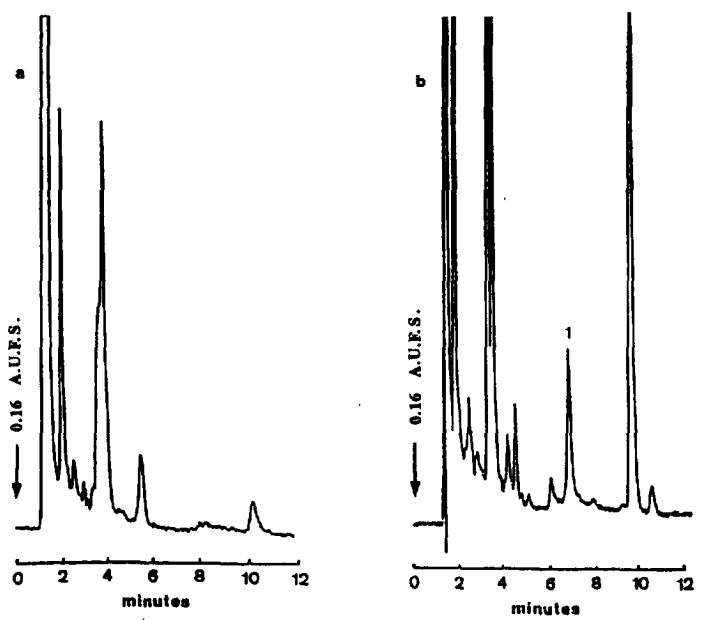

Fig. 2. Chromatograms of (a) blank myocardial tissue and (b) myocardial extract from a heart-transplant patient receiving 5.0 mg/kg daily of ganciclovir. For chromatographic conditions, see Experimental. Peak 1 = ganciclovir nucleotides.

from a heart-transplant patient under ganciclovir therapy.

Owing to the lack of commercially available standards for most of the active purine nucleotides, the sample treatment procedure was evaluated using GTP, 6TGMP and 6TIMP as standards. Under the conditions described, the degree of conversion of 6TIMP into 6-mercaptopurine, 6TGMP into 6-thioguanine and GTP into guanosine was 96, 100 and 96%, respectively. Mean analytical recoveries determined by adding known concentrations of standards to the tissue specimens before sample processing were $98.0 \pm 1.0\%$ and $101.0 \pm 2.0\%$ (mean $\pm$S.D.) for 6TIMP and GTP, respectively. The linearity (peak height versus concentration) was excellent up to 20 nmol/ml for 6TG and 6MP nucleotides and 40 μmol/l for ganciclovir nucleotides, with a correlation coefficient greater than 0.998. The minimum detectable amount, defined as a signal-to-noise ratio of 4, was 3.5 pmol for 6TG nucleotides and 2.0 pmol for 6MP nucleotides and ganciclovir nucleotides. The quantification limit was 2.5, 1.4 and 0.9 nmol/g of tissue for 6TG, 6PM and ganciclovir nucleotides, respectively, with a relative standard deviation (R.S.D.) of less than 15% under the analytical conditions described. The intra- and inter-day R.S.D.s were 1.0% and 4.4%, respectively, for ganciclovir nucleotides, 1.2% and 6.0%, 2.0% and 7.9% for 6TG and 6MP nucleotides, respectively.

4. Discussion

Sample preparation is essential for the determination of compounds in biological fluids. It requires several operations in order to free the analytes from matrix components and improve the specificity of the method. The specific operations are the following: (1) release of the compounds of interest from the biological matrix, (2) removal of proteins and other endogenous compounds and (3) procedures for liquid handling [12].

The sample treatment procedure described here combines in a single step the breakdown of the tissue matrix with the removal of macromolecules. This procedure leads to an improvement in analytical recoveries by reducing the loss of purine compounds owing to successive homogenization and extraction steps. Moreover, the conversion of nucleotide derivatives into their free bases or parent drug represents an alternative approach to the lack of available standards of active nucleotide analogues. With regard to stability studies of purine compounds in biological materials [8–10], the importance of sampling, storage and processing treatment for the accurate and reproducible determination of purine derivatives in biological samples must be emphasized.

In conclusion, the sample treatment procedure described for the determination of purine nucleotide analogues in lung and myocardial biopsy samples is simple and represents a suitable method for the investigation of the active nucleotide pool in tissues.

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