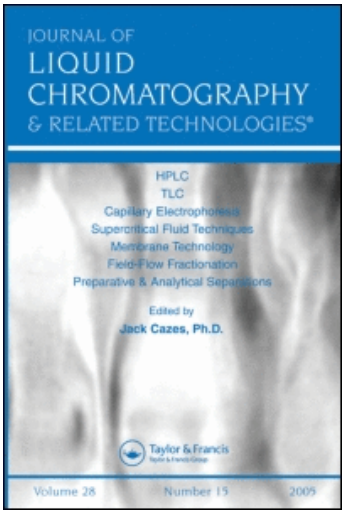

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Optimization of a Procedure for Extraction of Nucleotides from Plasma and Erythrocytes Prior to HPLC Analysis

Denise A. Van[a]; Phyllis R. Brown[b]
[a] Haverbeke Bredestraat, Ekeren, Belgium [b] Department of Chemistry, University of Rhode Island Kingston, Rhode, Island

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OPTIMIZATION OF A PROCEDURE FOR EXTRACTION OF NUCLEOTIDES FROM PLASMA AND ERYTHROCYTES PRIOR TO HPLC ANALYSIS

Denise A. Van Haverbeke
Bredestraat, 136
B-2070-Ekeren
Belgium

and

Phyllis R. Brown
Department of Chemistry
University of Rhode Island
Kingston, Rhode Island 02881

ABSTRACT

The efficiency of extraction of nucleotides from plasma and erythrocyte samples by means of the Khym TCA[1] - amine-Freon^{R} procedure was investigated.

Maximum efficiency of recovery was found when TCA concentrations greater than 9% were used to extract the nucleotides of cytosine, adenine and guanine from plasma and erythrocytes. The recovery from the erythrocyte matrix was 20% lower than that from the plasma matrix and 40% lower than the recovery of the same nucleotides from water. It was also found that using the exact amount of amine solution to neutralize the TCA resulted in increased recovery of the nucleotides.

INTRODUCTION

The use of microparticle chemically-bonded anion-exchange packings in HPLC has made possible the monitoring of nucleotides in biochemical studies with high speed, resolution and accuracy (1-8).

[1]See list of abbreviations.

Since the technique is very sensitive, sample preparation procedures used prior to chromatographic analysis are important and must not cause sizeable errors in the quantitative results. With biological fluids and tissues, proteinaceous material is usually removed by precipitation with either trichloroacetic acid (TCA) or perchloric acid (PCA). To protect the columns and prevent breakdown of the nucleotides, the acid has to be removed prior to HPLC analysis.

Five different sample preparation methods have been investigated for use prior to HPLC analysis by Brown and Miech (9). However, Brown (10) found that the TCA-TRIS procedure, which had been found to be very suitable with the pellicular packings, could not be used with the microparticle chemically-bonded anion-exchange columns. Therefore, there was a need for re-evaluation of the sample preparation procedures.

Khym (11) reported on a method in which the TCA or PCA was extracted into a solution of a water insoluble amine dissolved in Freon^{R} 113.[2] Chen et al. (12) investigated the Khym procedures for their efficiency in extracting nucleotides from aqueous solutions prior to HPLC analysis using microparticle chemically-bonded anion-exchange columns. The TCA - amine-Freon^{R} method showed excellent quantitative recovery. However, it was found that the concentrations of the amine solutions may change if the solution is not freshly prepared. Chen also found differences in recovery dependent on base structure and the number of phosphate groups present in the compounds.

In this study, the TCA - amine-Freon^{R} procedure was investigated for the recovery efficiency of nucleotides from plasma and red blood cells as compared to the recovery of nucleotides from water. Differences in recovery dependent on base structure and number of phosphate groups were studied in the two blood matrices. The influence of different TCA and amine concentrations on

[2]Freon^{R} is a registered trademark of E. I. duPont de Nemours & Co.

the recovery of the nucleotide standards from plasma and erythrocytes was also investigated.

MATERIALS

Apparatus

A Waters ALC 202 High Pressure Liquid Chromatograph (Waters Associates, Milford, MA) with a double beam UV detector operating at a wavelength of 254 nm was used. For identification purposes a Schoeffel Variable Wavelength Detector (Model SF 770 Spectroflow Monitor, Schoeffel Instrument Corp., Westwood, NJ) was used at a wavelength of 280 nm. The gradient accessory consisted of an electronic programmer and a second pump. Peak areas were integrated by means of an Auto-lab Minigrator (Spectra Physics, Santa Clara, CA). Samples were mixed on a Vortex-Genie K550-G (Scientific Industries Inc., Springfield, MA).

Columns

The columns used (Partisil PXS 10/25-SAX) supplied by Whatman (Clifton, NJ) are strong anion-exchange columns which are supplied pre-packed in 25 cm x 4.6 mm I. D. stainless steel columns. The ion-exchange moiety is quaternary ammonium ion which is bonded to the silica base through a Si-O-Si bond. The particle size is 10 microns.

Buffers

Aqueous buffers were prepared from reagent-grade potassium dihydrogen phosphate (Mallinckrodt, St. Louis, MO) and potassium chloride (Allied Chem., Morristown, NJ).

Chemicals

The nucleotides GMP, ADP, GDP and GTP were obtained from Sigma Chemical Company (St. Louis, MO) and AMP, CMP and ATP were obtained from PL Biochemicals Inc. (Milwaukee, WI).

Tri-n-octylamine (referred to subsequently as amine) was purchased from ICN Pharmaceuticals Inc., Life Sciences Group (Plainview, NY) and the Trichloroacetic Acid (TCA) from Sigma

Chem. Company. Freon^{R} 113 was obtained from Matheson Gas Products (Hackensack, NJ).

METHODS

1. Chromatography

The conditions for the HPLC separation of nucleotides, developed by Hartwick et al. (8) were improved by McKeag (13) and make possible the separation of the mono-, di- and triphosphate 5'ribonucleotides of adenine, guanine, hypoxanthine, xanthine, cytosine, uracil and thymine in 40 minutes. The initial eluent, 0.007M KH_2PO_4 and 0.007M KCl, was adjusted to pH=4.0 with HCl. The final eluent, 0.25M KH_2PO_4 and 0.5M KCl, was adjusted to pH=5.0 with potassium hydroxide.

A linear gradient from 0% of final eluent to 100% of final eluent was run in 35 minutes after 5 minutes of isocratic elution on the low concentration buffer. The flow rate was 2.0 ml per minute.

All the eluents were filtered on Millipore membrane filters (HA; Millipore, Bedford, MA) and degassed prior to use.

2. Blood Collection and Storage

All extraction efficiency studies were carried out on single lots of freshly collected whole blood (in acid-citrate-dextrose, ACD), from which the platelets had been extracted. The erythrocytes and plasma were separated by centrifugation at 1145 RCF for 10 minutes. The buffy coat was removed, and the erythrocytes were washed in normal saline prior to use.

3. Sample Preparation Procedure

One ml of doubly distilled water, plasma or erythrocytes and 1 ml of a 1mM standard solution of nucleotides were mixed vigorously with 2 ml of 6% TCA on a Vortex-Genie. The samples were centrifuged for 6 minutes at 1145 RCF. The erythrocytes are lysed in this step of the procedure. The supernatant was filtered through a Millipore membrane filter (GS; Millipore, Bedford, MA).

In the filtration of the red cell samples, a glassfiber filter (GF/F; Whatman, Clifton, NJ) was placed upon the membrane filter to keep it from clogging. To the filtrate, an equal amount of a 0.5M amine-Freon^{R}solution was added. The samples were vigorously mixed on a Vortex-Genie for 3 minutes at low speed. The supernatant was pipetted into a vial and adjusted to pH 7 with tris(hydroxy(methyl)aminomethane (Trizma base) (Sigma Chem. Company, St. Louis, MO).

Fresh solutions of 0.5M of the amine in Freon^{R} and 6% TCA in water were prepared daily before the sample preparations.

4. Influence of the Base Structure and the Number of Phosphate Groups

Four sets of standard solutions were prepared. The first solution contained AMP, CMP and GMP in a 1 mM concentration and was used to investigate the effect of the base structure. Two sets of solutions were used to investigate the effect of the number of phosphate groups present: a 1 mM solution of AMP, ADP, ATP and a 1mM solution of GMP, GDP, GTP. A 1mM solution of XMP was prepared to be used as an internal standard. The internal standard was added to the extracted samples before the chromatographic analysis. Known amounts of these standard solutions were added to water, plasma and erythrocyte matrices and recovered by the Khym procedure. Because we were determining efficiency of recovery, we were only interested in the recovery of the added standards. Therefore, we determined the amount of nucleotides present in the blood samples by preparing and analyzing samples to which we added water instead of the standard nucleotide solutions.

5. Influence of the TCA and Amine Concentrations

In order to find the effect of the TCA concentration on the recovery, a concentration range varying from 6% to 18% TCA was used during sample preparations. The recoveries of the nucleotides were determined for each TCA concentration.

To determine the exact amount of amine solution needed to neutralize the TCA used in the extraction procedure, a titration curve was set up. After adding a small amount of the amine solution to the TCA solution, the sample was vortexed, centrifuged at low speed for 1 minute and the pH was measured. From the titration curve, the exact amount of amine solution necessary to extract the TCA was determined. The samples were then prepared with the amount of amine determined from the titration curve and recoveries were calculated.

6. Calculations

The percentage recovery of a specific nucleotide can be calculated by means of the following equation:

$$\%R = \frac{A_1 \times C_2}{A_2 \times C_1} \times 100$$

$$A_1 = \frac{\text{area}_{\text{nucleotide extracted from blood sample}}}{\text{area}_{\text{XMP(not extracted)}}}$$

$$A_2 = \frac{\text{area}_{\text{nucleotide in standard solution(not extracted)}}}{\text{area}_{\text{XMP(not extracted)}}}$$

$$C_1 = \frac{\text{concentration of nucleotide in blood sample}}{\text{concentration of internal standard}}$$

$$C_2 = \frac{\text{concentration of nucleotide in standard solution}}{\text{concentration of internal standard}}$$

RESULTS

An example of the chromatograms of the extracted plasma and erythrocyte sample with and without added standards can be seen in Figure 1 and Figure 2.

The results of extractions of CMP, AMP and GMP from water, plasma and erythrocytes are shown in Figure 3 and the data are presented in Table I. If the percentage recovery for the nucleotides from water is compared with the values found by Chen et al.

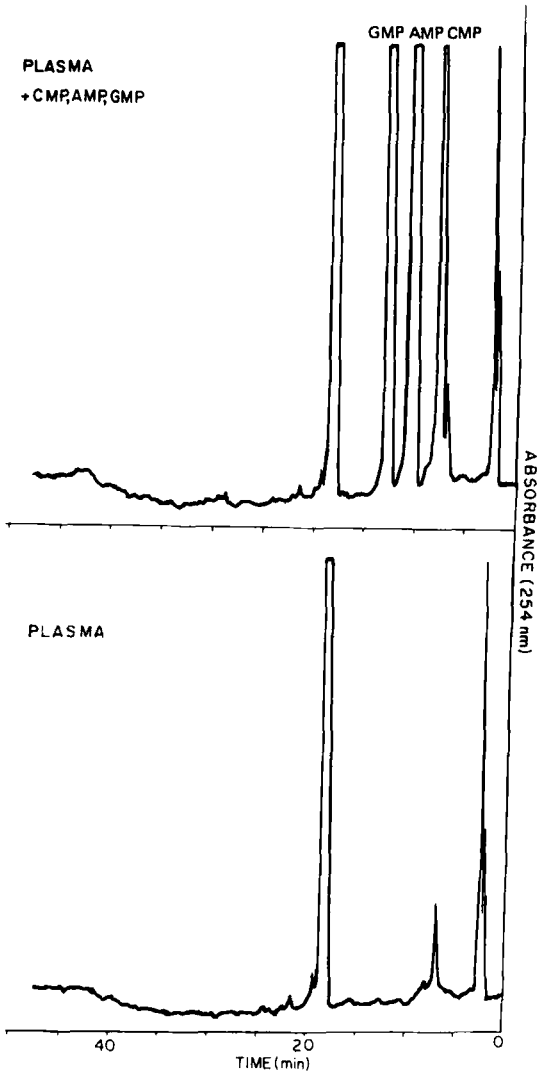

Figure 1 A. A chromatogram of 5 µl of an extract of human plasma to which CMP, AMP and GMP were added (approximately 0.25 mM in each).
B. A chromatogram of an extract of the same lot of plasma, to which these standards were not added.
The large peak at about 20 minutes is the internal standard, XMP. Chromatographic conditions were as follows: low strength eluent, 0.007 F. KH_2PO_4, 0.007 F. KCl, pH 4.0. High strength eluent, 0.25 F. KH_2PO_4, 0.50 F. KCl, pH 5.0. Gradient, 5 minutes isocratic, then 0% to 100% of the high strength eluent (linear), in 35 minutes. Flow rate, 2.0 ml/min. Detection is at 254 nm.

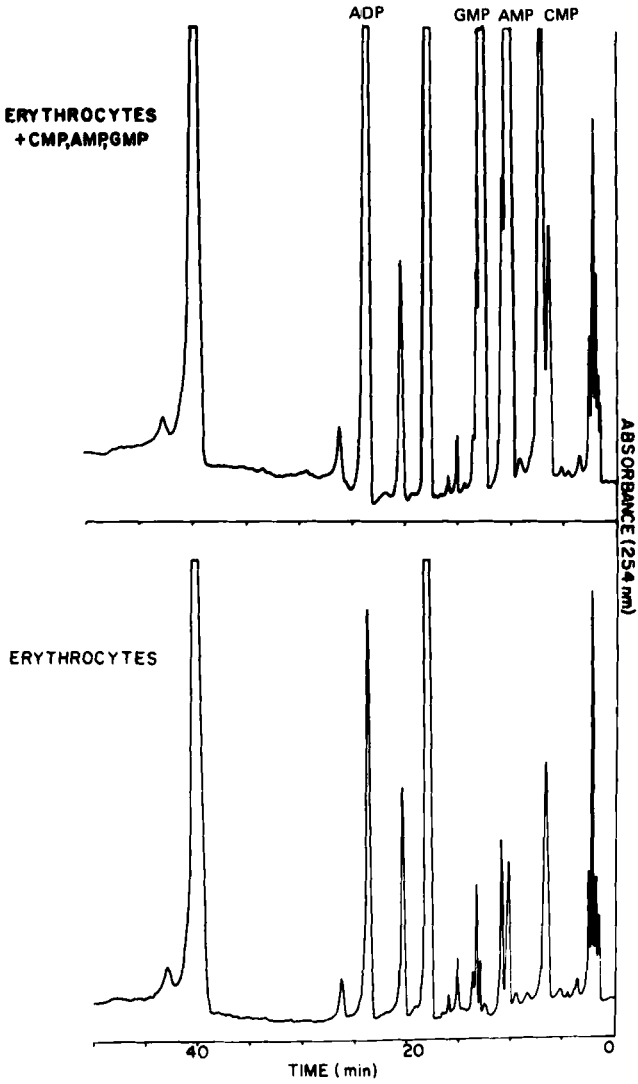

Figure 2 A. A chromatogram of 5 µl of an erythrocyte extract with added CMP, AMP and GMP.
B. A chromatogram of 5 µl of a blank erythrocyte extract. Same chromatographic conditions as in Figure 1. The upper chromatogram shows ADP as well as XMP co-injected with the extract for identification purposes.

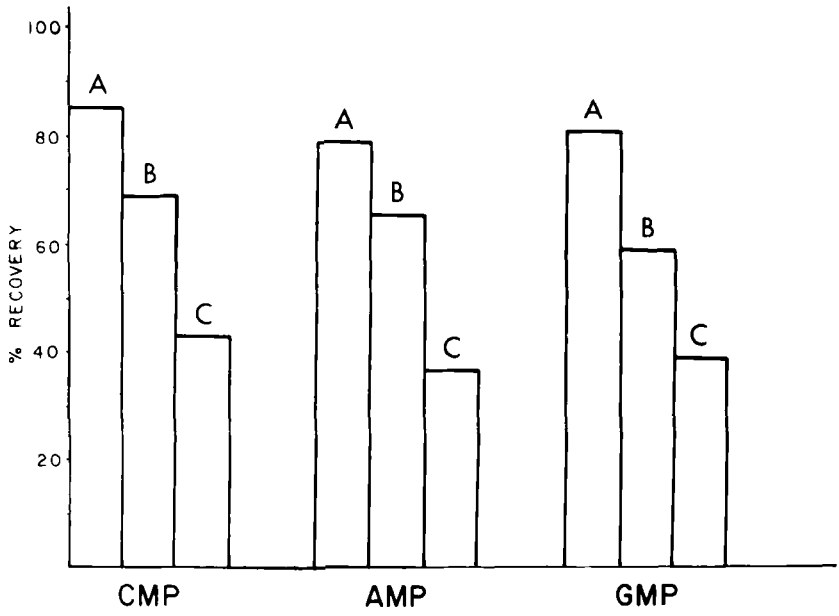

Figure 3 Bar graph showing the average recovery of the monophosphate nucleotides CMP, AMP and GMP from water (bar A), plasma (bar B) and erythrocytes, (bar C). The bar heights shown in Figures 3-5 represent the average of 3 independent samples.

(12), a decrease of 10% is observed. A possible reason for this loss is that a filtration step was incorporated in our procedure in order to compare, exactly, recovery factors of the nucleotides from water to the recovery factors from plasma and erythrocytes. Although Figure 3 indicates that the base structure of the nucleotides may cause a small difference in percentage recovery, the differences are not statistically significant. However, a significant decrease in recovery of both the adenine and the guanine triphosphate nucleotides was observed from the erythrocyte samples (Figures 4 and 5). There was not a similar decrease in recovery in the extraction from plasma.

Comparison of the recovery of AMP and GMP when a combined CMP-AMP-GMP standard solution was used (Figure 4) to their recovery in

TABLE I

EFFICIENCY OF THE TCA - AMINE-FREON PROCEDURE IN DIFFERENT MATRICES. INFLUENCE OF THE BASE STRUCTURE AND NUMBER OF PHOSPHATE GROUPS OF THE NUCLEOTIDES.

A. Standard Solution Containing CMP, AMP and GMP

MATRIX	PERCENTAGE RECOVERY		
	CMP	AMP	GMP
WATER	85.1 ± 2.9	78.9 ± 2.4	81.0 ± 3.4
PLASMA	68.7 ± 1.4	66.2 ± 1.9	59.1 ± 1.5
ERYTHROCYTES	43.0 ± 2.1	37.1 ± 2.5	39.0 ± 2.7

B. Standard Solution Containing GMP, GDP and GTP

MATRIX	PERCENTAGE RECOVERY		
	GMP	GDP	GTP
WATER	81.0 ± 3.4	77.6 ± 5.0	72.2 ± 6.1
PLASMA	59.1 ± 1.5	64.4 ± 1.4	60.4 ± 3.5
ERYTHROCYTES	39.0 ± 2.7	32.9 ± 3.2	23.0 ± 2.3

C. Standard Solution Containing AMP, ADP and ATP

MATRIX	PERCENTAGE RECOVERY		
	AMP	ADP	ATP
WATER	78.9 ± 2.4	76.2 ± 5.6	75.1 ± 2.7
PLASMA	66.2 ± 1.9	61.8 ± 2.9	61.8 ± 3.5
ERYTHROCYTES	37.1 ± 2.5	36.0 ± 1.3	17.9 ± 3.5

$n \geq 3$, ± 1 S.D.

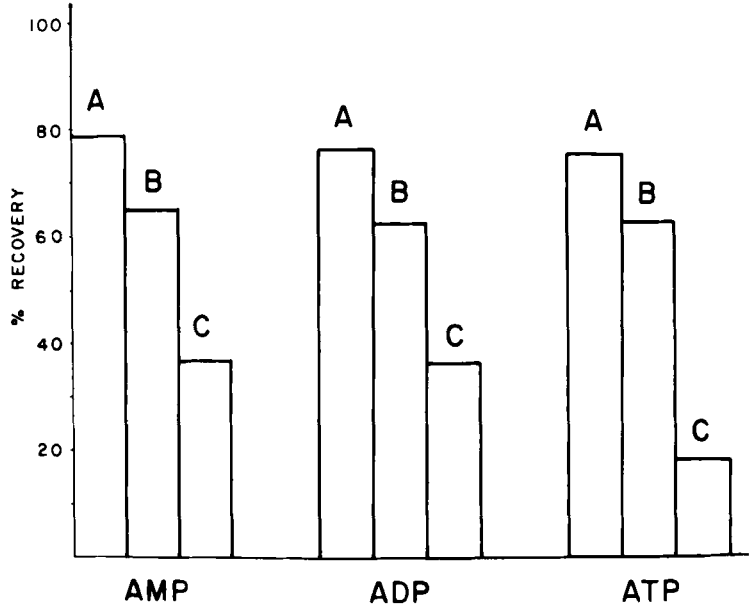

Figure 4 Bar graph of the percent recoveries of added AMP, ADP and ATP to water (bar A), plasma (bar B) and erythrocytes (bar C), showing the effect of the number of phosphate groups on the percent recovery.

the experiments when the combined GMP-GDP-GTP (Figure 5), and the combined AMP-ADP-ATP (Figure 4) standard solutions were used, showed recovery factors in the same range. This indicates that no breakdown of the tri- and diphosphate nucleotides occurred during the sample preparation.

Since it was found that recovery of nucleotides from plasma and erythrocytes is much lower than from aqueous solutions (Figure 3), it appears that efficiency of recovery is dependent on the number and kind of macromolecules present in the biological matrix. The recovery of nucleotides from plasma is 10-20% less than the recovery from water and only about 50% of the added nucleotides were recovered from the erythrocytes. Thus, it is postulated that hydrogen bonding to the proteinaceous precipitate may be one factor involved in the loss of the nucleotides. Therefore, we used in-

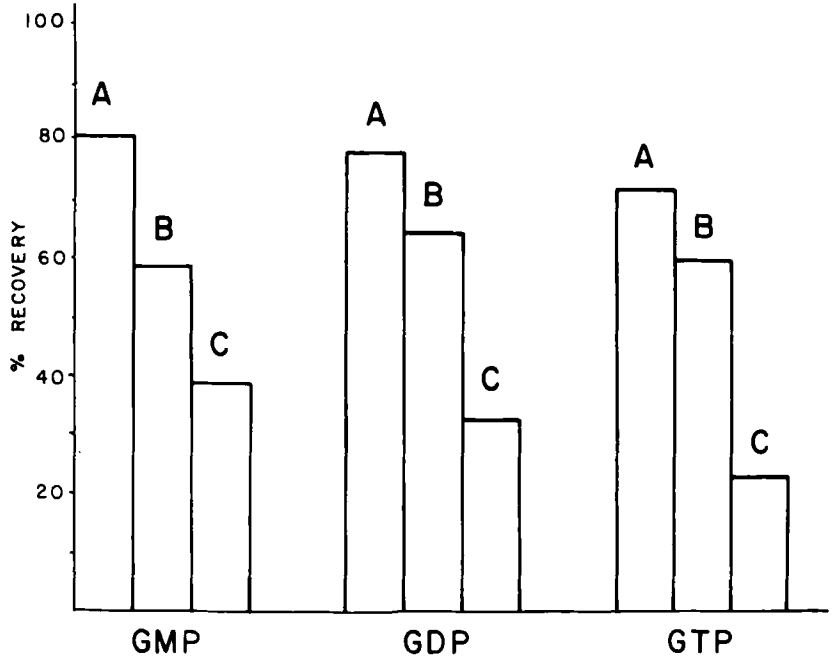

Figure 5 The percent recoveries of added GMP, GDP and GTP from water (bar A), plasma (bar B) and erythrocytes (bar C).

creasing concentrations of TCA to determine the optimal concentration of TCA for recovery of the nucleotides from the blood samples (Table II). Because breakdown of the nucleotides can occur if the acid concentration is too high, the samples were carefully monitored to see if any new peaks representing breakdown products appeared in the chromatograms or if there were drastic changes in peak areas. Since no changes were observed, it appears that little or no breakdown occurred in the acid range used (6-18%). Optimal TCA concentration was 9% or greater for extraction of nucleotides from either plasma or erythrocytes. (A value of 12% was chosen for all subsequent work.)

Another important factor in optimizing the recovery is the amount of amine solution used to remove the TCA from the solution

TABLE II

INFLUENCE OF THE TCA CONCENTRATION ON THE RECOVERY OF AMP-CMP-GMP

MATRIX	CONCENTRATION TCA	PERCENTAGE RECOVERY CMP	AMP	GMP
ERYTHROCYTES	6% TCA	43.0 ± 2.1	37.1 ± 2.5	39.0 ± 2.7
	9% TCA	64.8 ± 3.8	64.0 ± 1.9	59.8 ± 3.2
	12% TCA	67.6 ± 6.7	66.5 ± 6.6	67.6 ± 3.7
	15% TCA	70.4 ± 6.0	74.1 ± 0.2	75.9 ± 0.5
	18% TCA	65.0 ± 5.6	67.5 ± 2.3	69.8 ± 1.7
PLASMA	6% TCA	68.7 ± 1.4	66.2 ± 1.4	59.1 ± 1.5
	9% TCA	86.4 ± 6.7	65.6 ± 2.1	65.8 ± 1.2
	12% TCA	87.1 ± 5.8	68.5 ± 0.5	71.2 ± 2.9
	15% TCA	83.4 ± 5.6	68.0 ± 2.1	66.8 ± 2.2
	18% TCA	79.9 ± 1.8	65.7 ± 1.7	64.9 ± 2.5

$n \geq 3$

(Table III). By using more of the amine solution than necessary to extract the TCA, it appears that the nucleotides are also extracted into the amine layer. Using titration curves, it was found that the optimal amount of amine was different for plasma and erythrocytes, 1 ml being required for the plasma and 0.3 ml for the erythrocyte samples (Figure 6 and 7). With excess amine, the recovery of the nucleotides with 2 and 3 phosphate groups was on the average 3-5% lower than that of the monophosphate nucleotides, suggesting that polyphosphate nucleotides are more readily lost into the amine solution layer when excessive amounts of the amine solution is used to extract the TCA.

TABLE III

INFLUENCE OF EXCESS AMINE SOLUTION ON THE RECOVERY OF CMP, AMP, ADP AND ATP

MATRIX		PERCENTAGE RECOVERY			
		CMP	AMP	ADP	ATP
PLASMA	(1)	68.7 ± 1.4	66.2 ± 1.9	61.8 ± 2.9	61.8 ± 3.5
	(2)	75.3 ± 1.2	77.6 ± 0.9	78.5 ± 1.0	78.1 ± 0.7
ERYTHROCYTES	(1)	43.0 ± 2.1	37.1 ± 2.5	36.0 ± 1.3	17.9 ± 3.5
	(2)	47.0 ± 2.9	42.7 ± 1.8	53.5 ± 4.0	25.0 ± 8.2

(1) Excess amount of amine solution used to neutralize TCA.

(2) Exact amount of amine solution used to neutralize TCA.

6% TCA was used to precipitate the protein in both sets of samples.

$n \geq 3$, ± 1 S.D.

DISCUSSION

From our results it can be seen that the conditions for the extraction of added nucleotides to plasma and erythrocytes can be optimized to obtain maximum efficiency of extraction. The TCA - amine-Freon^{R} procedure as a sample preparation procedure in plasma and erythrocytes can be used but care must be taken that the TCA solutions are fresh and the concentrations kept constant to elimin-

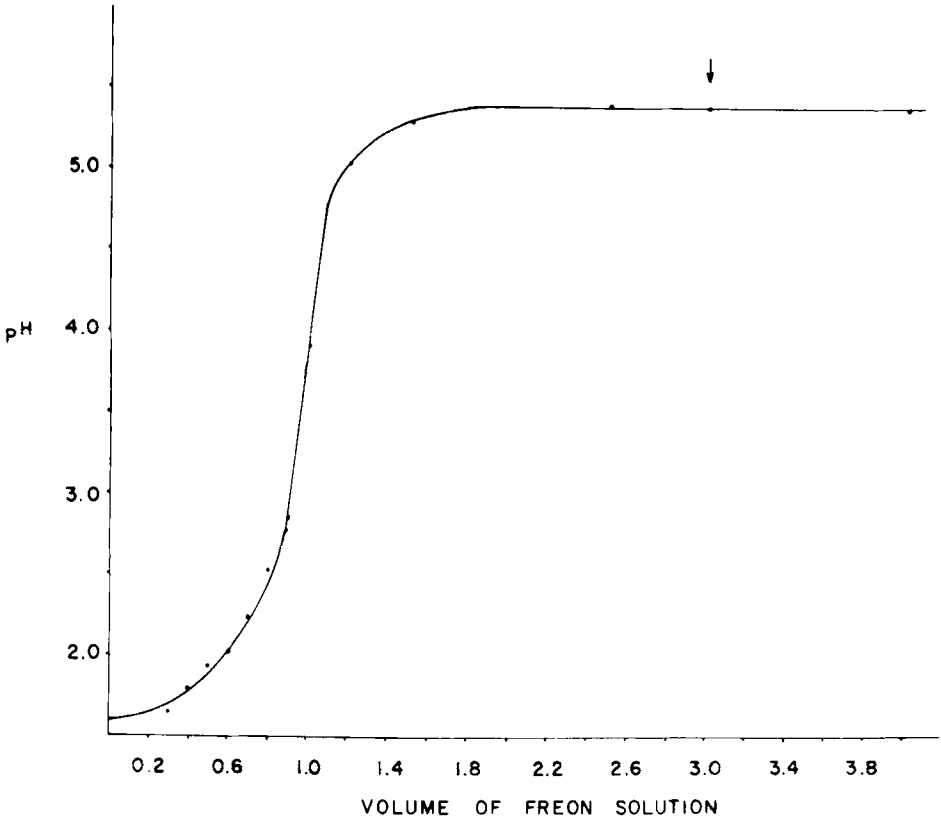

Figure 6 Titration curve showing the change in pH of the aqueous layer of extracted plasma as a function of the volume of amine solution added to the mixture. The arrows in Figures 6 and 7 represent the volume of 0.50 amine which is normally added to a sample (11,12). The equivalence point is at 0.98 ml of amine solution.

ate any variations in recovery (12). The optimal TCA and amine concentrations must be determined for each biological matrix, as the efficiency of recovery appears to be dependent on the type of macromolecules present in the samples. In the literature it has been assumed that the acid-soluble nucleotides and nucleosides are either extracted completely into the supernatant fluid or trapped in the precipitate. The possibility of adsorption on the precipi-

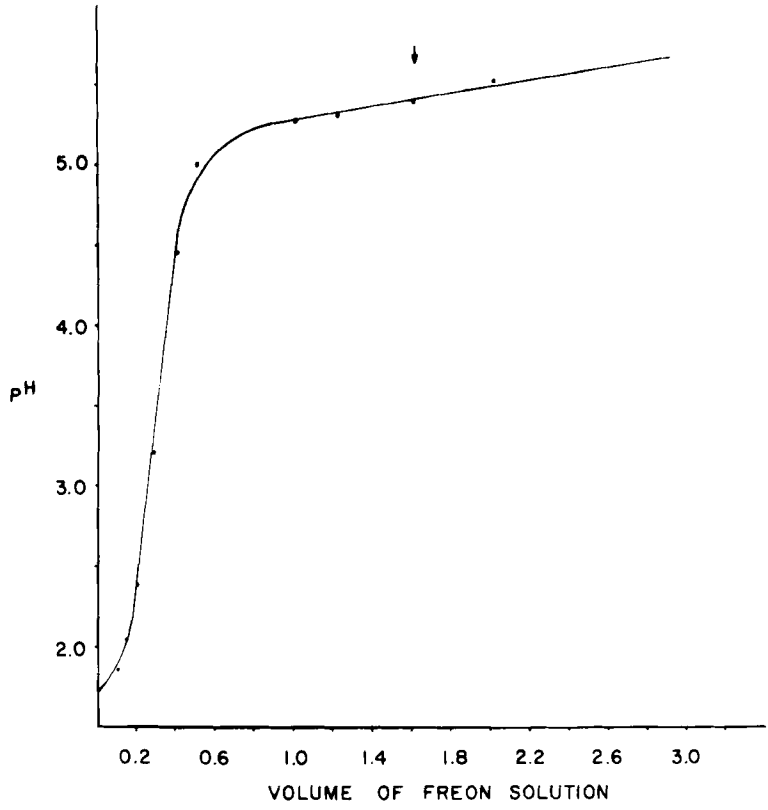

Figure 7 Titration curve of TCA with amine-Freon^{R} solution added to a sample of erythrocytes. Same general conditions as in Figure 6. The equivalence point is at 0.30 ml of added smine solution.

tate by means of some kind of bonding has been considered by Currie (15) who reported that xanthosine added to a tissue sample (liver homogenate) was incompletely recovered because of adsorption of the xanthosine molecules to the precipitate. In our studies with different TCA concentrations, we found better recoveries at higher acidities, suggesting that hydrogen bonding, which is a favored type of bonding for nucleic acids and their components, may be one of the factors involved in extraction efficiency. Therefore, the

first step of the extraction procedure and the precipitation of the protein with acid can cause sizeable errors in the quantitative results if the appropriate acidity is not used.

Our research, however, does not assume to answer the crucial question of the efficiency of recovery of the naturally occurring free nucleotides in cell extracts. What percentage of the free nucleotide pools are we actually extracting from the cells? Is a smaller percentage of the triphosphate nucleotides than monophosphates extracted from the cells as is indicated from our work with added nucleotides? Are we extracting the same percentage of pyrimidine nucleotides as purine pools? Are we adequately breaking the cell membranes to extract completely the nucleotides from the cells? On the other hand, in cellular material and tissue which contains DNA and RNA, will we break down the nucleic acids and obtain abnormally high values for the nucleotides? Thus, although our studies are beginning to give us insight into the conditions affecting optimization of nucleotide extraction procedures, our results do not give us absolute values of nucleotide concentrations in cells. However, the values we obtain can be used in comparative studies of normal and disease states. In using these procedures for future use in clinical laboratories, care must be taken to optimize and standardize the sample preparation procedures in order to obtain normal values or a normal range of values which are statistically valid.

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APPENDIX

List of Abbreviations

ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-phosphate
ATP	Adenosine 5'-triphosphate
CMP	Cytidine 5'-phosphate
GDP	Guanosine 5'-diphosphate
GMP	Guanosine 5'-phosphate
GTP	Guanosine 5'-triphosphate
TCA	Trichloroacetic acid
TRIS	Tris(hydroxy(methyl))aminomethane (Trizma base)
UMP	Uridine 5'-phosphate
XMP	Xanthosine 5'-phosphate
XTP	Xanthosine 5'-triphosphate
AMINE	Tri-n-octylamine

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