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Determination of the ultraviolet absorbance and radioactivity of purine compounds separated by high-performance liquid chromatography

Application to metabolic flux rate analysis

TILMAN GRUNE*, WERNER G. SIEMS and GERHARD GERBER
Institute of Biochemistry, Medical Faculty (Charité), Humboldt University Berlin, Hessische Strasse 3/4, O-1040 Berlin (Germany)
and
ROLF UHLIG
Department of Pediatrics, Medical Faculty (Charité), Humboldt University Berlin, Schumannstrasse 20/21, O-1040 Berlin (Germany)

ABSTRACT

A double detection system for the determination of adenine metabolism in biological tissues using isocratic ion-pair reversed-phase chromatography is presented. Two isocratic ion-pair separations were used: (i) 10 m*M* $NH_4H_2PO_4$, 2 m*M* tetrabutylammonium phosphate (PIC reagent A) and 18% acetonitrile for the determination of nucleotides and (ii) 50 m*M* KH_2PO_4, 1 m*M* PIC reagent A and 1% acetonitrile for the determination of monophosphorylated nucleotides, nucleosides and nucleobases. The parallel detection of ultraviolet absorbance at 254 nm and the radioactivity of separated purine compounds allows the detection of pool sizes and of the specific radioactivities in tracer kinetic experiments. The high-performance liquid chromatography methods were applied to the determination of flux rates during adenine nucleotide metabolism in suspensions of Ehrlich mouse ascites tumour cells. The pathways of adenine metabolism in cells during the proliferation and plateau phases of tumour growth were compared.

INTRODUCTION

Two different phases of growth of Ehrlich ascites tumour in the host can be distinguished: the proliferation or logarithmic phase and the stationary or plateau phase [1,2]. Cells in both growth phases show tremendous differences in the pattern of purines and the uptake of nucleotide precursors [2,3]. It is suggested that the transition of tumour cells from one growth phase into another is related to changes in the turnover rates of the pools of nucleotides, nucleosides and nucleobases. For this reason, the estimation of flux rates in tumour cells of the two growth phases is of biological importance. There are two general methods used for the measurement of metabolic flux rates: the first is based on the determination of concentrations in inhibitory experiments (*e.g.*, using modified nucleosides or nucleobases [4,5]), and the

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second is based on the determination of concentrations and the specific radioactivities in tracer experiments with radioactively labelled purine nucleotide precursors.

Many techniques have been developed for the chromatographic determination of the pool sizes of purine compounds [6–9]. The most common separation systems are ion-pair reversed-phase high-performance liquid chromatography (HPLC) for the separation of nucleotides or the full spectrum of purines [9–13] and reversed-phase HPLC for the determination of nucleosides and nucleobases [14,15]. Two strategies are used for the determination of purine compounds: (i) single-run gradient ion-pair reversed-phase HPLC [9,10] or (ii) the combination of short isocratic separation techniques for groups of metabolites [15,16].

For the assessment of metabolic pathways using tracer kinetics, *e.g.*, for purine metabolism, anion-exchange techniques for the separation of radioactively labelled nucleotides have been used in combination with the measurement of radioactivity in the individual fractions, which are collected, following HPLC [17,18]. The use of on-line detection of radioactivity of purine metabolites would facilitate this analytical procedure, and the aim of this study was to modify and apply two isocratic ion-pair separation techniques for the nucleotides, nucleosides and nucleobases of adenine metabolism for the parallel measurement of pool sizes and radioactivities without fractionation.

EXPERIMENTAL

Materials

Purine reference standards for HPLC were purchased from Boehringer (Mannheim, Germany). The reagents $NH_4H_2PO_4$ and KH_2PO_4 (HPLC grade) were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Acetonitrile and methanol (HPLC grade) were from Merck (Darmstadt, Germany). Tetrabutylammonium phosphate (PIC reagent A) was from Waters Assoc. (Milford, MA, U.S.A.). As a precursor of the flux rate experiments, [U-^{14}C]adenine from Amersham (Little Calfont, U.K.) was used (4 MBq/ml, 10.3 GBq/mmol). Further radioactively labelled compounds which were necessary as standards for the HPLC separations were also obtained from Amersham: [U-^{14}C]ATP (21.3 MBq/mmol), [U-^{14}C]ADP (20.1 MBq/mmol), [U-^{14}C]AMP (1.95 MBq/mmol), [U-^{14}C]GTP (16.9 GBq/mmol), [8-^{14}C]IMP (1.89 GBq/mmol), [U-^{14}C]Ado (18.6 GBq/mmol), [8-^{14}C]Ino (1.96 GBq/mmol), [8-^{14}C]Hyp (2.1 GBq/mmol), [6-^{14}C]Xan and [2-^{14}C]uric acid (1.87 GBq/mmol).

Cell line

Female mice of the ICR strain, weighing approximately 15–20 g, were used. The Ehrlich ascites tumour cells obtained from these animals 7–9 days after inoculation of the tumour were suspended in saline solution at a concentration of $5 \cdot 10^7$ cells per ml; 0.5 ml of this suspension were inoculated into the peritoneal cavity of a healthy animal to propagate the tumour.

Cell preparation

The animals were grouped in the following way: group I, the exponential phase, contained mice on the fifth day after tumour inoculation; group II, the stationary phase, contained mice on the twelfth day after tumour inoculation. The ascitic fluid

was aspirated and Ehrlich mouse ascites cells were washed with a cold (4°C) isotonic saline solution and incubated with continuous stirring at 37°C in a modified Eagle–Borsook solution containing glucose and the proteinogenic amino acids. The cytocrite was maintained at 2%. After a preincubation period of approximately 5 min, radiolabelled adenine was added to the cell suspension (1 μCi/ml of suspension at time zero). After 0.5, 1, 5, 10, 20, 30, 45, 60, 90 and 120 min, aliquots of 0.5 ml of suspension were taken and immediately prepared for HPLC analysis.

HPLC procedures

Sample preparation for HPLC. Aliquots of cell suspensions (0.5 ml) were added to the same volume of ice-cold 6% perchloric acid with stirring. The extracts were centrifuged for 10 min at 1200*g*. The supernatant was neutralized with 1.3 *M* potassium carbonate and centrifuged. The final supernatant was stored at −20°C. After thawing, 50 μl of the supernatant were determined by HPLC.

HPLC equipment. A Perkin Elmer (Norwalk, CT, U.S.A.) system consisting of a 410 pump system, an LC-95 variable-wavelength detector (adjusted to 254 nm), an LCI-100 integrator and a Rheodyne injector was used. The radioactivity detector was from Berthold (Wildbach, Germany). A 5-μm Nova-Pak C_{18} cartridge (100 × 8 mm I.D.) with an RCM 8 × 10 module from Waters Assoc. was also used.

Analytical schedule

Method A. Nucleotides were determined by an isocratic ion-pair reversed-phase HPLC technique. The buffer contained 10 m*M* $NH_4H_2PO_4$, 2 m*M* PIC reagent A and 18% acetonitrile. The flow-rate was 1.5 ml/min.

Method B. Monophosphorylated nucleosides, nucleosides and nucleobases were determined by an isocratic ion-pair reversed-phase technique. The eluent was a buffer containing 50 m*M* KH_2PO_4 (pH 5.1), 1 m*M* PIC reagent A and 1% acetonitrile. The flow-rate was 1.5 ml/min. Peak identification was performed by coelution of the extract with standard mixtures. Therefore, some of the biological samples were divided. One aliquot was analyzed without addition of standard compounds, and to other aliquots reference compounds were added for comparing HPLC runs.

RESULTS AND DISCUSSION

Two HPLC separation systems were used. Both systems were isocratic and they had the advantage of an identical quenching effect of the elution buffer during one run, which is important for the precise measurement of radioactivity.

The first method presented in this paper allows the determination of the mono-, di- and triphosphorylated adenine and guanine nucleosides. As described previously, this procedure does not allow the separation of IMP and GMP [15]. Deoxypurine compounds were excluded prior to this study. Fig. 1 shows chromatograms of the separation of nucleotides (Fig. 1a, measurement of pool sizes; Fig. 1b, measurement of radioactivity). The decreased concentration of acetonitrile (18%) and the lower flow-rate (1.5 ml/min) compared with the separation method reported by Grune *et al.* [15] (20% acetonitrile, 2 ml/min) increase the separation time and improve the detection of radiolabelled nucleotides in the 400-μl flow cell of the radioactivity detector.

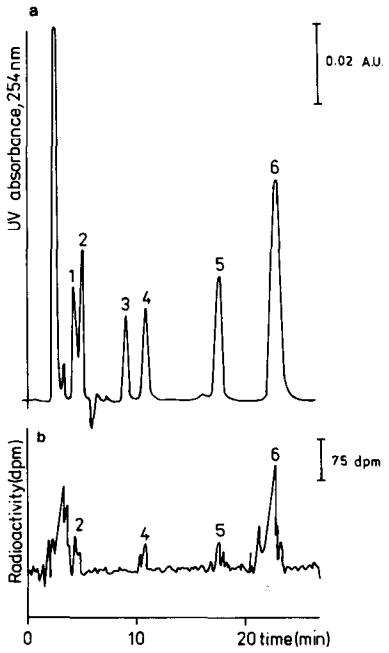

Fig. 1. Chromatograms of separations of nucleotides in extracts of Ehrlich mouse ascites cell suspensions. (a) Measurement of pool sizes; (b) detection of radioactivity. Injection volume, 50 μl; column, Nova-Pak C_{18} cartridge (100 × 8 mm, 5 μm particle size); mobile phase, 10 m*M* $NH_4H_2PO_4$, 2 m*M* PIC reagent A, 18% acetonitrile; flow-rate, 1.5 ml/min. Peaks: 1 = IMP + GMP; 2 = AMP; 3 = GDP; 4 = ADP; 5 = GTP; 6 = ATP.

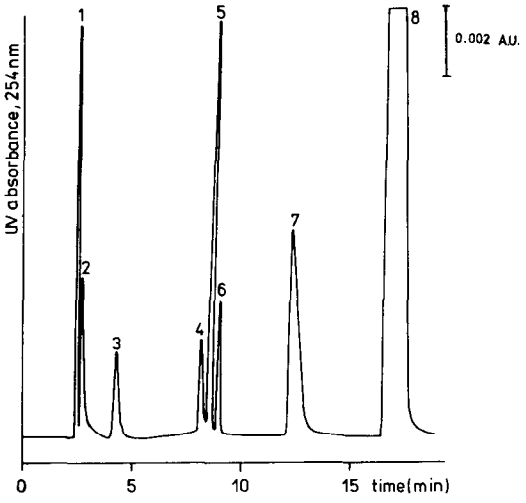

Fig. 2. UV absorbance detection of nucleotide degradation products using an isocratic ion-pair reversed-phase HPLC. The concentration of nucleoside and nucleobase standards was in the range 5–15 μ*M* and the concentration of AMP (peak 8) was 0.5 m*M*. Injection volume, 50 μl; column, Nova-Pak C_{18} cartridge (100 × 8 mm, 5 μm particle size); mobile phase, 50 m*M* KH_2PO_4 (pH 5.1), 1 m*M* PIC reagent A, 1% acetonitrile; flow-rate, 1.5 ml/min. Peaks: 1 = Hyp; 2 = Xan; 3 = uric acid; 4 = Ade; 5 = Ino; 6 = Guo; 7 = Ado; 8 = AMP.

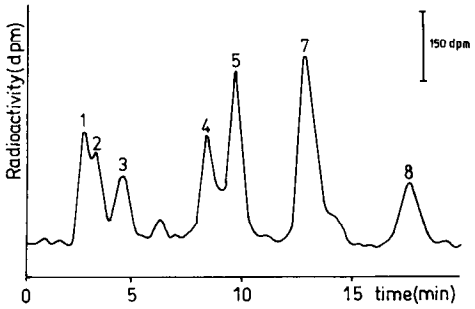

Fig. 3. Determination of radioactivity of authentic radioactive labelled standards of nucleotide degradation products. For chromatographic conditions and peak numbers, see Fig. 2.

The determination of the concentrations and radioactivities of the nucleotide degradation products was performed by an isocratic ion-pair reversed-phase separation method, as described by Togusov *et al.* [16]. However, the eluent in this instance contains a higher concentration of phosphate buffer and only 1% acetonitrile. This method allows the detection of nucleobases, nucleosides and monophosphorylated nucleosides, as shown in Figs. 2 (UV absorbance) and 3 (radioactivity). The difficulty of determining nucleoside levels by reversed-phase HPLC (a decreasing baseline from the front peak of the nucleotides) can be avoided using this method. The nucleotides were either separated very late, or, for example the triphosphorylated nucleosides, were retained at least partially on the column. Therefore, after about fifteen separations the column needs to be rinsed with 20% acetonitrile for 10 min (flow-rate 1.5 ml/min) to remove the remaining nucleosides.

Table I shows the concentrations of purine compounds and Table II the specific

TABLE I

CONCENTRATIONS OF PURINE COMPOUNDS IN SUSPENDED EHRLICH MOUSE ASCITES CELL SUSPENSIONS

Values are given as mean ± S.D. (n = 4).

Purine compound	Concentration (mmol/l cells)	
	Exponential phase	Stationary phase
ATP	4.61 ± 0.23	2.68 ± 0.19
ADP	1.55 ± 0.17	1.09 ± 0.16
AMP	0.65 ± 0.12	0.51 ± 0.10
GTP	0.95 ± 0.23	0.35 ± 0.21
IMP + GMP	0.31 ± 0.07	0.15 ± 0.06
	Concentration (μmol/l cell suspension)	
	Exponential phase	Stationary phase
Ado	0.45 ± 0.11	0.59 ± 0.17
Ade	0.98 ± 0.27	1.44 ± 0.22
Hyp	1.55 ± 0.26	1.44 ± 0.33
Uric acid	3.37 ± 0.29	5.47 ± 0.35

TABLE II

SPECIFIC RADIOACTIVITIES OF PURINE COMPOUNDS OF EHRLICH MOUSE ASCITES CELLS OF DIFFERENT GROWTH PHASES

[^{14}C]Adenine was added to the suspension and 30 min after incubation samples were taken for analysis. Values given are means (n = 4); dpm/pool is the radioactivity of metabolites calculated for the suspension volume of 1 ml, despite the distribution of the metabolites either only inside (ATP, ADP, AMP, GTP, GMP, IMP) or inside and outside (Ado, Ade, Hyp, uric acid) the tumour cells. N.D. = not detected.

Purine compound	Exponential phase		Stationary phase	
	dpm/nmol	dpm/pool	dpm/nmol	dpm/pool
ATP	1545	141 477	725	38 971
ADP	915	28 373	545	11 923
AMP	2365	30 746	2410	24 564
GTP	995	63 162	N.D.	N.D.
IMP + GMP	2325	14 407	4030	11 923
Ado	45 509	20 479	24 044	14 186
Ade	485 985	476 265	842 145	1 212 688
Hyp	18 946	29 366	6900	9936
Uric acid	6289	21 197	2522	13 795
Nucleic acids and others	–	1 394 528	–	882 014
Total	–	2 220 000	–	2 220 000

and total radioactivities of the pools after 30 min of incubation of the Ehrlich ascites tumour cells in the presence of ^{14}C-labelled adenine. The growth-dependent changes in the concentration of purine compounds reported previously [2] are confirmed. The distribution of the radiolabelled purine precursors shows a higher radioactivity in most components of purine metabolism in the cells during the proliferating phase of tumour growth, suggesting higher enzyme activities and metabolic flux rates in these cells compared with those of the plateau phase.

The methods presented in this paper allow the determination of adenine nucleotides and their degradation products, in addition to the determination of their ^{14}C-labelling, without fractionation of the eluent. It is therefore possible to calculate metabolic flux rates in the pathways of adenine metabolism.

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