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# OPTIMIZATION OF THE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF PURINE DERIVATIVES IN ERYTHROCYTES, THYMOCYTES AND LIVER MITOCHONDRIA

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## SUMMARY

Various methods are described for the analysis of purine derivatives in biological samples by ion-pair high-performance liquid chromatography (HPLC) with both gradient and isocratic systems. A new approach is proposed that is suitable for the separation of nucleic acid constituents in different cells with a specific enzymatic activity pattern. The ion-pair HPLC methods were developed for the analysis of erythrocytes, lymphocytes and mitochondria acid-soluble fractions in clinical and experimental studies of normal and altered nucleotide metabolism. The results of studies of purine metabolite redistribution in mouse liver mitochondria during a 30-min incubation at 37°C and data on purine metabolic alterations in mouse thymocytes during hepatoma growth are discussed.

## INTRODUCTION

Despite the rapid advances in chromatographic techniques for the determination of nucleic acid constituents, the choice of an appropriate high-performance liquid chromatographic (HPLC) procedure for the separation of purines and pyrimidines depends strongly on the biological samples concerned. This problem is associated with the variety of purine and pyrimidine metabolic profiles in different cells and tissues and also with the purpose of the investigation. This necessitates the development of appropriate methods for the separation and analysis of nucleic acid constituents in each individual instance. Ion-pair HPLC affords

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the possibility of developing tissue-specific chromatographic procedures [1,2]. In this study, HPLC was used to separate and identify purine nucleotides, nucleosides and bases in mouse liver mitochondria in a single analysis under gradient conditions. Major oxypurines actively metabolized in red blood cells and the products of purine catabolism were examined in erythrocytes of newborn infants with perinatal hypoxia using an isocratic system with tetrabutylammonium phosphate (TBA). Additionally, the application of pentane- and heptanesulphonic acids made it possible to increase the resolution of hypoxanthine and guanine and also of cytosine and cytidine in mouse thymocytes.

## EXPERIMENTAL

### *Chemicals*

Nucleic bases, nucleosides and their mono-, di- and triphosphates were purchased from Sigma (St. Louis, MO, U.S.A.), potassium dihydrogenphosphate and ammonium dihydrogenphosphate from Prolabo (Paris, France), TBA (PIC reagent A) from Beckman (San Ramon, CA, U.S.A.), pentanesulphonic acid (PIC reagent B5) and heptanesulphonic acid (PIC reagent B7) from Waters Assoc. (Milford, MA, U.S.A.), carboxyatractylosid (CAT) and oligomycin from Boehringer (Mannheim, F.R.G.) and HPLC-grade acetonitrile (CromAR) from Promochem (Wesel, F.R.G.). Reverse-osmosis water from a Milli-R/Q water purifier (Millipore, Bedford, MA, U.S.A.) was passed through a Norganic cartridge (Waters Assoc.) for the efficient removal of organic contaminants.

### *Instrumentation*

Two HPLC systems were used for the assay of the nucleotide content of the biological samples.

System A (System Gold) consisted of programmable solvent delivery module 126, scanning detectory module 167 and Model 210A manual injector (all from Beckman). An IBM PC AT with Gold chromatography software/interface was used for the module control, storing raw chromatographic data and subsequent baseline correction and peak-area calculations. Separations were accomplished with a Beckman Ultrasphere IP (5 $\mu$m) column (250 mm$\times$4.6 mm I.D.) under gradient conditions and an Ultrasphere ODS (5 $\mu$m) column (150 mm$\times$4.6 mm I.D.) in the isocratic mode.

System B was assembled from the following Waters liquid chromatograph modules: Model 510 solvent delivery system, Model 441 absorbance detector, Model 481 Lambda-Max LC spectrophotometer, Model U6K manual injector and Model 730 data module peak-area integrator. Isocratic separation was performed using a $\mu$Bondapak (10 $\mu$m) stainless-steel column (300 mm$\times$3.9 mm I.D.) (Waters Assoc.).

In both system A and system B two wavelengths (254 and 280 nm) were used simultaneously for better peak identification.

*Sample preparation*

The mitochondria were isolated from the liver of male C3HA mice as described previously [3], and 50 μl of the acid-soluble fraction (ASF) of mitochondria suspension were used for the HPLC analysis.

Lymphocytes were isolated from mouse thymus by centrifugation in Ficoll, and 50 μl of lymphocyte ASFs were used for HPLC analysis [4].

## RESULTS AND DISCUSSION

*Ion-pair gradient HPLC analysis of purine nucleotides, nucleosides and bases in mouse liver mitochondria*

We previously developed a method for the simultaneous separation of nucleotides, nucleosides and bases using TBA as an ion-pair modifier under acetonitrile gradient conditions [4,5]. This chromatographic procedure was optimized in order to obtain the best possible resolution of the early eluting nitrogen bases and nucleosides using an Ultrasphere IP column. The mobile phase was modified by adding 3% of acetonitrile to the initial buffer and using concave gradient elution. Fig. 1 shows the separation of purine compounds in mouse liver mitochondria.

Mouse liver mitochondria contain a purine 5′-nucleotidase (EC 3.1.3.5), AMP deaminase (EC 3.5.4.6), adenosine deaminase (EC 3.5.4.4) [6,7] and deoxyguanosine kinase (no EC number) [8]. However, little is known about the concentrations of purine nucleosides and bases in mitochondria. It was found that these concentrations were about one order of magnitude lower than those of adenosine

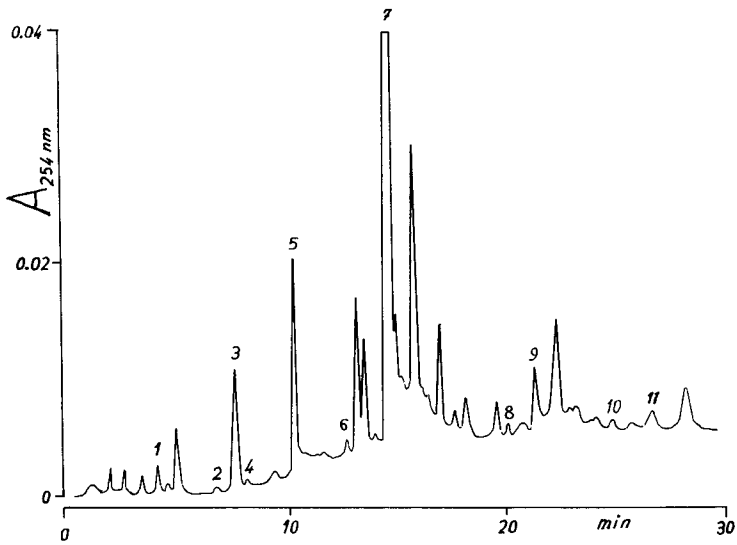


Fig. 1. Gradient separation of purine compounds in mouse liver mitochondria by ion-pair HPLC on an Ultrasphere IP column (system A). Mobile phase: 10 m*M* $KH_2PO_4$-2 m*M* TBA-3% acetonitrile, pH 5.25 (buffer A); 10 m*M* $KH_2PO_4$-2 m*M* TBA-20% acetonitrile, pH 7.25 (buffer B). Concave gradient (No. 4, 10 min). Flow-rate, 1.0 ml/min. Detection, 254 nm. Peaks: 1 = hypoxanthine/guanine; 2 = adenine; 3 = inosine; 4 = guanosine; 5 = adenosine; 6 = IMP/GMP; 7 = AMP; 8 = GDP; 9 = ADP; 10 = GTP; 11 = ATP.

TABLE I

NON-INDUCED PURINE NUCLEOTIDE CATABOLISM IN MOUSE LIVER MITOCHONDRIA

| Metabolite | Concentration (mean ± S.D.) (nmol/mg of protein) | |
|---|---|---|
| | Control ($n=6$) | Incubation (30 min) ($n=3$) |
| ATP | 1.68 ± 0.23 | 1.03 ± 0.10 |
| ADP | 1.33 ± 0.10 | 0.53 ± 0.23 |
| AMP | 3.55 ± 0.42 | 0.90 ± 0.47 |
| GTP | 0.06 ± 0.03 | 0.03 ± 0.01 |
| GDP | 0.04 ± 0.01 | < 0.01 |
| GMP/IMP | 0.31 ± 0.04 | 0.01 ± 0.01 |
| Adenosine | 0.24 ± 0.12 | 1.41 ± 0.41 |
| Inosine | 0.03 ± 0.02 | 2.18 ± 0.28 |
| Hypoxanthine/guanine | 0.16 ± 0.02 | 0.41 ± 0.08 |
| Adenine | < 0.01 | < 0.01 |
| Guanosine | < 0.01 | < 0.01 |

nucleotides and were in the same range as those of guanosine nucleotides (Table I). These studies dealt with the analysis of the breakdown products following incubation for 30 min at 37°C in the presence of the inhibitors of ATPase (oligomycin) and adenyl translocase (CAT). Table I shows that the AMP concentration decreased significantly after the treatment, whereas those of ADP and ATP remained almost unchanged. Moreover, the purines released were mostly adenosine and inosine, their concentrations increasing five- to twenty-fold. The low hypoxanthine and guanine contents and trace amounts of adenine could be due to the AMP deaminase, adenosine deaminase and 5′-nucleotidase activities, which led to a sharp increase in adenosine and inosine levels.

*Isocratic HPLC analysis of oxypurines in the erythrocytes of newborn infants with chronic perinatal hypoxia*

This investigation concentrated on the levels of hypoxanthine and other purine metabolites in the erythrocytes from the umbilical cord blood of newborn infants with perinatal hypoxia using isocratic reversed-phase chromatography with 1.0 m*M* TBA in the mobile phase. Fig. 2 shows a chromatogram of a mixture of standard purine compounds. The optimum conditions found for the Ultrasphere ODS column (relatively high ionic strength, unlike that reported in ref. 2, and a low concentration of an organic modifier) permit the successful separation of almost all purine bases, nucleosides and nucleoside monophosphates within 10 min.

Some researchers have suggested that perinatal hypoxia should be assessed by the elevation of the hypoxanthine level in the plasma of newborn infants [9,10]. However, other workers showed that as a criterion of hypoxia this parameter was not sufficiently reliable [11]. In this study, we focused on the alterations in the levels of hypoxanthine and some other purine derivatives occurring in the erythrocytes from the umbilical cord blood of newborn infants with perinatal hypoxia.

It is known that human erythrocytes are highly specialized cells, participating

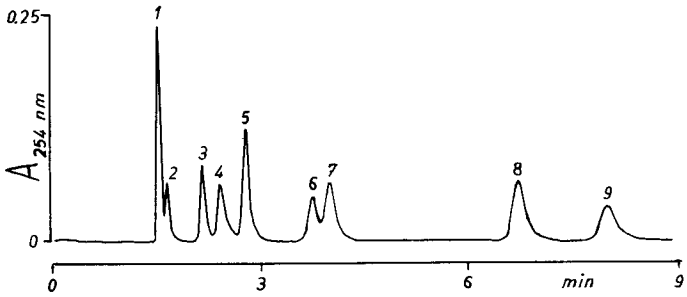


Fig. 2. Separation of standards on an Ultrasphere ODS column (system A). Mobile phase: 60 m*M* $KH_2PO_4$-1 m*M* TBA-1% acetonitrile, pH 5.1. Flow-rate, 1.2 ml/min. Detection, 254 nm. Peaks: 1 = hypoxanthine; 2 = xanthine; 3 = uric acid; 4 = adenine; 5 = inosine; 6 = GMP; 7 = IMP; 8 = adenosine; 9 = AMP.

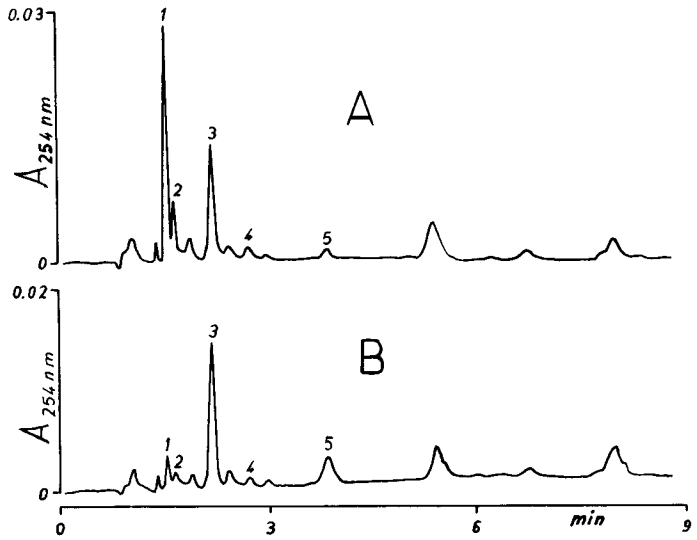


Fig. 3. Chromatograms of oxypurines from newborns' erythrocytes ASF with (A) favourable and (B) complicated period of early adaptation. Conditions as in Fig. 2. Peaks: 1 = hypoxanthine; 2 = xanthine; 3 = uric acid; 4 = inosine; 5 = IMP.

in the transport of preformed purines between tissues [12]. The availability of the oxypurine cycle, which is remarkable for the uptake of hypoxanthine and the accumulation of IMP, accompanied by the release of hypoxanthine, appears to be specific to metabolic properties of the red blood cells [13].

Fig. 3 illustrates chromatograms of the erythrocytes from the umbilical cord blood of newborn infants with the favourable and complicated periods of early adaptation. Table II shows the content of oxypurines in the erythrocytes from two groups of infants. There is a reciprocal relationship between hypoxanthine and IMP in the erythrocytes of infants from the two groups, which may be due to the effects of hypoxia on the oxypurine cycle. Apparently, hypoxia leads to an imbalance between the formation of IMP from hypoxanthine and the dephosphorylation of IMP.

TABLE II

CONTENT OF PURINE COMPOUNDS IN ERYTHROCYTES DERIVED FROM THE UMBILICAL CORD BLOOD OF NORMAL NEWBORN INFANTS AND TWO GROUPS OF INFANTS WITH CHRONIC PERINATAL HYPOXIA

| Metabolite | Concentration (mean ± S.D.) (μmol per 100 ml of cells) | | |
|---|---|---|---|
| | Control ($n=10$) | Group 1 ($n=14$) | Group 2 ($n=9$) |
| IMP | 0.24 ± 0.05 | 0.19 ± 0.04 | 0.79 ± 0.06 |
| Inosine | 0.78 ± 0.13 | 0.52 ± 0.11 | 0.36 ± 0.09 |
| Hypoxanthine | 2.85 ± 0.48 | 5.12 ± 0.51 | 0.89 ± 0.17 |
| Xanthine | 0.57 ± 0.16 | 0.41 ± 0.06 | 0.33 ± 0.08 |
| Uric acid | 5.44 ± 0.87 | 4.29 ± 0.52 | 4.98 ± 0.92 |

*Ion-pair isocratic HPLC analysis of purine nucleosides and bases in mouse thymocytes*

In order to evaluate the role of the bases and their nucleosides in thymocytes during tumour immunosuppresion, we developed an ion-pair reversed-phase method for the separation and quantitation of the bases hypoxanthine, xanthine, adeninine and guanine and their nucleosides inosine, adenosine and deoxyadenosine [4]. The use of alkylsulphonates (PIC B5 and PIC B7) as ion-pair reagents allowed the separation of guanine from the peak of hypoxanthine (usually co-eluted). Moreover, a modification of the $C_{18}$ phase to a dynamic cation-exchange retention mechanism resulted in the possibility of determining the weak base cytosine and its nucleoside cytidine. Fig. 4 illustrates the application of this method to the acid-soluble pool of normal mouse thymocytes and thymocytes of the fifth

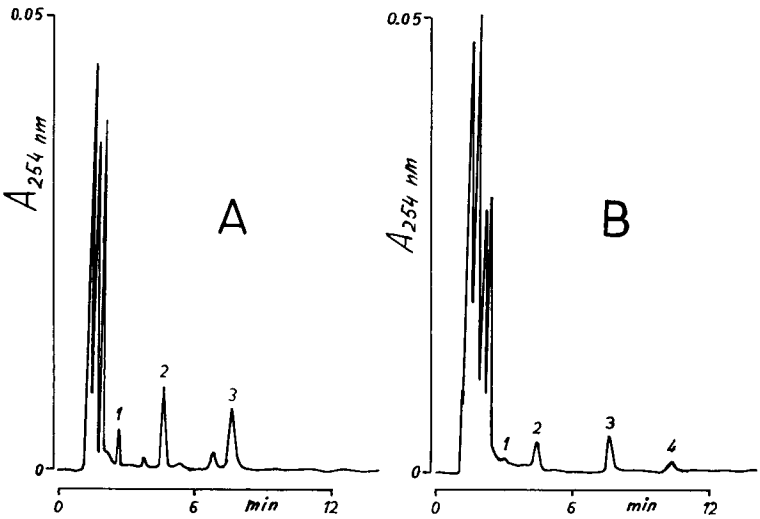


Fig. 4. Chromatograms of (A) normal mouse thymocytes ASF and (B) thymocytes of the fifth day of hepatoma growth. System B, μBondapak $C_{18}$ column. Mobile phase, 10 m*M* $NH_4H_2PO_4$–2 m*M* PIC B5–2.5 m*M* PIC B7–8% acetonitrile, pH 3.3. Flow-rate, 2.0 ml/min. Detection, 254 nm, 0.05 a.u.f.s. Peaks: 1 = hypoxanthine; 2 = guanine; 3 = adenine; 4 = adenosine.

day of hepatoma growth. As a result of hepatoma growth, the concentrations of adenine and guanine were halved and the pools of hypoxanthine and inosine were depleted in thymocytes within five days of tumour growth. In contrast, the appearance of adenosine seems to reflect the six-fold decrease in the activity of adenosine deaminase in the thymocytes during the highest growth period of hepatoma 22 [14]. These changes may be accompanied by alterations in the differentiation and maturation of mouse thyomocytes during immunosuppresion.

In conclusion, employment of ion-pairing reagents in reversed-phase HPLC makes it possible to develop and improve the separation methods for the cell and organelles with different nucleic acid metabolism patterns.

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