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FAST REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF NUCLEOTIDES IN RED BLOOD CELLS

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SUMMARY

A method for the accurate determination of ATP, ADP, AMP, NADP and NAD in 20 min in red blood cell extracts by reversed-phase high-performance liquid chromatography is described. Experimental parameters affecting the separation are discussed.

INTRODUCTION

In the framework of a study on metabolism in human and other mammalian erytrocytes we were interested in the simultaneous determination of ATP, ADP, AMP, NADP and NAD in perchloric acid (PCA) extracts of red blood cells. As a large number of samples had to be analysed, a high analysis speed was required for a routine method. Further, because the relative concentrations of the five compounds in erythrocyte extracts may differ widely, baseline separation is imperative for their accurate quantitation.

Traditionally, nucleotides have been determined by ion-exchange high-performance liquid chromatography (HPLC)[1–6]. However, this technique does not fit our needs as the compounds of interest either are only partially separated[6] or require lengthy analysis times (> 40 min). Moreover, it is often necessary to use high-concentration buffers and/or fast gradient elution, which require considerable analytical work.

Good results have been obtained by reversed-phase liquid chromatography (RPLC)[7–11], which shows some advantages over the ion-exchange mode because of its reproducibility, versatility and ease of operation[12]. Studies on the retention mechanism of nucleotides, nucleosides and related bases in RPLC have been published by Van Haastert[13] and by Zakaria *et al.*[14]. However, the analysis time is a limiting factor[11].

In this paper we describe an improvement of the RPLC method that allows a rapid and accurate determination of selected nucleotides in blood cell extracts in 20 min.

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EXPERIMENTAL

Apparatus and materials

A Varian 5000 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a microprocessor control unit, a single-piston reciprocating pump and a variable-wavelength UV detector (Model UV-50) employing a 10-μl flow cell was used. The detector was set to monitor absorbance at 254 nm. Samples were injected by means of a Valco sample injector valve equipped with a 25-μl injection loop. Retention times and peak areas were obtained using a Shimadzu Chromatopac R-1B electronic integrator.

A 5-μm Supelcosil LC-18 column (25 cm × 4.6 mm I.D.) (Supelco, Bellefonte, PA, U.S.A.) with an efficiency of 7000–8000 theoretical plates per 10 cm was used throughout. A stainless-steel guard column (2 cm × 4.6 mm I.D.) packed with pellicular reversed-phase material was used to protect the analytical column.

Analytical-reagent grade potassium dihydrogen phosphate was obtained from Merck (Darmstadt, F.R.G.), nucleotide standards of the highest grade available from Sigma (St. Louis, MO, U.S.A.) and Boehringer (Mannheim, F.R.G.) and methanol (HPLC grade) from Hoechst (Frankfurt, F.R.G.). Water, either of HPLC grade from Carlo Erba (Milan, Italy) or doubly distilled, was used in the preparation of buffer, standard and sample solutions.

Standards were prepared as 5 m*M* stock solutions (stored at −20°C) by dissolving a weighed mass of the dried material in 0.1 mol/l potassium dihydrogen phosphate buffer solution (pH 6). Working standards of various concentrations were prepared by dilution of the stock solutions.

Buffer solutions, after preparation and pH adjustment, were filtered through a 0.22-μm filter (Millipore, Bedford, MA, U.S.A.).

Procedure

In the final procedure, gradient elution at a flow-rate of 1.3 ml/min, which developed a back-pressure of 100–150 atm, was used. The temperature was 18–22°C. The first eluent was a 0.1 *M* potassium dihydrogen phosphate solution (buffer 1) and the second was 0.1 *M* potassium dihydrogen phosphate solution containing 10% (v/v) methanol (buffer 2). The chromatographic conditions were as follows: 4 min at 100% of the first buffer and then a linear gradient up to 100% of the second buffer in 9 min; the gradient was then returned to the first buffer in 1.0 min and the initial conditions restored in 6 min. Before use, the column was conditioned by running the gradient programme through it twice. Water was passed through the column at 0.3 ml/min overnight and regeneration was carried out weekly with a 30-min water wash followed by a 100% methanol wash and finally a 30-min water wash. The flow-rate used during column regeneration was the same as in the analytical step.

In isocratic runs, the column was allowed to equilibrate with the mobile phase for 1 h at a flow-rate of 1.3 ml/min before injection.

Perchloric acid (PCA) extracts of erythrocytes were obtained as reported by Beutler[15]. A 1-ml volume of 10% (w/v) perchloric acid was added to 1 ml of freshly drawn whole blood. The extract was then neutralized with potassium carbonate and centrifuged immediately before injection. The final volume was adjusted to 2 ml.

RESULTS AND DISCUSSION

Table I gives the capacity ratios (k') of ATP, ADP, AMP, NADP and NAD for different pH values and buffer concentrations. A baseline separation in the shortest time is obtained with a compromise of the following critical experimental factors:

(a) The ATP capacity ratio should be high enough to allow separation from other compounds that may be present in appreciable concentrations in erytrocyte extracts (GTP, GDP, etc.) and all of which are eluted before ATP.

(b) The buffer concentration should be as low as possible to avoid routine problems.

(c) A buffer pH close to neutrality is preferable because the column show a short analytical life at *ca.* pH 3.

(d) NAD should have a reasonable k' value so that it is possible to use a low methanol content and/or a slow gradient programme to fasten the elution of AMP, NAD and NADP.

(e) As a consequence of the last point, AMP and NAD should have the largest difference in k' values.

All these conditions are met by using 0.1 *M* buffer of pH 6 and a gradient programme towards the same buffer containing 10% methanol, as described under Experimental.

Fig. 1 shows the separation of a standard mixture of nucleotides carried out in 13 min using the simple gradient programme described under Experimental. Considering that a regeneration time of 7 min is required, baseline separation of ATP, ADP, AMP, NADP and NAD is achieved in 20 min. This is a substantial improvement over reported separations. In fact, by using high efficiency microparticulate anion-exchange columns, the same analysis time is required, but the pairs AMP–NAD and ADP–NADP are only partially separated while a reversed-phase separation[11] of the five compounds requires 1 h. The chromatogram of a red blood cell extract shows that the five compounds are completely separated from the interfering compounds which are eluted before the ATP peak. Further, ADP, which is usually present at concentration levels up to 12 times lower than that of ATP, as experimentally measured, can be determined with good accuracy. The gradient used does not cause excessive baseline drift, so that NADP, NAD and in particular AMP can also be easily determined. While retention times were very reproducible in the short time period, some changes were observed after prolonged routine use of the column (over 1 month). This could be compensated for by changing the methanol content of buffer 2 or by slight changes to the gradient programme.

Two advantages arise from the use of 0.1 *M* buffers at pH 6. First, although the column should be stable in the pH range 2.5–7, rapid deterioration (few days) was observed when using buffers at pH 3, whereas under the conditions given, the column has been used for several months without adverse effects. On the other hand, the use of relatively low molarity buffers avoids time-consuming inconveniences. In fact, when 0.3 or 0.5 *M* buffers were employed some problems arose with the apparatus used (clogging of connection lines and loops, etc.), while longer times were obviously required to clean the system from salt residues.

The calibration graphs, obtained by injecting 25 μl of standards of different concentrations, were linear in the range up to 15,000 pmole for absorbance at 254

TABLE I

CAPACITY RATIOS FOR DIFFERENT pH VALUES AND BUFFER CONCENTRATIONS

Compound	*Buffer concentration (mol/l)*															
	0.02				*0.05*				*0.1*				*0.3*			
	pH															
	6	*5*	*4*	*3*	*6*	*5*	*4*	*3*	*6*	*5*	*4*	*3*	*6*	*5*	*4*	*3*
ATP	0.7	0.7	1.2	1.0	1.0	1.1	1.3	0.8	1.4	1.5	1.3	1.2	2.0	1.9	1.6	1.1
ADP	1.1	1.1	1.2	1.0	1.4	1.5	1.5	0.8	1.9	2.0	1.5	1.0	2.3	2.3	2.0	1.1
AMP	3.3	4.0	3.5	2.7	3.8	4.9	4.2	2.3	4.2	5.5	4.3	2.7	4.5	5.6	5.1	2.9
NADP	3.3	4.0	4.2	5.2	4.6	6.1	5.8	4.2	6.0	7.8	6.7	5.6	8.1	10.5	11.2	7.6
NAD	14.0	13.7	13.0	12.0	17.6	17.2	15.5	9.6	21.8	20.0	16.2	11.9	24.6	22.7	22.5	14.3

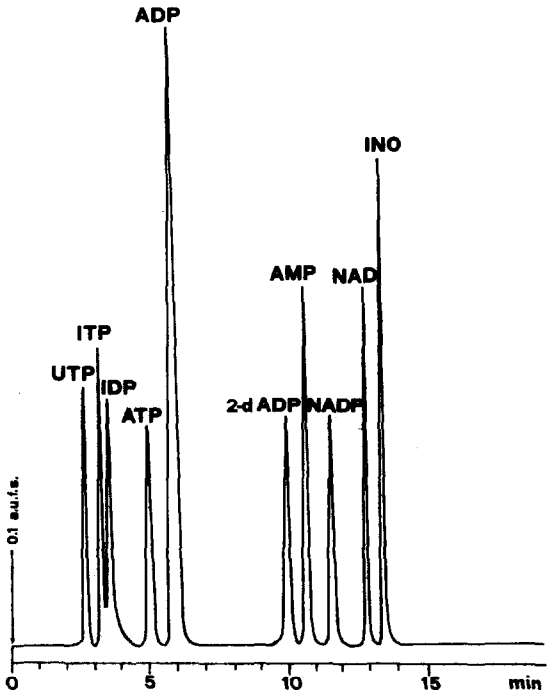

Fig. 1. Separation f a standard mixture of nucleotides using the chromatographic conditions given under Experimental. Column: Supelcosil LC-18, 5 μm (25 cm × 4.6 mm I.D.).

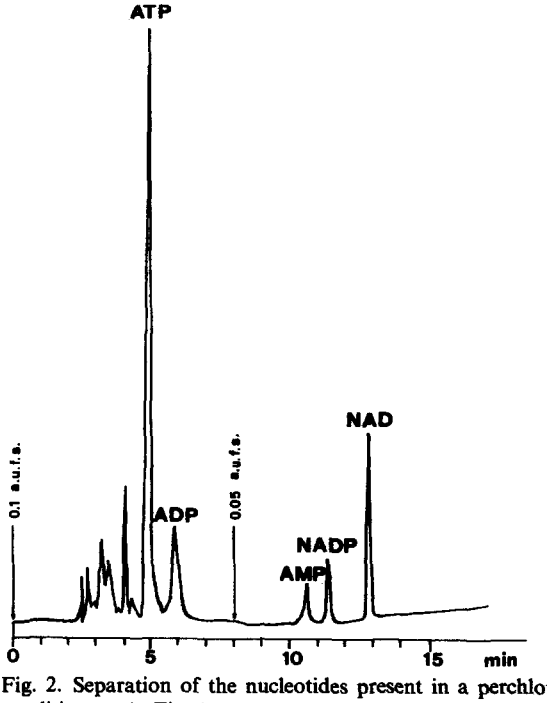

Fig. 2. Separation of the nucleotides present in a perchloric acid extract of erytrocytes. Experimental conditions as in Fig. 1.

TABLE II

PRECISION OF THE METHOD CALCULATED ON TWO POOLED EXTRACTS

Compound	*Concentration (μmol/l red blood cell extract)*	*Relative standard deviation (%)*		
		1 day	*6 days*	*60 days*
ATP	1110	2.0	2.5	2.5
ADP	103	2.0	2.5	4.0
AMP	10	3.5	4.4	5.0
NADP	34	2.4	2.7	3.7
NAD	55	2.7	3.3	3.3

nm. This range includes the amounts actually injected when extracts are analysed. Calibration of the standards was also carried out by using molar absorptivities at 254 nm of $15.4 \cdot 10^3$ for ATP, ADP and AMP and $18.0 \cdot 10^3$ l mol^{-1} cm^{-1} for NADP and NAD. The difference between the actual and theoretical concentrations was within the experimental error. Good reproducibility is obtained in the quantitation, as shown in Table II, which gives the run-to-run precision, the day-to-day precision for a period of a week and the long-term precision, measured on two pooled extracts. Daily values are the means of nine analyses, weekly values are the means of daily duplicate analyses and the monthly values are the means of weekly duplicate analyses. There is no substantial difference between the data collected on the same day and over a period of 2 months. The high efficiency of the column coupled with the low baseline noise enhance the sensitivity of the method. This is important for the measurement of AMP, NADP and NAD, which are present in the actual samples analysed at picomole levels. The detection limit for these three compounds was found to be about 2 pmole.

The concentrations of ATP, ADP, AMP, NADP and NAD found in the red blood cell extracts of five normal human adults were 1065 ± 60, 95 ± 12, 8 ± 2, 32 ± 4, and 51 ± 7 μmol/l, respectively, and the ATP/ADP and ATP/AMP ratios were 11.2 and 130, respectively.

In conclusion, the method here described, based on RPLC chromatography, allows the rapid and accurate determination of nucleotides in erythrocytes. The sensitivity of the method is such that it can be applied to small amounts of erythrocytes (*ca.* 25–50 μl).

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