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Aldehyde and nucleotide separations by high-performance liquid chromatography

Application to phenylhydrazine-induced damage of erythrocytes and reticulocytes

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ABSTRACT

High-performance liquid chromatographic (HPLC) techniques were used for the determination of aldehydes as lipid peroxidation products and of nucleotide concentrations in rabbit red blood cells exposed to phenylhydrazine hydrochloride. Aldehydes were determined by derivatization to dinitrophenylhydrazones, followed by thin-layer chromatographic and HPLC separation with methanol water on an ODS column. Nucleotides and their derivatives were measured by ion-pair reversed-phase HPLC gradient elution with 10 mM ammonium phosphate buffer containing 2 mM tetrabutylammonium phosphate on ODS columns. The sensitivity of mature erythrocytes was compared with that of reticulocytes against the toxicological effects of phenylhydrazine. For both cell types an increase in aldehyde concentrations, especially of 4-hydroxynonenal, and a decline in both energy-rich nucleoside triphosphate and NAD$^+$ were demonstrated.

INTRODUCTION

The use of phenylhydrazine has long been known to result in the oxidation of cellular compounds and lysis of red blood cells. Phenylhydrazine has been used to produce haemolytic anaemias resulting in reticulocytosis [1]. Known effects of phenylhydrazine treatment include attacks on haem proteins, loss of membrane fluidity, changes in protein breakdown, Heinz body formation and ATP depletion [2–4].

The action of phenylhydrazine seems to be mediated by the production of drug intermediates and activated oxygen species [5]. These radicals induce peroxidation of unsaturated fatty acids of red blood cell membranes. The lipid peroxidation results in

the formation of a wide variety of secondary products. Some of these secondary products, the aldehydes, are highly cytotoxic [6]. A method for the determination of aldehydes was applied with the aim of correlating changes in aldehyde amounts with the breakdown of nucleotides during phenylhydrazine treatment. Reversed-phase high-performance liquid chromatographic (RP-HPLC) techniques were used for the determination of aldehydes as a parameter for the balance between free radical-generating and antioxidative systems and of nucleotides and their degradation products as criteria for the energy metabolism in rabbit red blood cells.

The sensitivity of mature erythrocytes was compared with that of reticulocytes concerning the toxicological effects of phenylhydrazine.

EXPERIMENTAL

Chemicals

All reference standards of the highest analytical grade available were purchased from Boehringer (Mannheim, F.R.G.) and Sigma (St. Louis, MO, U.S.A.). $NH_4H_2PO_4$ was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.), aceto-nitrile, methanol, *n*-hexane and dichloromethane from Merck (Darmstadt, F.R.G.) and tetrabutylammonium phosphate (PIC reagent A) from Waters Assoc. (Milford, MA, U.S.A.).

Thin-layer chromatographic (TLC) plates (silica gel 60 F_{254}, 0.2 mm thickness) were obtained from Merck. 2,4-Dinitrophenylhydrazine (DNPH) was purchased from Union Chimique (Belgium).

Cell preparation

Red blood cells were drawn from the ear vein of rabbits, washed twice and incubated at 37°C with a haematocrit value of about 20% in a physiological saline solution buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 7.4) at 37°C. The incubation solution contained 50 mM HEPES, 120 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 1 mM Na$_2$HPO$_4$ and 5 mM glucose. Phenylhydrazine hydrochloride was used at a concentration of 20 mM. Reticulocytes were produced by bleeding as described [7].

Determination of aldehydes

Aldehydes were determined as described previously [8,9]. After incubation 2.5 ml of a suspension of red blood cells (+ 2.0 ml of 0.9% NaCl + 0.5 ml of 10 mM EDTA) were mixed with 5.0 ml of DNPH solution (solution in 1 mM HCl, extracted twice with 15 ml of *n*-hexane, 1.8 mM solution adjusted by absorbance, $\varepsilon = 14\ 500\ 1$ mol^{-1} cm^{-1}) and samples were incubated for 2 h at room temperature in the dark and for 1 h in an ice-bath in the dark. Subsequently, extraction with 7 ml of dichloro-methane, centrifugation at 900 g (three times), evaporation and dissolution in 1 ml of dichloromethane were carried out.

TLC. TLC plates were eluted with dichloromethane (comparison with known standards [8]). Two individual fractions were scraped off (zones I and III) as desribed [8]. That was followed by extraction with 10 ml of methanol three times, evaporation and dissolution in 1 ml of methanol.

HPLC. A system from DuPont (Wilmington, DE, U.S.A.) was used, consisting of a model 8800 pump module, a model 8800 series gradient liquid chromatographic system, a Model 850 column compartment, a variable-wavelength detector (350 nm) and a Spectra-Physics (Santa Clara, CA, U.S.A.) SP 4100 integrator.

The eluent was methanol–water (4:1, v/v) in an isocratic run at a flow-rate of 0.9 ml min^{-1}. The column was 250 × 4.6 mm I.D. filled with 5 μm LiChrosorb (Merck, Darmstadt, F.R.G.). Peak identification was performed by coelution with known standards.

Determination of nucleotides

Nucleotides and nucleotide degradation products were determined as described previously [9].

Extraction procedure. The samples were deproteinized (6% perchloric acid), centrifuged (8 min, 1200 g) and neutralized with triethanolamine–potassium carbonate. After filtration 50 μl of the supernatant were analysed by HPLC.

HPLC. A system from Perkin-Elmer (Norwalk, CT, U.S.A.) was used, consisting of a Model M410 pump system, a model LC95 variable-wavelength detector (254 nm), a model LCI-100 integrator and a Rheodyne model 7125 injector. The column used was Supelco ODS (150 × 4.6 mm I.D.) with a precolumn (20 × 4.6 mm I.D.).

$NH_4H_2PO_4$ (10 mM) containing 2 mM tetrabutylammonium phosphate (TBA) was used as buffer A and buffer A–acetonitrile (80:20) as buffer B. The gradient was linear from 100% A to 70% B in 5 min followed by an isocratic separation with 30% A–70% B in 8 min and a slight convex gradient to 100% B in 20 min. The final steps of this run were an isocratic separation with 100% B in 7 min, a linear gradient to 100% A within 2 min and isocratic pumping of 100% A for 10 min. The flow-rate was 1 ml min^{-1}. The peak identification and quantification were performed as described [10].

RESULTS AND DISCUSSION

Aldehyde determination

The DNPH derivatives of aldehydes were separated in a polar and a non-polar fraction by an extraction [8]. The non-polar carbonyl compounds were separated by TLC in two zones (zones I and III [8]). Zone I contained 4-hydroxynonenal, 4-hydroxyoctenal and 4-hydroxyhexenal and zone III contained pentanal, 2,4-hexadienal, hexanal, 2,4-heptadienal, octenal and 2,4-decadienal. Chromatograms of standard mixtures for these two zones are shown in Figs. 1 and 2. The chromatogram in Fig. 3 shows the separation of compounds of zone I in a reticulocyte-rich suspension with phenylhydrazine treatment at time zero and after 3 h of incubation.

The concentrations of 4-hydroxynonenal and 4-hydroxyoctenal after 3 h of phenylhydrazine treatment increased in reticulocyte-rich suspensions up to maximum values of 6 and 0.8 μM, respectively. In erythrocyte suspensions the corresponding maximum values of 4-hydroxynonenal and 4-hydroxyoctenal were 1 and 0.1 μM after 3 h of treatment.

We could not find any accumulation of other aldehydes. Also in experiments with hydrogen peroxide treatment of rabbit erythrocytes 4-hydroxynonenal was detected as the main aldehyde compound [9]. In phenylhydrazine-driven radical reac-
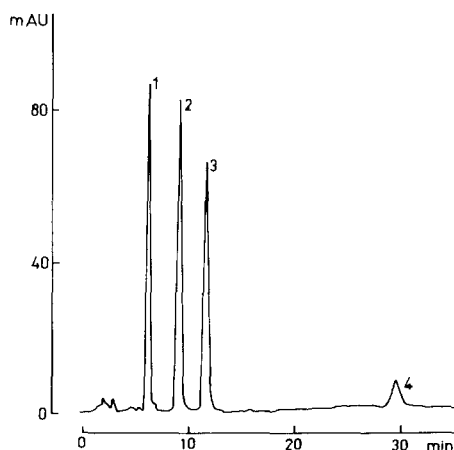
Fig. 1. Chromatogram of a standard mixture of 2,4-dinitrophenylhydrazones of aldehydes (zone I). Peaks: 1 = 4-hydroxyhexenal; 2 = 4-hydroxyoctenal; 3 = 4-hydroxynonenal; 4 = 4-hydroxyduodecenal.

tions the radical damage is probably catalysed by phenyl free radicals [1], but also by other oxidative processes, *e.g.*, H_2O_2 and O_2^- formation leading to $\cdot OH$ radical generation [11]. This results in a multitude of carbonyl compounds and other secondary and tertiary products [12]. It can be concluded that the oxidative damage induced by phenylhydrazine hydrochloride is higher in reticulocytes than in erythrocytes and 4-hydroxynonenal is the main product of lipid peroxidation in both cell types.

The chromatographic method presented allows the assessment of phenylhydrazine-induced changes in cells by determination of secondary lipid peroxidation products.

Nucleotide determination

The detection of hypoxanthine, xanthine, uric acid, inosine, adenine, adenosine, NAD^+, IMP, AMP, GDP, UDP, ADP, GTP, UTP and ATP was possible by
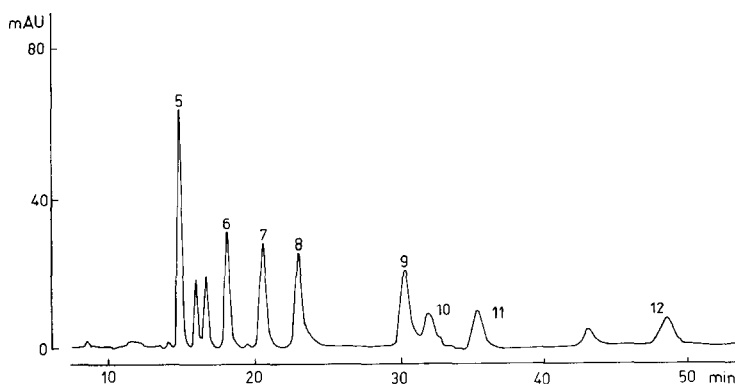

Fig. 2. Chromatogram of a standard mixture of 2,4-dinitrophenylhydrazones of aldehydes (zone III). Peaks: 5 = pentanal; 6 = 2,4-hexadienal; 7 = hexanal; 8 = 2,4-heptadienal; 9 = heptanal; 10 = octenal; 11 = 2,5-nonadienal; 12 = 2,4-decadienal.
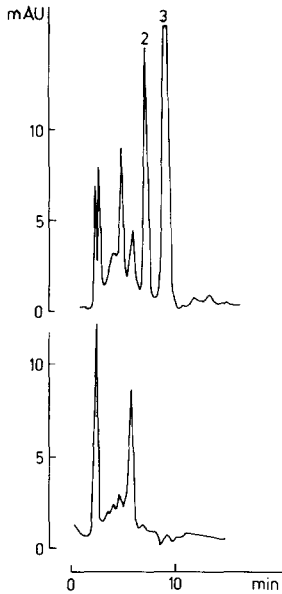
Fig. 3. Chromatograms of lipid peroxidation products of reticulocytes at time zero (bottom) and after incubation with phenylhydrazine for 3 h (top) (zone I). Peak numbers as in Fig. 1.

application of gradient ion-pair RP-HPLC [9,10]. The proposed method yields a separation of nucleotides, nucleosides and nucleobases comparable to those reported by other workers [13,14]. Most of these metabolites can be detected in red blood cells. In Fig. 4 a chromatogram of an extract of a reticulocyte-rich suspension is presented.

The nucleosides and nucleobases, which are present at low concentrations in red blood cells, have short retention times.

The nucleotide and nucleotide degradation product concentrations are given in Table I. The control values were stable with respect to all parameters for the whole incubation time of 90 min. In agreement with previous studies, the differences in the pool sizes in erythrocytes and in reticulocytes (reticulocyte-rich red blood cell suspensions with about 40% of reticulocytes were used for the experiments) could be demonstrated [10].

ATP is degraded to the same extent in rabbit reticulocytes and erythrocytes, by about 80%. There is a temporary increase in ADP and AMP. The ATP:AMP ratio decreases to 10–20% of the initial value after 90 min. GTP is also degraded faster in the reticulocytes than in the erythrocytes. There is a great loss of purines during phenylhydrazine incubations, by 40–55% after 90 min of incubation. The adenosine and hypoxanthine concentrations are increased in the presence of phenylhydrazine. This accumulation of the pool size accounts for the 5-fold increase of the initial for hypoxanthine. The adenosine concentration increases 3-fold in reticulocyte suspensions and 2-fold in erythrocyte suspensions. NAD^+ is degraded. Therefore, the method allows changes in the purine nucleotide metabolism, which are important for the evaluation of the action of phenylhydrazine to monitor.
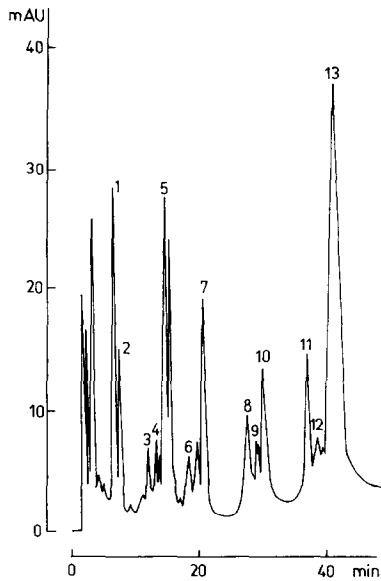
Fig. 4. Chromatogram of an acidic extract of reticulocyte-rich red blood cell mixture. Peaks: 1 = hypoxanthine; 2 = xanthine; 3 = inosine; 4 = adenosine; 5 = NAD^+; 6 = IMP; 7 = AMP; 8 = GDP; 9 = UDP; 10 = ADP; 11 = GTP; 12 = UTP; 13 = ATP.

TABLE I

CONCENTRATIONS OF NUCLEOTIDES AND NUCLEOTIDE DEGRADATION PRODUCTS IN RABBIT RETICULOCYTE-RICH SUSPENSIONS (39.5% RETICULOCYTES) AND ERYTHROCYTES AFTER 90 min OF INCUBATION (mmol PER 1 OF CELLS, MEAN ± S.D., n = 5)

Metabolite	Initial value		20 mM phenylhydrazine	
	Erythrocyte	Reticulocyte	Erythrocyte	Reticulocyte
ATP	1.523 ± 0.052	2.877 ± 0.221	0.463 ± 0.080	0.503 ± 0.202
ADP	0.210 ± 0.030	0.298 ± 0.042	0.267 ± 0.075	0.330 ± 0.099
AMP	0.113 ± 0.017	0.135 ± 0.024	0.134 ± 0.011	0.263 ± 0.062
Sum AdN	1.846 ± 0.099	3.311 ± 0.287	0.864 ± 0.166	1.096 ± 0.363
GTP	0.203 ± 0.043	0.433 ± 0.044	0.156 ± 0.057	0.149 ± 0.040
GDP	0.074 ± 0.012	0.096 ± 0.008	0.121 ± 0.015	0.129 ± 0.040
GMP + IMP	0.052 ± 0.021	0.065 ± 0.012	0.049 ± 0.008	0.114 ± 0.040
Ado	0.018 ± 0.002	0.018 ± 0.002	0.037 ± 0.001	0.055 ± 0.018
Hyp	0.019 + 0.004	0.046 + 0.006	0.103 ± 0.028	0.216 ± 0.049
Sum purines	2.212 ± 0.181	3.961 ± 0.359	1.330 ± 0.275	1.789 ± 0.550
NAD^+	0.065 ± 0.004	0.115 ± 0.013	0.022 ± 0.003	0.021 ± 0.008
ATP/ADP	7.25 ± 0.25	9.65 ± 0.74	1.73 ± 0.30	1.52 ± 0.61
ATP/AMP	13.48 ± 0.46	21.31 ± 1.67	3.46 ± 0.60	1.91 ± 0.77
GTP/GDP	2.74 ± 0.58	4.51 ± 0.46	1.29 ± 0.47	1.16 ± 0.31

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