Journal of Chromatography, 339 (1985) 380—387
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2495

Note

High-performance liquid chromatographic method for the analysis of purine and pyrimidine bases, ribonucleosides and deoxyribonucleosides in biological fluids

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(First received August 27th, 1984; revised manuscript received November 21st, 1984)

Purine and pyrimidine bases, nucleosides and nucleotides have biological importance as components of nucleic acids, coenzymes and mediators of hormone action.

Several studies have revealed disorders in purine and pyrimidine metabolism in inborn errors of metabolism [1—4] and neoplastic diseases [5, 6]. More recently, interest has been focused on the role of purine and especially deoxypurine in hereditary immunodeficiency diseases [7—9] such as adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) deficiency.

Several methods for the analysis of bases and nucleosides by high-performance liquid chromatography (HPLC) have been described in the literature [10—17]. Ion-exchange chromatography has been used to separate bases and nucleosides [16, 17]; however, reversed-phase chromatography is at present the most commonly used liquid chromatographic mode for the separation of bases and nucleosides [10, 11, 13—15].

In this paper is presented a rapid reversed-phase HPLC procedure for the analysis of purine and pyrimidine bases, the corresponding ribonucleosides and major deoxyribonucleosides, with high sensitivity and selectivity.

0378-4347/85/$03.30

Also, ultra-rapid treatment of the blood samples was used to prevent metabolic conversion of purines during the sample preparation. In order to obtain the optimal separation of all compounds, the influence of the pH, the concentration of the organic modifier and the effect of the ionic strength on retention behaviour of the purine and pyrimidine compounds were examined.

This method was applied to the analysis of bases and nucleosides in plasma and cell samples and was used as an analytical tool to determine purine metabolites in ADA deficiency.

EXPERIMENTAL

Reagents

The bases and nucleosides were purchased from Sigma (St. Louis, MO, U.S.A.). Potassium dihydrogen phosphate, perchloric acid, and methanol were obtained from E. Merck (Darmstadt, F.R.G.).

Apparatus

The liquid chromatograph consisted of a Chromatem 800 (Touzart et Matignon, Vitry, France) equipped with a variable-wavelength absorbance detector (Pye Unicam PU 4020; Philips, Bobigny, France) operating at 254 nm and an SP 4100 integrator (Spectra Physics, Orsay les Ulis, France). The column (15 cm × 4.6 mm I.D.) and the precolumn (5 cm × 4.6 mm I.D.) used as a guard column were packed with Hypersil ODS 3 μm (Shandon, Cheshire, U.K.) by the slurry packing technique as described by Coq et al. [18].

Chromatographic conditions

The analyses were performed with the gradient elution mode using 0.02 *M* potassium dihydrogen phosphate, pH 6.0 and 0.02 *M* potassium dihydrogen phosphate, pH 6.0—methanol (40:60) as eluents. The concentration of methanol in the elution solvent was varied from 0% to 12% over a period of 20 min. The gradient proceeded through four steps: 0% for 3 min, 0% to 5% in 2 min, 5% to 12% in 10 min, and 12% for 5 min. The flow-rate was 1.5 ml/min.

Sample collection and treatment

Blood was collected in a heparinized tube. In accordance with our previous findings, this tube was immediately centrifuged to prevent metabolic changes which occur when plasma is left in contact with the erythrocytes [19]. Plasma was decanted and kept in an ice bath. The leucocytes and the upper layer of erythrocytes were removed; 500 μl of the remaining erythrocytes were then rapidly deproteinized by 50 μl of 35% perchloric acid (PCA). Plasma was treated in the same way. The deproteinized samples were centrifuged for 10 min at 1500 *g* at 4°C. After centrifugation, the supernatants were removed and adjusted to pH 6—7 with 5 *M* sodium hydroxide. The extracts were stored at −20°C awaiting analysis.

Lymphocytes were isolated from freshly drawn heparinized blood on a one-step Ficoll—Isopaque gradient [20] by centrifugation at 700 *g* for 35 min. Lymphocytes were washed three times in a sodium chloride medium. The cell

pellet was resuspended ($1 \cdot 10^6$ to $5 \cdot 10^6$ cells per ml) in a sodium-buffered saline medium and an aliquot was removed for cell number determination. The lymphocyte suspension was treated in the same way as plasma and erythrocytes.

In order to minimize the slight dilution caused by PCA deproteinization, an ultrafiltration procedure described by other authors [21, 22] was tested. The plasma samples were filtered through an Amicon Micropartition system MPS 1 (Amicon, Paris, France) at 500 *g* for 20 min. Using this method, we found changes in the metabolite content. On the chromatogram of a plasma spiked with adenosine and deoxyadenosine, we observed an increase of inosine and deoxyinosine peaks and a simultaneous decrease of adenosine and deoxyadenosine peaks. The recoveries of adenosine and deoxyadenosine added to plasma were approximately 96% using PCA deproteinization and only 73% by the ultrafiltration procedure.

RESULTS AND DISCUSSION

Chromatographic separation

In order to optimize the chromatographic separation of sixteen bases and nucleosides, the influence of the pH, the concentration of the organic modifier and the ionic strength of the mobile phase on the capacity ratio (k') were investigated.

Fig. 1 shows the effect of the pH of the mobile phase on the retention of the bases, the ribonucleosides and the deoxyribonucleosides, the pH ranging from 4.5 to 6.5. In the pH range considered, the majority of the compounds are in their neutral form, so they can be analysed by reversed-phase chromato-

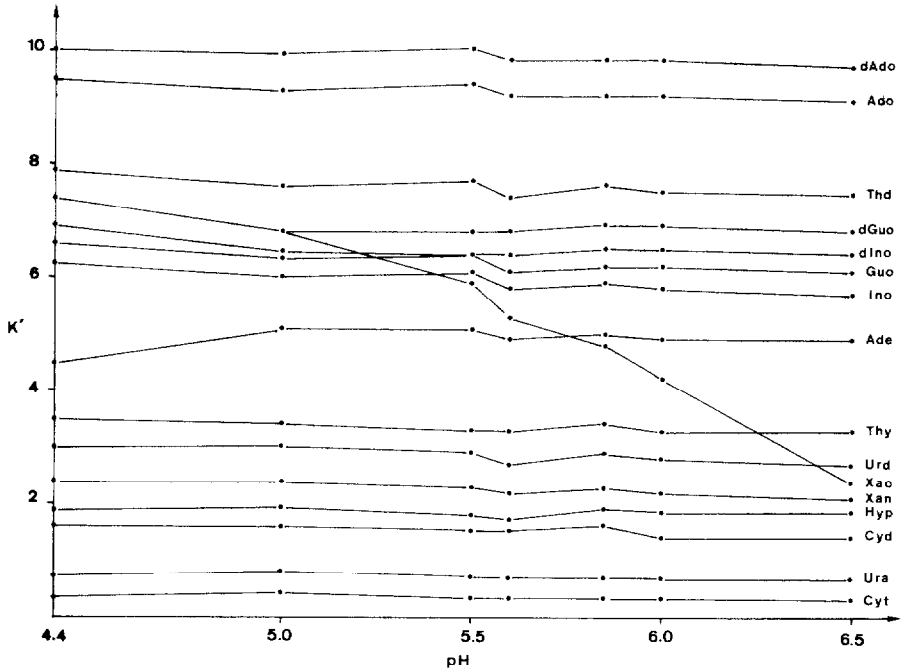

Fig. 1. Effect of the pH of the mobile phase on the retention of bases and nucleosides. Chromatographic conditions are described in Experimental. For abbreviations, see Fig. 3.

graphy. The retention behaviour of the bases and nucleosides is related to their pK values [23]. Xanthosine, which has a pK_a of 5.7, shows important variations of k' when the pH of the eluent increases from 4.5 to 6.5. The retention of the other compounds whose pK_b values are below 4.5 (weak bases) and pK_a values above 6.5 (weak acids) did not change significantly in this pH range. Fig. 1 shows that the separation of all the bases and nucleosides can be attained at pH 6.0.

The influence of the percentage of organic modifier (methanol) in 0.02 M potassium dihydrogen phosphate, pH 6.0 is represented in Fig. 2. The retention of bases and nucleosides decreases as the concentration of methanol is

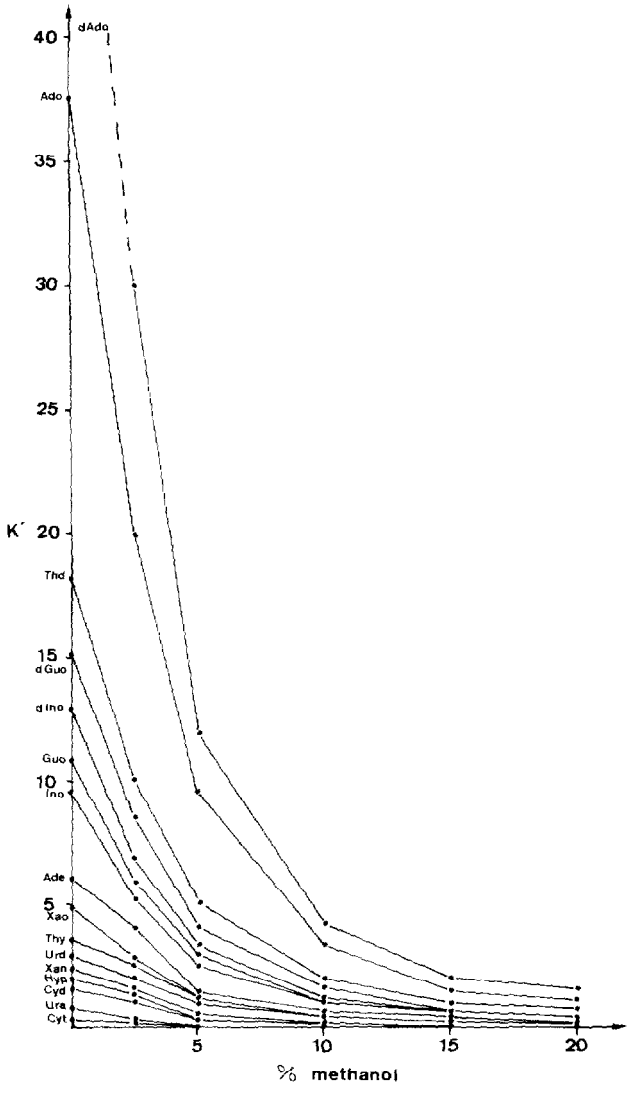

Fig. 2. Effect of the concentration of methanol on the retention of bases and nucleosides. Stationary phase: Hypersil ODS 3 μm. Mobile phase: 0.02 M potassium dihydrogen phosphate pH 6.0 and 0.02 M potassium dihydrogen phosphate—methanol (0—20%). For abbrevations, see Fig. 3.

increased. The influence of the concentration of the organic modifier on the degree of retention of these compounds was used to establish the gradient elution system.

The retention of the compounds was not significantly influenced by the ionic strength of the buffer over the range 0.01—0.10 *M* potassium dihydrogen phosphate.

Using these data, the optimal mobile phase composition for the separation of bases, ribonucleosides and deoxyribonucleosides was determined. The corresponding chromatogram is shown in Fig. 3. The separation of the sixteen compounds can be achieved in 20 min. The chromatogram in Fig. 3 shows that adenine tails in this chromatographic system. Most compounds containing an amino group tail badly on alkyl silica packings [24, 25]. The tailing peak shape of adenine may be related to the interaction of the adenine amino group with residual silica hydroxyls. The data reported by Zakaria and Brown [26], who

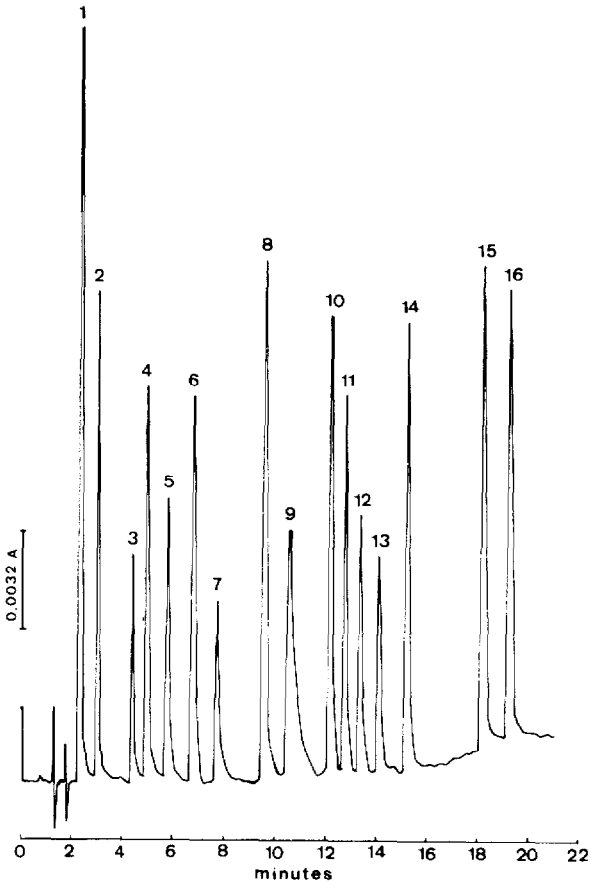

Fig. 3. Chromatographic separation of bases and nucleosides. Chromatographic conditions are described in Experimental. Peaks: 1 = cytosine (Cyt), 2 = uracil (Ura), 3 = cytidine (Cyd), 4 = hypoxanthine (Hyp), 5 = xanthine (Xan), 6 = uridine (Urd), 7 = thymine (Thy), 8 = xanthosine (Xao), 9 = adenine (Ade), 10 = inosine (Ino), 11 = guanosine (Guo), 12 = deoxyinosine (dIno), 13 = deoxyguanosine (dGuo), 14 = thymidine (Thd), 15 = adenosine (Ado), 16 = deoxyadenosine (dAdo).

have found that the 6-aminopurine substituent interacts with residual silanols of the alkyl—silica bonded phase, give support to this hypothesis.

Recovery and detection limit of adenine and purine nucleosides

The recoveries of adenine, inosine, deoxyinosine, guanosine, deoxyguanosine, xanthosine, adenosine and deoxyadenosine added to plasma were 97% ± 4. The relationship between the concentration and the peak area of these compounds was linear over the range 0.25—20 μmol l^{-1}. The minimum quantity detectable was 2.5 pmol for guanosine, deoxyguanosine, adenosine, deoxyadenosine, 3.5 pmol for xanthosine, inosine, deoxyinosine and 5 pmol for adenine.

Applications

This method was applied to the analysis of purine nucleosides and adenine in plasma and cell samples from healthy subjects. Neither xanthosine, guanosine and inosine nor deoxyribonucleosides were detected in plasma and erythrocyte samples. Adenine was found at a concentration of 1.0 ± 0.7 μmol l^{-1} in plasma and at a concentration of 1.9 ± 0.6 μmol l^{-1} in erythrocytes. Inosine was the only nucleoside found in lymphocytes and at a concentration of 1.2 ± 0.7 nmol

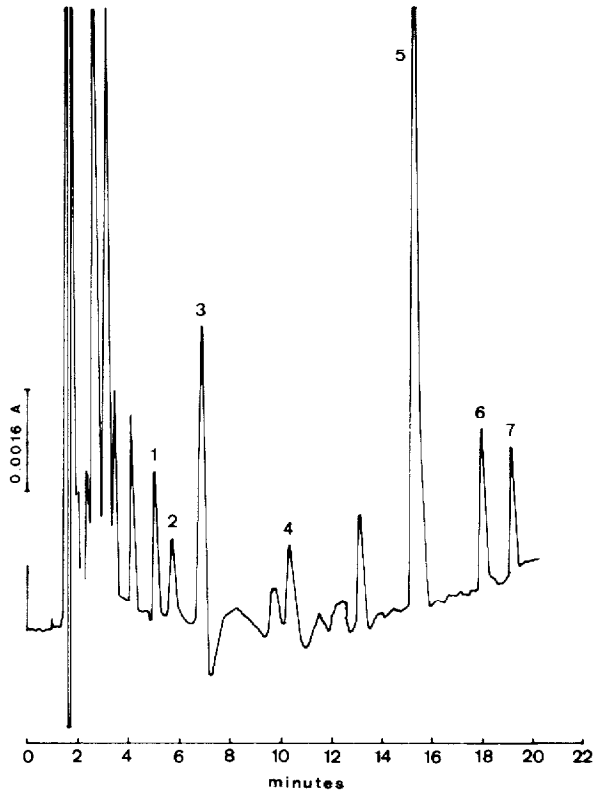

Fig. 4. Chromatogram of plasma sample from a patient with ADA deficiency who had undergone a bone marrow transplant. Chromatographic conditions are described in Experimental. Injection volume: 20 μl. Peaks: 1 = hypoxanthine, 2 = xanthine, 3 = uridine, 4 = adenine, 5 = peak observed when using PCA deproteinization, 6 = adenosine, 7 = deoxyadenosine.

per 10^6 cells (n = 6). Adenosine was not detected in erythrocyte and in lymphocyte cells, whereas in plasma the concentration of this compound was found to be 0.8 ± 0.4 μmol l^{-1} (n = 6). Peak identification of adenosine and inosine was confirmed by the enzymatic reaction with adenosine deaminase and purine nucleoside phosphorylase, respectively.

Also, using this HPLC method, purine nucleosides and adenine were determined in biological fluids and cells of an ADA-deficient patient who had undergone a bone marrow transplant ten years previously. The patient has since recovered normal immunological functions.

Adenosine deaminase deficiency results in a severe combined immmunodeficiency disease [7, 27]. The ADA deficiency leads to an accumulation of adenosine, deoxyadenosine and adenine deoxynucleotides in plasma, erythrocytes and lymphocytes [27].

The chromatogram of a plasma sample from the child who had undergone bone marrow transplant is shown in Fig. 4. In plasma, the adenine and adenosine concentrations were increased (2.2 and 2.3 μmol l^{-1}, respectively). Also, deoxyadenosine was found at a concentration of 1.8 μmol l^{-1} whereas this compound was not detectable in plasma samples from healthy subjects. In erythrocyte samples, adenosine and deoxyadenosine were not detected and adenine was present at a concentration of 1.5 μmol l^{-1}. In lymphocytes, adenosine and deoxyadenosine were undetectable; furthermore, inosine was not detectable whereas this compound was present in lymphocytes from healthy subjects. Our results show that in the patient studied, the bone marrow transplant had not entirely corrected the purine metabolism disorder.

With the rapid reversed-phase procedure described in this paper, the purine and pyrimidine bases, the corresponding ribonucleosides and major deoxyribonucleosides can be separated with high selectivity. A rapid PCA deproteinization of blood samples was used in order to prevent purine metabolic changes during the sample treatment.

The HPLC method described is an accurate analytical tool for the determination of purine metabolite levels in healthy subjects and in patients with purine metabolism deficiency.

ACKNOWLEDGEMENTS

We should like to thank P. Baltassat for his interest in our work in the field of purine metabolism, Dr. G. Souillet who kindly entrusted us with the biochemical study of his patient and Mrs. Adla for her secretarial assistance.

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