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RAPID DETERMINATION OF ADENINE NUCLEOTIDES IN BRAIN TISSUE BY ION-PAIRED REVERSED-PHASE COLUMN LIQUID CHROMATOGRAPHY UNDER ISOCRATIC CONDITIONS

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SUMMARY

A rapid method for analysis of adenine nucleotides (AMP, ADP and ATP) in nervous tissue based on ion-paired reversed-phase column liquid chromatography under isocratic conditions is described. An optimal composition of elution buffer was 25 m*M* potassium phosphate and 4% triethylamine adjusted to pH 6:5 with phosphoric acid. Typical separation time did not exceed 10 min with a 10-cm long compact glass cartridge packed with 5-μm silica C_{18}. The method was employed to determine ATP, ADP and AMP concentrations in rat brain extracts and values thus obtained were compared with those published elsewhere.

INTRODUCTION

For the estimation of nucleotides in biological materials by column liquid chromatography (LC), ion-paired reversed-phase chromatography on octyldecyl-silica resins [1] or ion-exchange chromatography on microparticulate anion-exchange resins [2] are the most frequently used techniques.

To obtain an optimal resolution of nucleotides with both separation modes a gradient elution has to be used, with increasing concentration of organic modifier in the first method, or with increasing ionic strength in the latter method. In both respective systems, nucleotides are eluted in order of increasing polarity of molecules, i.e. mono-, di-, and triphosphates. All three adenine nucleotides strongly influence a number of reactions that regulate both production and utilization of energy within the cell. A ratio of their concentrations, i.e. energy charge potential [3], expressed as EC = (ATP + ½ADP)/

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(ATP + ADP + AMP) is a convenient measure of the energy state under both physiological and pathological conditions. Previous methods for determination of these substances by LC on ion exchangers and reversed phases as well as by enzymatic and fluorometric methods are, however, time-consuming and rather complicated.

In this paper, a rapid method for the determination of adenine nucleotides in the brain based on ion-paired reversed-phase LC under isocratic conditions is described. Triethylamine, known as the eluent in preparative ion-exchange chromatography and recently used in the form of volatile triethylammonium bicarbonate in preparative reversed-phase LC of adenine analogues [4], was found to be the most suitable ion-pairing reagent.

Optimization of chromatographic conditions is discussed in detail and practical application of the method is demonstrated by determination of AMP, ADP and ATP concentrations in the rat brain.

EXPERIMENTAL

Apparatus

A DuPont Model 848 liquid chromatograph (DuPont Instruments, Wilmington, DE, U.S.A.), a Rheodyne Model 7120 injector valve (Berkeley, CA, U.S.A.) with a 50-μl sample loop, either a DuPont fixed-wavelength detector at 254 nm or a Pye Unicam 4020 (Pye Unicam, Cambridge, U.K.) variable-wavelength detector and chart recorder OH 814 (Radelkis, Budapest, Hungary) were used. An analytical compact glass cartridge (CGC) column, either 150 × 3 mm I.D. or 100 × 3 mm I.D., pre-packed with Separon Six C_{18}, particle size 5 μm (Laboratorní Přístroje, Prague, Czechoslovakia) connected "butt-to-butt" with a short 15 × 3 mm I.D. guard column, was simply repacked after each 30—50 analyses with Separon $6C_{18}$ (particle size 10 μm) by use of a stainless-steel piston as suggested in the literature [5]. Saturation pre-column (50 × 4.6 mm I.D.) packed with 30-μm silica Silasorb (Lachema, Brno, Czechoslovakia) was connected between a high-pressure pump and an injector.

The eluent for optimal separation of nucleotides, consisting of 25 m*M* potassium dihydrogen phosphate with 4% triethylamine adjusted to pH 6.5 with concentrated phosphoric acid, was freshly prepared daily and filtered through a 0.22-μm Millipore filter (Millipore, Bedford, MA, U.S.A.).

Chemicals

Nucleotides ADP, ATP, cAMP, CDP, CTP, UTP, GTP, NADP and GDP were obtained from Serva (Heidelberg, F.R.G.), AMP, CMP, IMP, UMP, GMP, UDP, UDP-Glu, acetyl-CoA and nicotinamide mononucleotide were from Sigma (St. Louis, MO, U.S.A.), S-adenosylmethionine from Calbiochem-Behring (La Jolla, CA, U.S.A.) and NAD from Lachema. Triethylamine was purchased from Merck (Darmstadt, F.R.G.) and was used without additional purification. All other chemicals were of analytical-reagent grade; water was redistilled from potassium permanganate solution.

Extraction procedure

Male Wistar rats (250—300 g) were anaesthetized intraperitoneally with

pentobarbital (50 mg/kg) (Spofa, Prague, Czechoslovakia). The skin on the head was incised and the head was dipped into liquid nitrogen for 3 min. Frozen brain was freed from the skull and powdered, under constant cooling with liquid nitrogen. The powdered tissue was extracted according to the procedure originally proposed for yeast in 1 *M* formic acid saturated with 1-butanol [6]. The method was modified for the brain tissue as follows: approximately 200 mg of the powdered tissue was transferred into a polyethylene test-tube containing 1 ml of 1 *M* formic acid in a mixture of acetone—butanol—water (8:1:1) cooled to −70°C. The powdered tissue was evenly stirred in the solution, test-tubes were warmed to −5°C and 1 ml of the solution containing 1 *M* formic acid and 1 m*M* ethylene glycol bis(2-aminoethyl ether)-N,N′-tetraacetic acid (EGTA) in water saturated with butanol was added. The tissue was homogenized in a Potter-Elvehjem homogenizer with a PTFE piston and the homogenate was centrifuged at 4000 *g* for 10 min. The pellet was re-extracted with 0.5 ml of the extraction solution and pooled supernatants were lyophilized overnight. Dried extracts were dissolved in 200—1000 μl of water and centrifuged; the clear supernatant was used for LC analysis.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

An effect of the composition of the elution solution (salts, ionic strength, pH, concentration of ion-pairing reagent and organic modifier) on the retention of nucleotides was studied. Substitution of sodium or potassium cations for ammonium cations in the elution solution has no effect on resolution; among the counter-ions followed ($H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}, SO_4^{2-}, CH_3COO^-, $HCOO^-$) $H_2PO_4^-$ was found to be the most suitable. $(NH_4)_3PO_4$ or $(NH_4)H_2PO_4$ used originally were changed for the chemically purer KH_2PO_4 that gave lower blank values in the UV detector and its use did not require frequent column regeneration. Similar effects were found with nucleotide separation by anion-exchange LC [2]. Ionic strength of the buffer has no significant effect on chromatographic resolution in the range 0.1—1.2 and, therefore, a concentration 25 m*M* KH_2PO_4 was used in all experiments.

Ion-suppression LC does not give perfect resolution of nucleotides, especially for ADP and ATP [7, 8], whereas ion-paired chromatography with tetrabutylammonium cation as the ion-pairing reagent, and with gradient elution [1], results in a change in the retention order of nucleotides according to the content of the phosphoric acid moiety and not according to the base type. Correspondingly, triethylamine has been chosen as a surfactant, as it is one of the weakest cationic surface-active substances used in reversed-phase LC [9] and, thus, it could be expected not to induce very marked changes in the retention characteristics of adenine nucleotides. An effect of triethylamine and pH on the capacity factor k' for ATP is given in Fig. 1. According to current opinion, the surfactant effect on the retention in reversed-phase LC cannot be unambiguously attributed either to ion-pair formation or to the mechanism of dynamic ion-exchange chromatography and, therefore, in agreement with suggested broader ion-interaction chromatography [10], a thermodynamic

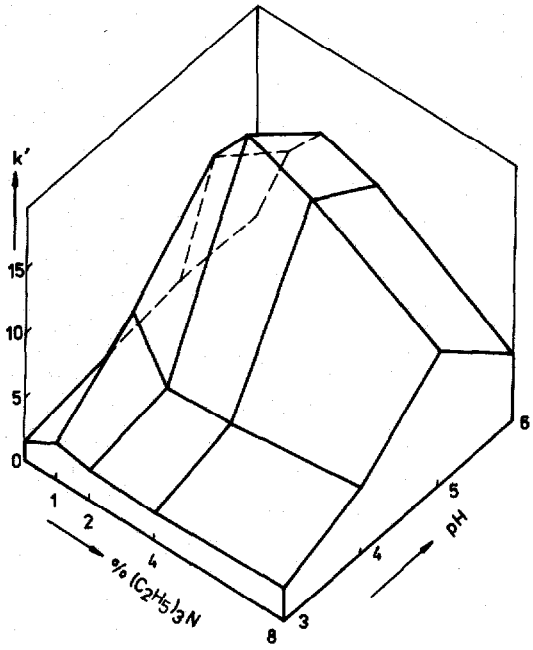
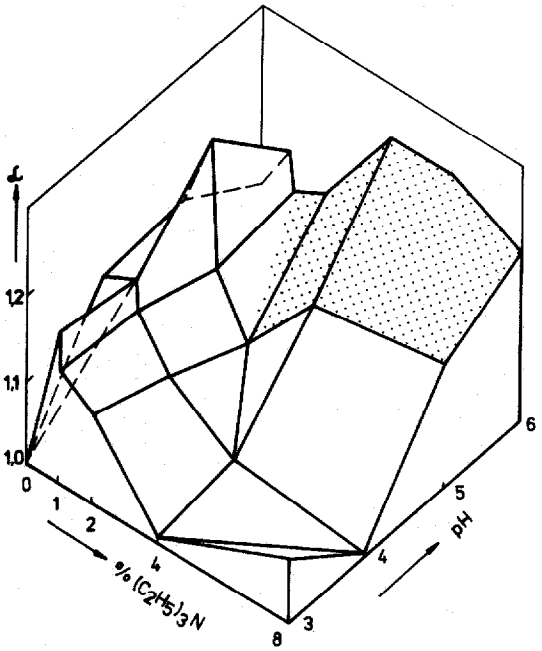


Fig. 1. Influence of ion-pairing reagent, triethylamine, and pH on ATP retention. Chromatographic conditions: a 15-cm CGC Separon $6C_{18}$ column; eluent 0.1 *M* $(NH_4)_3PO_4$; flow-rate 0.5 ml/min.

model for retention was proposed [11]. The dependence of the capacity factor (k') value on triethylamine content in the elution buffer (shown in Fig. 1) is in agreement with the above model. The ascending part of the curve represents a contribution of Langmuir's adsorption, while the descending part represents a contribution of interfacial tension effects according to the Szyszkowski equation. Similar proportionalities were found for GTP, AMP and ADP. The proportionality of k' versus pH plotted in Fig. 1 showed a maximum in pH range 4—6, i.e. between the two ATP pK_a values (4.0 and 6.5). This fact is also known for the behaviour of zwitter-ionic solutes in reversed-phase LC [12, 13], which enables the conclusion to be drawn that the reversed phase preserves its hydrophobic character in spite of ion-interaction effects due to triethylamine application and, simultaneously, the separation in reversed-phase mode is asserted.

Nucleotides differ from each other in polarity and pK_a value, therefore optimal conditions for separation have to be found. In Fig. 2, a two-factor window diagram [14] is shown for four nucleotides: GTP, AMP, ADP and ATP. In the diagram, two maxima are seen: the first one (α_{min} = 1.23) for 0.8% $(C_2H_5)_3N$ at pH 5.0. An order of elution for GTP, ADP, ATP and AMP is similar to that in ion-suppression LC except there is a change in the position of ADP and ATP. In the second maximum, the optimal chromatographic performance as determined by the separation ratio (α) value of the least resolved pair (α_{min} = 1.24) was found for 4% $(C_2H_5)_3N$ at pH 6.0. The dotted area around this maximum indicates the chromatographic conditions with nucleotides eluted in the order GTP > AMP > ADP > ATP, and represents also the area of



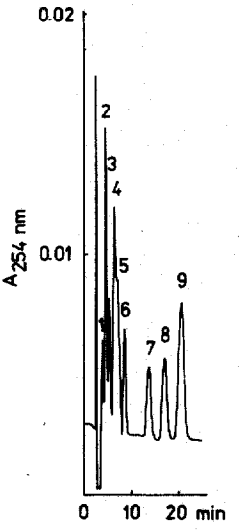


Fig. 2. A two-factor window diagram for GTP, AMP, ADP and ATP.

Fig. 3. Separation of a standard nucleotide mixture on a 15-cm CGC Separon $6C_{18}$ column under optimal conditions. Eluent: 0.1 *M* $(NH_4)_3PO_4$ containing 4% triethylamine at pH 6.0; flow-rate 0.5 ml/min. Peaks: 1 = CMP; 2 = CDP + CTP + UMP; 3 = UDP; 4 = UTP + GMP; 5 = IMP + GDP; 6 = GTP; 7 = AMP; 8 = ADP; 9 = ATP.

strongest ion-interaction effect. A chromatogram of nucleotide standards under these optimal conditions, obtained with a 15 cm long column, is shown in Fig. 3.

The addition of a 3% concentration of the organic modifier (acetonitrile or methanol) caused a decrease of 65% in the k' value of adenine nucleotides, as well as an overlapping of the peaks of AMP and NAD. Therefore, to shorten the analysis time, it was more advantageous to use the shorter column rather than to vary the solvent strength. A chromatogram of the separation of (a) standard nucleotide mixture, (b) rat brain extract and (c) the same brain extract with the addition of standards as in a, in a 10-cm column, is shown in Fig. 4. To achieve better resolution of AMP and NAD, the pH of the eluent was increased to 6.5.

Peaks were identified by their retention times, by co-elution with added standards and by comparing absorbance ratios at different wavelengths (A_{254}/A_{240}, A_{254}/A_{260}, A_{254}/A_{276}) with those of the standards [15]. It was found that mono-, di-, and triphosphates of uracil, cytosine, guanine, NAD, NADH, UDP-Glu, Acetyl-CoA, S-adenosylmethionine and nicotinamide mononucleotide do not interfere in the analysis of adenine nucleotides. The retention time of NADP is equal to that of ADP; its effect on the accuracy of ADP determination, however, can be neglected since its concentration is more than 100 times lower as compared to ADP [16].

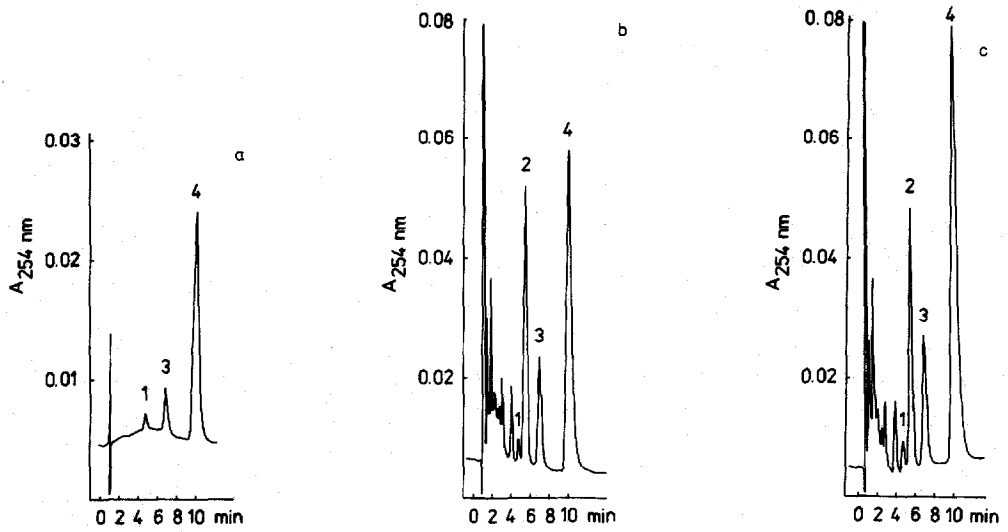


Fig. 4. Chromatographic separation on a 10-cm Separon $6C_{18}$ column. Eluent: 25 m*M* potassium phosphate, 4% triethylamine at pH 6.5; flow-rate 1 ml/min; (a) 20 μl of a standard mixture; (b) 20 μl of the rat brain extract; (c) 20 μl of brain extract (see b) + 20 μl of standard mixture (see a). Peaks: 1 = AMP (0.064 nmol); 2 = NAD; 3 = ADP (0.315 nmol); 4 = ATP (3.3 nmol).

Calibration, reproducibility and comparison with literature data

AMP, ADP and ATP standards were analysed in the concentration range 10 pmol to 25 nmol. Under constant chromatographic conditions, calibration graphs can be plotted simply by measuring the peak height. Calibration curves were calculated by linear regression analysis: AMP: $y = 0.0205\ x + 0.0012$; ADP: $y = 0.0193\ x + 0.003$; ATP: $y = 0.0182\ x + 0.011$. The reproducibility of calibration curves, however, can easily be affected by gradual ageing of the column, by flow-rate fluctuations and by measurement at different sensitivities of the detector, therefore, for practical purposes, a method of direct com-

TABLE I

ADENINE NUCLEOTIDES IN THE RAT BRAIN

Values are given in nmol/mg wet weight ± S.E.M.

Method	AMP	ADP	ATP	Total	Energy charge potential
Immersion in liquid nitrogen LC, present method (n = 10)	0.058 ± 0.002	0.288 ± 0.009	3.01 ± 0.09	3.36	0.940
Immersion in liquid nitrogen Fluorometric analysis [17]	0.041 ± 0.002	0.258 ± 0.001	2.99 ± 0.02	3.29	0.949
Freeze blowing Fluorometric analysis [19]	0.038 ± 0.004	0.382 ± 0.036	2.65 ± 0.25	3.07	0.925
Surface freezing Ion-exchange LC [20]	0.008 ± 0.002	0.158 ± 0.008	2.83 ± 0.07	3.00	0.969

parison to the standard, with a similar nucleotide content to that in the sample, has been chosen. Standards were analysed following five to seven analyses of tissue samples. The error of measurement for parallel analyses of brain extract depends on the peak height, but for peaks higher than 25 mm it was lower than 2%.

Determined values of AMP, ADP and ATP and their standard error of the mean (S.E.M.) depend both on the method of killing the animals [17, 18] and on the method of nucleotide extraction [6]. Literature data on adenine nucleotide concentrations in brain tissue are rather contradictory. Table I shows that ATP, ADP and AMP values, as determined by this procedure, are in good agreement with data obtained by other methods that are generally accepted and used. The concentration of AMP is higher than that given in some other studies, probably as a consequence of the method of tissue extraction rather than that of animal killing.

CONCLUSION

Ion-paired reversed-phase LC with triethylamine as the weak ion-pairing reagent allows the determination of AMP, ADP and ATP in brain tissue under isocratic conditions. Optimization of chromatographic parameters shortened the time of analysis to about 10 min.

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