MICROANALYSIS OF FREE RIBONUCLEOTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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The existing method of separating nucleotides on anion-exchange columns has been improved. It has been shown that the displacement of the base line of the UV detector connected with gradient elution of the sample can be decreased by purifying the eluent. A method for the effective purification of KH_2PO_4 is described. A method has been developed for the optical compensation of the displacement of the base line which consists in superposing a negative concentration gradient of a nonionogenic optically dense component of the mobile phase. A composition of the mobile phase and conditions of chromatography are given which permit the pool of free ribonucleotides in an extract from $1.0 \cdot 10^{-6}$ and fewer cells to be determined.

Information on the quantitative composition of nucleotides in biological objects is of considerable interest in the study of cell metabolism. The appearance and rapid development of the technique of high-performance liquid chromatography (HPLC) has considerably broadened the possibilities of chromatographic analysis. At the present time, the quantitative determination of free nucleotides by the HPLC method on ion-exchange resins using a UV detector is generally adopted [1-4]. A necessary condition for the simultaneous determination of nucleotides with different degrees of phosphorylation is a gradient elution regime. The displacement of the base line (BL) of the detector connected with the gradient regime is a factor which limits the sensitivity of the method in the region of low concentrations of nucleotides [2, 3]. The selection of the composition of the mobile phase and the purification of the eluents from UV-absorbing impurities enable the magnitude of the displacement of the BL to be decreased, which is essential for medicobiological investigations in which only a small amount of material to be analyzed is available.

A buffer solution of potassium or ammonium dihydrogen phosphate is most frequently used for the HPLC of nucleotides [1-7]. Schmuckler [8] has described a procedure for purifying KH_2PO_4, but the lengthiness and laboriousness of this method limit its application. We have investigated various possibilities for decreasing the displacement of the base line and have proposed a method of optical compensation which permits working without a displacement in the range of sensitivity of the UV detector of 0.01 optical unit for the scale and above.

To free the salt from UV-absorbing impurities we have used the method of repeated recrystallization from supersaturated aqueous solution in combination with precipitation from aqueous methanol. This method was developed on the basis of our experimental results according to which in the recrystallization of KH_2PO_4 from aqueous solutions the impurities remain in the mother liquor and the crystals are relatively purified. However, at a low rate of growth of the crystals the impurities reprecipitate with them and the quality of purification falls. In addition, the residual level of UV absorption of the recrystallized salt can be lowered by precipitation from aqueous methanol (Table 1).

Three different batches of KH_2PO_4 were purified by three recrystallizations and one reprecipitation with methanol. One batch required additional recrystallization after precipitation from aqueous ethanol. In this way, several portions of purified KH_2PO_4 were obtained the level of UV absorption of which permitted its use for HPLC at a sensitivity of the UV detector of 0.08 optical units for the scale (Table 2). A chromatogram of the salt solution (without a sample) had two isolated peaks in the regions of concentrations of 0.25

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TABLE 1. Efficacy of Various Stages in the Purification of KH_2PO_4

Purification procedure	Object	UV absorption*
1. Initial reagent		0.337
2. First recrystallization from supersaturated aqueous solution	Crystals taken after 3 h	0.037
	Crystals taken after 24 h	0.081
3. Second recrystallization from supersaturated aqueous solution	Crystals	0.035
4. Third recrystallization from water	Crystals	0.027
5. Precipitation with methanol	Crystals	0.015

*The UV absorption of a 1.0 M solution in a quartz cell with an optical path length of 1.0 cm and a wavelength of 254 nm.

TABLE 2. Efficacy of a Complete Cycle of Purification for Various Batches of Reagent

Batch of salt	Purity grade	Absorption*	
		initial	after the cycle of purification
1	ultrapure	0,337	0,015†
2	cp	0,092	0,029
3	cp	0,095	0,021
4	cp	0,112	0,034

*UV absorption of a 1.0 M aqueous solution at a wavelength of 254 nm with a path length of 1.0 cm (optical units).
†The purification of this batch by Schmukler's method [8] gave a product with a UV absorption of 0.035.

and 0.4 M and a uniformly rising base line (Fig. 1). The retention times of the peaks were reproducible.

In order to separate nucleotides we used an eluent consisting of an aqueous solution of KH_2PO_4 and KCl in equal concentrations. The partial replacement of phosphate by chloride with no change in the total molarity lowers the displacement of the base line and has a favorable influence on its form (Fig. 1). A chromatogram of this eluent had practically no peaks and the form of the base line accurately corresponded to the form of the gradient. The linear relationship between the difference in the optical densities in the starting and the final buffers and the displacement of the base line permits the assumption that by superposing a negative concentration gradient of an optically dense component not interacting with the stationary phase of the column and not affecting the selectivity of separation a base line with no displacement can be obtained. We tested a number of such compounds, and from these we selected acetone. Although there are reports of the successful separation of a mixture of ketones on anion-exchange resins [9], we were unable to detect any interaction of the acetone with the material of the column. A chromatogram of acetone in the

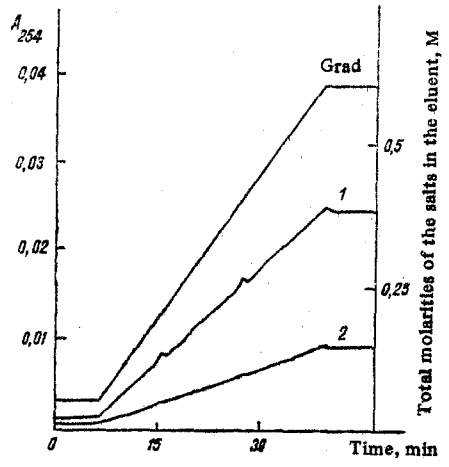

Fig. 1

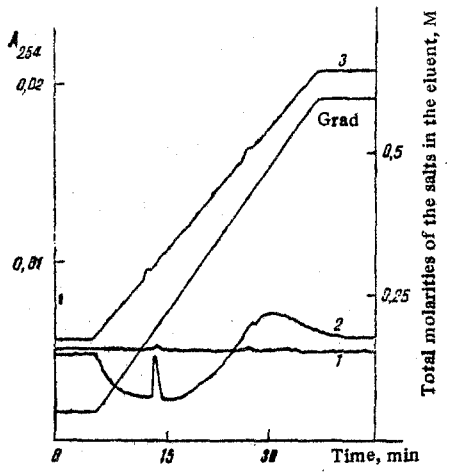

Fig. 2

Fig. 1. Form of the base line of the UV detector on the elution of a mobile phase with concentration gradients (Grad) from 4.0 mM KH_2PO_4 to 0.6 M KH_2PO_4 (1) and from 2 mM KH_2PO_4 + 2 mM KCl to 0.3 M KH_2PO_4 + 0.3 M KCl (2).

Fig. 2. Change in the form of the correctly compensated mobile phase (1) with a lowering of the temperature of the column by 12°C (2) and a lowering of the pH of the final buffer by 1.0 unit (3). Form of the concentration gradient — "Grad".

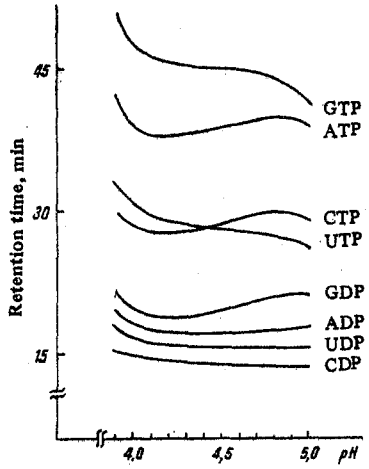

Fig. 3. Dependence of the retention times of nucleoside di- and triphosphates on the pH of the final buffer.

given solvent system consisted of a single narrow peak issuing with the solvent front. The high optical density and the convenient absorption maximum of aqueous solutions of acetone (about 260 nm) permits it to be used for optical compensation in very low concentrations (about 0.003%), which has no effect on the process of chromatographic separation.

Acetone was added to a low concentration of the buffer (starting buffer) in an amount ensuring the equivalence of the optical density of the starting and final buffers, which was checked spectrophotometrically at the wavelength of the UV detector. When the correctly compensated mobile phase was chromatographed there was no displacement of the baseline and no foreign peaks were recorded (Fig. 2).

As already mentioned, the method of optical compensation is applicable only under the condition of a linear dependence of the readings of the UV detector on the concentration of the salt in the eluent, i.e., when the following equation is satisfied:

$$A = k \cdot C + A_0 (I - C),$$

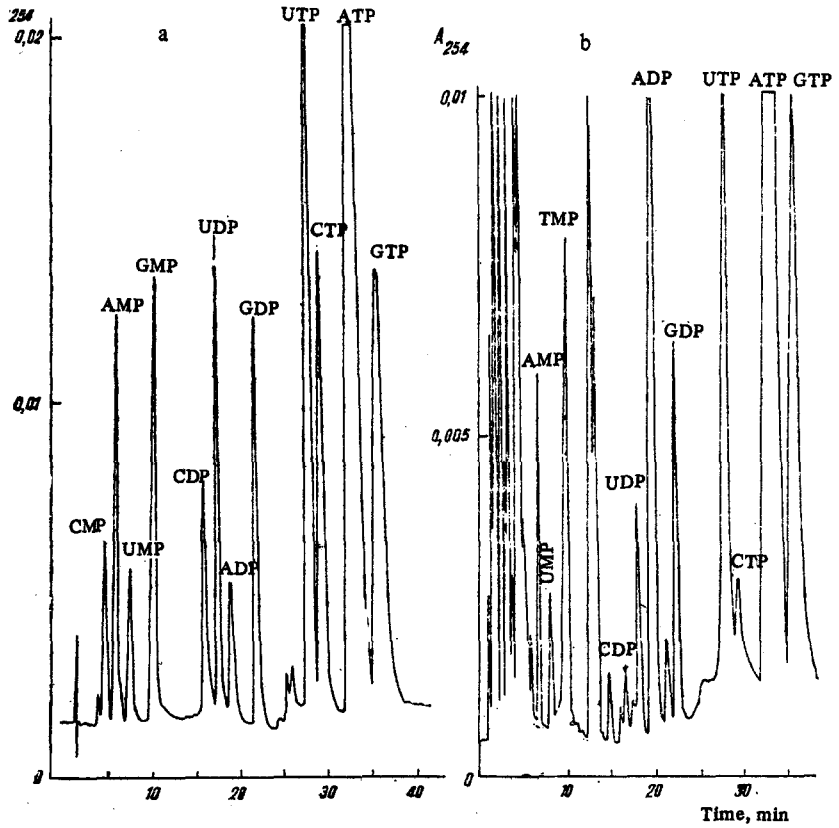

Fig. 4. Chromatogram of standard nucleotides (a) and chromatogram of an acid-soluble extract of human myeloblastic cells ($3.0 \cdot 10^6$) (b).

where A is the reading of the UV detector;

A_0 is the specific extinction of the starting buffer;

C is the proportion of the final buffer in the composition of the mobile phase; and

k is a proportionality factor which depends on the degree of purity of the salt and the conditions of chromatography. The condition of linearity is observed if the UV-absorbing impurities of the eluent do not interact with the material of the column. The displacement of the base line is described by the equation

$$\Delta A = k \cdot C + A_0 (1 - C) - A_0 = C (k - A_0).$$

It is easy to see that the addition of the compensator to the starting buffer lowers the value of ΔA, and when $A_0 = k$ the displacement of the base line is equal to zero. The magnitude k thus characterizes the partial extinction of the final buffer in the eluate.

In a real chromatographic system, as the result of the partial interaction of the impurities with the ion-exchange resin in the matrix, a deviation from linearity is observed. These deviations are greater the higher the optical density of the final buffer.

The procedure for the purification of the salt that has been described and the addition of chloride to the eluent, which probably suppresses the interaction of the impurities with the column, and also raising the temperature of the column permit the form of the baseline to be approximated to the ideal form. Raising the pH of the column buffer from 3.5 to 5.0 has no effect on linearity but appreciably decreases the magnitude k.

In practice, even at a temperature of 35°C and with a difference in the optical densities of the starting and final buffers not exceeding 0.01 optical units, with the use of an optical compensator a base line of satisfactory form is obtained (Fig. 2).

For the chromatographic separation of ribonucleoside mono-, di-, and triphosphates we used a concentration gradient of phosphate–chloride buffer with an optical compensator.

The composition and program of the mobile phase were selected experimentally in the light of literature information [3, 5, 6]. To improve the selectivity of separation we studied the dependence of the retention times of ribonucleoside di- and triphosphates in the column on the pH of the final buffer. As can be seen from Fig. 3, the retention curves of UTP and CTP have a point of inflection at pH 4.4. At pH 3.9 and 4.9 all the peaks are well separated (Fig. 4a), but the sequence of elution of UTP and CTP is different. A final buffer with pH 4.9 is preferable, since it gives a smaller value of k and sharper and more symmetrical peaks of the triphosphates.

On the whole, for the HPLC of the ribonucleotides using strong anion-exchange resins it is possible to recommend the following composition and program of the mobile phase. Binary system of solvents: starting buffer 2.0 mM KH_2PO_4 + 2.0 mM KCl, pH 3.0; final buffer 0.3 M KH_2PO_4 + 0.3 M KCl, pH 4.9. Linear gradient of from 0 to 100% concentration of the buffer for 20 min with delaying of gradient for 4 min and isocratic elution with the final buffer until the GTP is eluted (about 20 min). Rate of flow of the eluent, 2.0 ml/min; temperature of the column, 35°C.

The use of these conditions of separation for the analysis of free ribonucleotides in solid extracts of animal and human cells has given satisfactory results. Figure 4b shows a chromatogram of an acid-soluble extract from human myeloblastic cells. The use of eluents purified by the proposed method and of optical compensation of the displacement of the base line permits working at a sensitivity of the UV detector of 0.01 optical units on the scale and above. At such a sensitivity of UV photometry we have obtained good results on analyzing an acid-soluble extract from $1.0 \cdot 10^6$ cells (about 1.0 mg).

EXPERIMENTAL

A Spectra Physics SP-8000 high-pressure liquid chromatograph and SP-8310 UV detector (254 nm) and a Whatman anion-exchange resin column with Partisil-PXS-19 SAX, 4.6 mm × 25.0 cm, were used. As the standard we employed the ribonucleoside monophosphates UMP, CMP, GMP, and AMP (Serva) and the corresponding ribonucleoside di- and triphosphates (Reanal). The acid-soluble cell extract was obtained by the usual method [10], and the cells were treated with perchloric acid.

Purification of the Salt. Recrystallization from a supersaturated aqueous solution was carried out in the following way: A 5.0-M KH_2PO_4 solution prepared with heating to 95°C was carefully and rapidly cooled in an ice bath, after which it was vigorously stirred. The fine crystals of the salt that deposited were filtered off on a paper filter and dried in a dry-heat chest at 120°C for 3-4 h.

The reagent that had been recrystallized in this way was dissolved with heating to 80°C in water to saturation. To the hot solution so prepared, methanol was added in small portions with the continuous stirring to give a water:methanol ratio of 2:1. The fine crystals that then deposited were filtered off on a paper filter (Filtrak No. 389, GDR) and dried.

The quality of purification of the KH_2PO_4 was determined from the UV absorption of a 1.0 M solution of the salt on SF-16 and Varian Cary-219 spectrophotometers.

SUMMARY

A procedure has been developed for the effective purification of KH_2PO_4 and a composition of the mobile phase giving the minimum displacement of the baseline of the UV detector in the gradient elution of a sample with a phosphate buffer in the process of TPLC analysis has been proposed.

A method of optical compensation has been developed which permits working under a gradient elution regime with a high sensitivity of the UV detector with no displacement of the base line. The method gives good results in the analysis of nucleotides in small amounts of biological material.

The use of the modifications of the method of HPLC analysis of ribonucleotides that have been described permits the sensitivity of the determination to be raised by an order of magnitude.

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INFLUENCE OF THE POTENTIAL OF THE OXIDIZING SYSTEM ON THE EXOTHERMIC STAGES IN THE OXIDATION OF LIGNIN

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It has been shown that on the oxidation of soda lignin by the potassium ferri-ferrocyanide system thermal and luminous energy due to exothermic processes are evolved. The heat effect and also the total amount of light emitted are determined by the potential of the oxidizing system.

The development of the theory of soda and sulfate cooks and also of oxidative methods for the delignification and bleaching of lignocellulose materials raises the question of the investigation of redox processes in lignin. It is known [1] that lignin is a biopolymer of stereoirregular structure with various types of substitution of the phenylpropane structural units. The oxidation potential of model compounds of the structural unit of lignin are different and, consequently, it may be expected that the individual fragments in lignin [2] likewise differ in relation to their redox potentials. In actual fact, when using various oxidizing systems the oxidation processes take place differently. In view of the inhomogeneity and different properties of the individual fragments, it is general laws of the occurrence of redox processes in lignin that must be studied.

The oxidation of lignin is accompanied by the evolution of luminous energy (chemiluminescence), which shows the existence of exothermic or endothermic stages [3]. We have considered that the dependence of the evolution of thermal and luminous energy in the titration of an alkaline solution of soda lignin by a potassium ferri-ferrocyanide redox system on the potential of this system.

On titration, both thermal and luminous energy were evolved. The intensity of luminescence (I) in the process of titration passed through a maximum with a following fall (Fig. 1). The total amount of light emitted (S) and the dependence of the rise in temperature on the volume of titrant have sigmoid forms. The thermal effect of the process has been calculated from the results of calorimetry. With an increase in the potential of the oxidizing system from E = 0.223 V to E = 0.300 V the thermal effect, like the total amount of light emitted, rose to ΔH = 51.1 kcal/nominal mole of lignin and $S = 2.17 \cdot 10^{-8}$ lm·sec. With a rise in the potential of the oxidizing system they fell. The positive heat effect and the evolution of luminous energy indicate the existence of exothermic stages.

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