Use of High-Pressure Liquid Chromatography for Monitoring Nucleotide Concentration in Human Blood: A Preliminary Study with Stored Blood Cell Suspensions

Phyllis R. Brown, R. E. Parks, Jr., and Jane Herod

The development of high-pressure liquid chromatography has made available a new technique for monitoring nucleotide concentrations in biological materials sensitively, accurately, and rapidly. Because there appears to be a correlation between ATP concentrations and viability of stored blood, a preliminary study was carried out on blood cell suspensions to evaluate the technique for possible use in blood banks, clinical laboratories, and research on preservatives for blood.

Additional Keyphrases: viability of stored blood • blood banking • ATP, ADP, AMP • blood preservation

The recent development of high-pressure liquid chromatography (HPLC) has introduced a new technique for monitoring nucleotide concentrations in biological materials (1, 2). Other methods are available for the determination of ATP (3) and other nucleotides; however, an advantage in using HPLC is that the major nucleotides can be determined at one time. In addition, the analyses are accomplished with speed, sensitivity, accuracy, and ease of operation.

A decrease in the concentrations of organic phosphate, especially that of ATP, is closely associated with the loss of viability of stored blood (4–7). The relationship between viability and ATP was investigated by Gabrio et al. (8, 9) and they found that the post-transfusion survival time of stored blood was substantially increased when the ATP concentrations were elevated through incubation with adenosine. Because there appears to be a correlation between ATP concentrations and erythrocytic survival, it seems likely that the viability of stored blood may be evaluated by monitoring adenine nucleotide concentrations.

At present the condition of stored blood is usually judged by time-consuming in vivo tests in which the fate of the erythrocytes in the circulation of the recipient is observed. The index of viability of stored blood is the percentage of injected erythrocytes that survive 24 to 48 h after transfusion. This index of viability is also referred to as the "post-transfusion survival" (10).

Since there is no convenient and rapid "in vitro" method for monitoring the viability of blood, blood banks usually discard blood stored for more than 21 days. In addition, rapid monitoring techniques are needed in research to evaluate the efficiency of blood preservation. Much research is in progress, not only to prolong the storage life of blood but also to improve the quality of stored blood, thus making it possible to stockpile large volumes of blood against the possibility of disaster and to store blood of rare types for emergencies (10). This paper describes a preliminary study, with high-pressure liquid chromatography, of the nucleotide concentrations in human blood cell suspensions stored over a period of 5 months.

It should be emphasized that this investigation was undertaken mainly to evaluate the technique for blood storage work. Thus, the blood samples used were those readily available to us in large quantity. This blood was not whole blood but blood from which the plasma-rich platelets had been removed. Therefore, the blood referred to in this study consisted of erythrocytes and leukocytes suspended in acid-citrate–dextrose (ACD).
**Materials and Methods**

Human blood, obtained from the Hematologic Research Division of the Memorial Hospital, from which the platelet-rich plasma had been removed, was stored in ACD for various periods of time. It was deproteinized by adding 1 volume of sample of 2 volumes of cold trichloroacetic acid solution (120 g/liter) with rapid stirring on a vortex-type mixer. The suspensions were centrifuged and 0.5 ml of the supernatant fluid was neutralized with a saturated solution of tri(hydroxymethyl)aminomethane. These nucleotide extracts were frozen immediately.

Twenty-microliter samples of the solutions were analyzed by high-pressure liquid chromatography by use of a Varian LCS 1000 (2). The column was 1 mm × 3 m, stainless-steel, and packed with a pellicular anion-exchange resin (Varian Aerograph LFS). The low concentration eluent of the linear gradient was 0.015 molar KH$_2$PO$_4$, the high concentration eluent was 0.025 molar KH$_2$PO$_4$ in 2.2 molar KCl. The pH of both solutions was adjusted to 4.5 with KOH. The flow rate of the eluent into the column was 12 ml/h and the flow rate of the high-concentration eluent into the low-concentration buffer was 6 ml/h. Column temperature was 75°C.

To determine the possibility of more rapid analyses, we did a few analyses with higher flow rates—the flow rate of the eluent into the column was increased to 24 ml/h and that of the high concentration eluent into the low concentration to 12 ml/h.

Nucleotide concentrations were determined by multiplying peak height times width at half height and comparing this area to that of a known concentration of the nucleotide (2).

**Results**

As has been reported previously (4–7), ATP concentrations in the stored blood decreased with time of storage (Figures 1, 2, 3). The AMP and ADP concentrations increased in blood that was stored up to three weeks and then decreased with storage time. After three months, the concentrations of all the adenine nucleotides had decreased to a point where they were negligible. A large peak with a retention time of 7 min was seen in a chromatogram of blood stored for long periods. This peak contained nucleosides and bases that are not retained on the column under the operating conditions described.

When the samples were analyzed with use of flow rates of 24 ml/h and 12 ml/h, the retention times of the adenine nucleotides were decreased (Figure 4) without loss of resolution of AMP, ADP, and ATP peaks. This increased speed of analysis was possible with human blood cell suspensions because the adenine nucleotides are the only ones present in significant concentrations. If a pure leukocyte sample or a sample of the whole blood of other species is to be analyzed, care must be taken to maintain good resolution of all peaks, because guanosine nucleotide and (or) UTP may be present in appreciable quantities (13, 14). With increased flow rates, appropriate calibration factors must be determined, because it has been found that peak areas are affected by flow rates (15).

**Discussion**

HPLC is a new technique with great potential for monitoring the nucleotide levels of stored blood. It can be valuable because the whole spectrum of nucleotides in cell extracts can be analyzed at one time. Thus, dynamic reactions can be monitored and the formation and (or) degradation of various nucleotides can be followed concurrently. For example, the decrease in ATP concentrations in stored blood with time can be followed at the same time as alterations in AMP and ADP concentrations. These data may be useful in evaluating the condition of stored blood, because the decrease of AMP and ADP may indicate the start of irreversible degradations that lead to a loss of erythrocytic viability. In addition, this technique can be used in investigations of preservatives for blood—for example, in examining the use of ribonucleosides as preservatives. In investigating various preservatives to be used with ACD, Gabrio et al. (16) found that the most efficient ribonucleoside preservative was inosine, followed by adenosine, guanosine, and xanthosine in descending order.
The post-transfusion survival with inosine and ACD averaged 82% after 6–7 weeks storage. With HPLC, we were able to monitor rapidly and sensitively the in vitro metabolism of these compounds and other adenosine analogs in fresh and stored human erythrocytes and to determine whether nucleotides are formed, which nucleotides are produced (the mon-, di-, or triphosphate nucleotide), and the effect of the presence of exogenous quantities of ribonucleosides on ATP concentrations (17). It was found that when erythrocytes from fresh human blood were incubated in vitro with adenosine or inosine, large concentrations of the monophosphate nucleotide accumulated. When erythrocytes were incubated with guanosine, under the same conditions, a quantity of GTP was formed that exceeded the ATP level by several fold, and there was no change in the ATP concentration (18). The experiments with guanosine suggest the possibility that erythrocyte survival may be improved by storing extra high-energy phosphate bonds in the form of GTP. Because there is a large amount of nucleoside diphosphate kinase (EC 2.7.4.6) in erythrocytes (19), the GTP may feed into the ATP system. Further investigations on the metabolism of ribonucleosides in fresh and stored blood are under way.

It must be emphasized that this investigation was a preliminary study, to determine the possible potential of HPLC in monitoring the viability of stored blood and in research on blood preservatives. Further work is required on a large number of stored blood samples to analyze statistically the validity of our results. In addition, in vivo assays of the post-transfusion survival of stored blood should be performed by chromium tagging (20) and the data obtained from the in vivo assay of viability correlated with the results of the nucleotide analysis. Thus from a graphic correlation between ATP concentrations and post-transfusion survival, the viability of a stored sample may be monitored by measuring the sample’s ATP concentration. It is also possible that a correlation will be found between erythrocytic survival and the concentrations of nucleotides other than ATP. The availability of HPLC may make possible the identification of such correlations, if they exist.

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References


