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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ASCORBATE-2-PHOSPHATE, ADENINE AND HYPOXANTHINE IN STORED HUMAN BLOOD*

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SUMMARY

A high-performance liquid chromatographic procedure was developed to assay for adenine, hypoxanthine, and ascorbate-2-phosphate in both red blood cells and plasma. The samples were diluted and filtered through Centriflo filter cones to remove most of the blood proteins before injection onto the column. The application of the technique to an experimental blood preservation study is illustrated.

INTRODUCTION

When human blood is stored in blood banks it is collected in a citrate anticoagulant and given nutrient supplementation to maintain cellular viability and function throughout the storage period [1]. During the development of new preservation systems we wanted to monitor the levels of two chemical additives: adenine and ascorbate-2-phosphate (AsP), as well as the level of hypoxanthine, a metabolite of ATP, during six weeks of blood storage. Adenine is utilized by the red blood cells to help maintain the cellular ATP level, (which correlates with retention of viability), and ascorbate-2-phosphate assists in the maintenance of red cell 2,3-diphosphoglycerate (2,3-DPG) (which correlated with the oxygen-delivery function of the cells) [1]. Hypoxanthine indicates how much of the adenine nucleotide pool is lost during storage.

Reports on the high-performance liquid chromatographic (HPLC) analysis of simple aqueous solutions of adenine are available [2]. AsP analysis by HPLC

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has also been described for simple mixtures in water solutions [3]. In this paper we present an HPLC method to assay for adenine, AsP and hypoxanthine in both the red cells and the supernatant plasma during six weeks of blood bank storage at 4°C. The red cells were stored in an additive solution, with and without residual plasma, to determine if the plasma enzymes (phosphatases) would modify the utilization or distribution of AsP during red cell storage.

EXPERIMENTAL

Blood storage study

All blood was drawn into citrate–phosphate–dextrose (CPD) solution contained in standard plastic blood bags. Four units of packed red cells were each divided in half, and one half of each was washed with isotonic saline to remove residual plasma. The final hematocrit of each half unit was 80%. To each half unit (about 150 ml) we added (by sterile filtration) an additive solution (50 ml) containing adenine, AsP, glucose, sodium phosphate and sodium chloride [1]. The units were stored at 4°C for 42 days and assayed weekly for adenine, AsP and hypoxanthine. Other metabolic assays of interest (data not reported here) were done by microfluorometric enzyme assays [4]. At the end of the study all blood units were cultured to insure that sterility had been maintained.

Sample preparation and recovery studies

At weekly intervals, 1-ml blood samples were removed from the storage bags and centrifuged by a double centrifugation technique (2800 *g* for 10 min, then 26 000 *g* for 10 min) to separate plasma and red cells. Hematocrits of the final packed red cells were measured. Red cells were lysed by adding three volumes of cold water. Plasma samples were also diluted with three volumes of cold water. All samples were deproteinated by centrifugal filtration using Amicon (Danvers, MA, U.S.A.) CF-25 Centriflo cones, with centrifugation at 1000 *g* for 30 min at 4°C. Samples were stored frozen and then thawed and filtered through 0.22-μm filters before the HPLC analysis.

Sample recovery from the preparation procedure was evaluated by adding aliquots of each standard compound (0.01–0.9 mmol/l) to red cell lysates, supernatant plasma, and plasma diluted with isotonic saline ($n=4$ each). The recovery samples were then processed by filtration as described above and analyzed by HPLC with comparison to known concentrations of pure standards. Samples of fresh CPD blood were used for the recovery studies.

Instrumentation and chromatographic conditions

All analyses were done on a 25 cm × 4.6 mm Ultrasil-AX anion-exchange column, 10-μm particle size (Altex, Berkeley, CA, U.S.A.) with a Waters Assoc. (Milford, MA, U.S.A.) HPLC system consisting of Model 510 pumps, a Model 721 controller, a Model 450 detector, and a WISP autosampler. The mobile phase was HPLC-grade water in pump A and 0.3 *M* potassium dihydrogen phosphate buffer at pH 4.4 in pump B. Flow-rates varied from 2.0 to 3.5 ml/min and the delivery from pump B varied from 0 to 100% (see Table I). Separations were

TABLE I

GRADIENT AND FLOW PROGRAMS FOR THE RED CELL LYSATES AND PLASMA ASSAYS

Time (min)	Flow-rate (ml/min)	Percentage pump A	Percentage pump B
Red cell lysates			
0	1.90	100	0
7	2.20	81	19
8	3.00	35	65
12	3.50	30	70
14	3.50	0	100
24	3.50	0	100
27	3.00	100	0
30	3.00	100	0
31	1.90	100	0
Plasma			
0	1.90	100	0
7	2.20	81	19
8	3.00	35	65
11	4.00	35	65
14	3.00	100	0
18	1.90	100	0

performed at room temperature with UV detection at 254 nm. The system pressure was monitored on the second channel of the data station. The gradients were all linear with analysis times of 18 min for the plasma samples and 31 min for the red cell samples. Details of the flow programs are shown in Table I.

Calculations

Calculations of the final concentrations for the compounds of interest were made using the statistics and spreadsheet capabilities of MINITAB (Minitab Project, University Park, PA, U.S.A.). Sample peak integration counts were converted to concentrations using linear regression of the counts from standards run at several concentrations. The sample concentrations were then corrected for dilution, percentage recovery (from the recovery studies), and percentage plasma contamination of the red cell lysates.

RESULTS

Typical chromatograms for red cell lysates and plasma or supernatant runs are shown in Fig. 1. The two types of samples were analyzed under different conditions because of interfering peaks in the red cell lysates. The AsP seen in the lysate trace is from contaminating supernatant solution. No intracellular AsP was found in the red cell lysates of any samples. The ascorbic acid (AA) shown in Fig. 1B was added to the sample to show its elution position. A peak with the same retention time as AA was seen in some samples, but was impossible to quantify due to its (chemical?) instability.

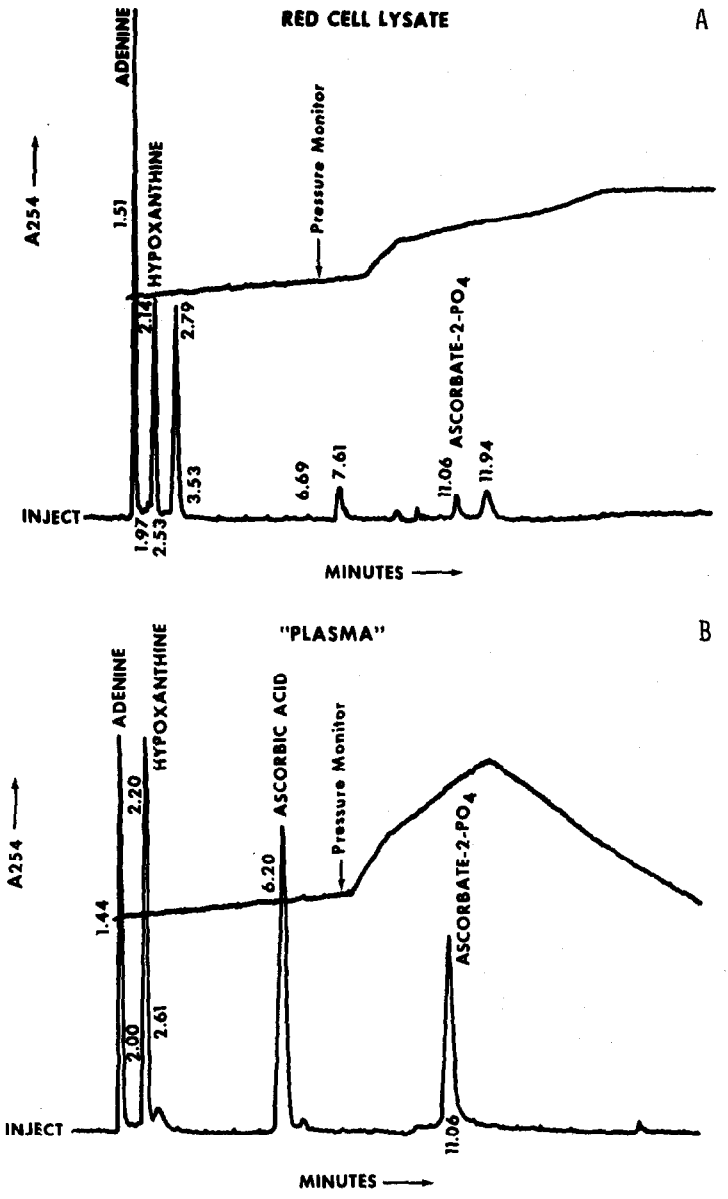

Fig. 1. HPLC tracings (absorbance at 254 nm versus time) for samples from red cell lysate (A) and supernatant plasma (B). Values indicate peak retention times (min). Ascorbic acid was added to the plasma sample (B).

Fig. 2 shows the results of the recovery studies for AsP and adenine additions to supernatant solution with and without added plasma. Hypoxanthine recoveries (data not shown) were similar to those for adenine. The three solutions shown represent CPD plasma (25% plasma), plasma diluted with additive solution (8.3% plasma), and water (straight additive solution). These dilutions include the 1:4 water dilution made before deproteinizing with the Centriflo cones. Similar stud-

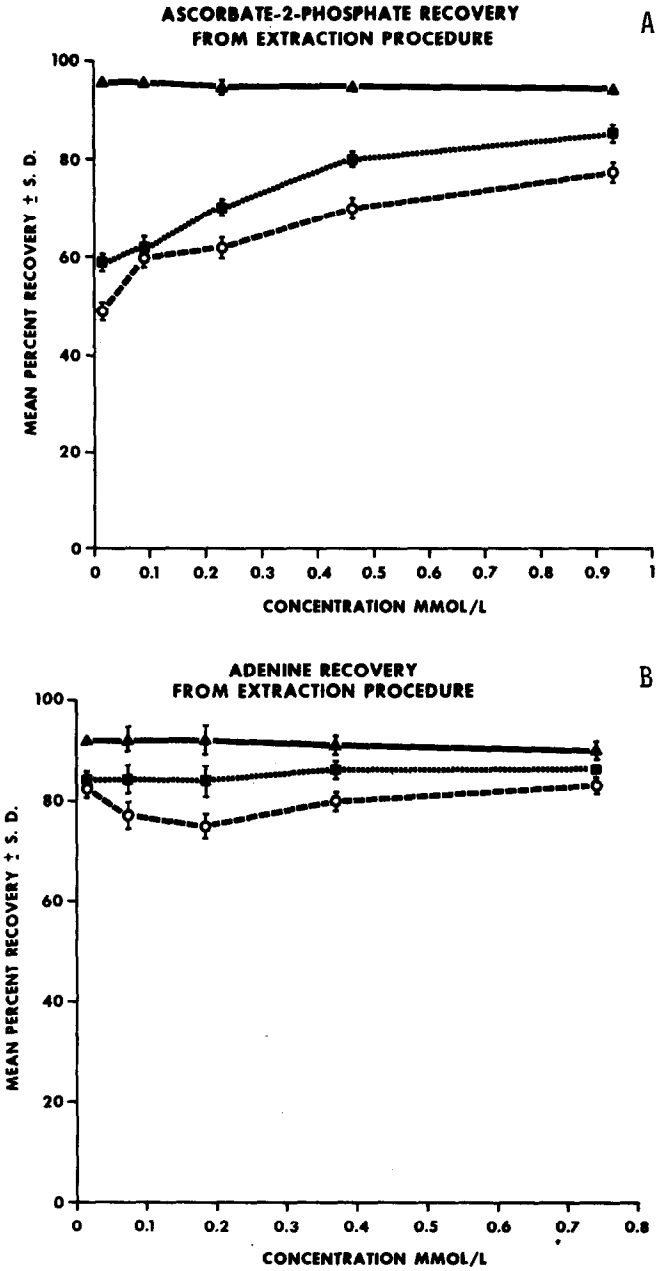

Fig. 2. Recovery curves for addition of various amounts of AsP (A) and adenine (B) added to water (saline) and two dilutions of plasma which were then processed with Centriflo cones and chromatographed. Recoveries of adenine and hypoxanthine were constant from the red cell lysates (76% and 85%, respectively). Key: △—△, water; ■- - -■, 8.33% plasma; ○- -○, 25% plasma.

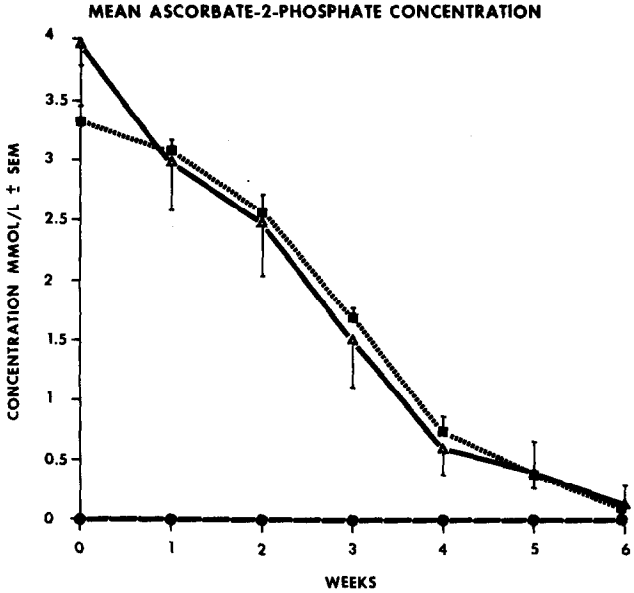

Fig. 3. Changes in AsP concentration during blood storage for 42 days in an experimental preservative solution. Key: △—△, ascorbate-2-phosphate in plasma; ■- -■, ascorbate-2-phosphate in saline supernatant; ●- -●, ascorbate-2-phosphate in red blood cells.

ies were done with red cell lysates. Maximal recovery, above 90%, was seen in the protein-free samples. Recovery was also constant over the entire concentration range in the protein-free samples. When protein was present in the sample, recovery was affected inversely by protein concentration (Fig. 2B). Relative recoveries of AsP were also dependent on AsP concentrations (Fig. 2A), i.e., the percentage recovery versus AsP concentration profile has a positive slope when protein is present in the supernatant solution. Appropriate adjustments for recovery were made in calculating the results in the blood storage studies.

Changes in AsP concentration during blood storage are shown in Fig. 3. The supernatant from washed and unwashed red cells showed similar loss of AsP. No AsP was found in the lysates of either set of red cells.

Adenine concentrations during red cell storage are shown in Fig. 4. The decrease during storage reflects incorporation of adenine into the adenosine nucleotide pool by the red cell [5]. The concentrations of adenine in red cells and plasma are similar since adenine freely enters and egresses the red cells to maintain equilibrium.

During red cell storage the size of the adenosine nucleotide pool decreases since AMP is irreversibly deaminated and converted to hypoxanthine [5]. The generation of hypoxanthine is illustrated in Fig. 5, which is nearly a mirror image of the cellular ATP levels (data not shown).

DISCUSSION

Conceptually, because of sample specificity considerations and ease of analysis, measuring the levels of certain chemical nutrients in stored blood is best done

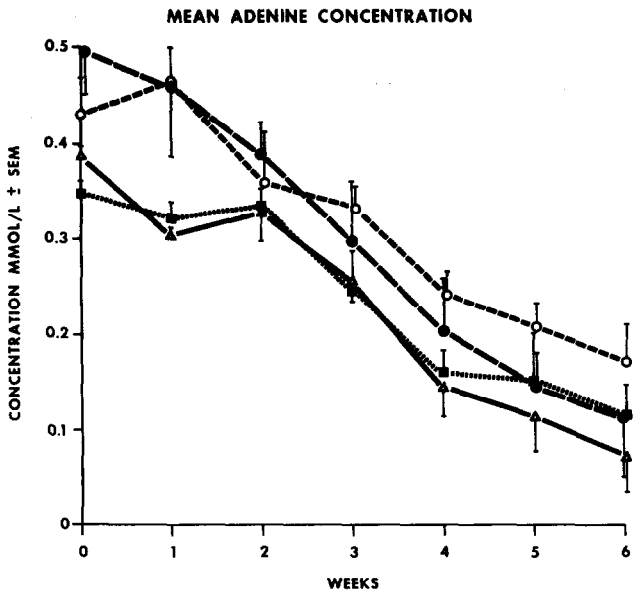

Fig. 4. Changes in adenine concentration during blood storage for 42 days in an experimental preservative solution. Key: △—△, adenine in plasma; ■- -■, adenine in saline supernatant; ●- -●, adenine in red blood cells from plasma suspension; ○- -○, adenine in red blood cells from saline suspension.

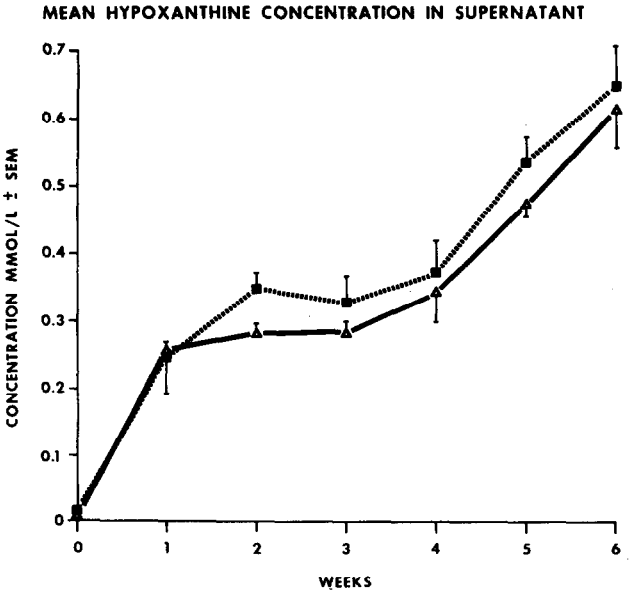

Fig. 5. Changes in hypoxanthine concentration during blood storage for 42 days in an experimental preservative solution. Key: △—△, hypoxanthine in saline supernatant; ■- -■ hypoxanthine in plasma.

using HPLC. However, as is frequently the case, it was significantly more difficult to measure the compounds in a biological system than to measure them in a simple water solution. With blood as the starting sample, non-specific binding and the co-elution of undefined peaks during HPLC made it necessary to develop one sample-processing procedure and two HPLC run procedures to quantify adenine, AsP, and hypoxanthine.

Three methods were tried to deproteinize the samples: boiling, acid precipitation, and the Centriflo cones. The cones gave the best recovery with the least contamination of the HPLC column. Recovery of known quantities of adenine and hypoxanthine from the cone was high and constant throughout the concentration range of interest. At low concentrations of AsP a higher proportion of AsP was lost, probably due to binding with the trapped protein in the cones. Concentration-dependent adjustments to the calculations were therefore made when analysing unknown samples for AsP.

The determination of levels of adenine and AsP during blood storage was successful. Adenine utilization in this red cell preservative was somewhat slower than in currently used preservative solutions like CPDA-1 (CPD adenine), where all the adenine is gone at 42 days [5]. The utilization of AsP during red cell storage is demonstrated by Fig. 3, although the catabolic products of the compound are unknown [1]. The amount of AsP used in this study was optimized to best retain 2,3-DPG concentrations for 42 days [1]. The finding that AsP is lost at a near-linear rate and does not approach zero until day 42 is consistent with the optimization data [1]. Changes in hypoxanthine concentration during blood storage are identical to those seen in other preservation studies [6], indicating that the adenine nucleotide pool loss seen with this preservative is similar to that seen with other preservatives currently in clinical use.

The development of this HPLC procedure has allowed us to quickly assess several important parameters in the nutritional status of red blood cells during preservation. The use of Centriflo cones has proved simple and reliable in removal of contaminating proteins, and does not introduce new chemicals to possibly complicate the separation or contaminate the column. This analytical method may have additional applications in situations where clinical blood samples need to be analyzed for confirming or monitoring disorders of purine metabolism.

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