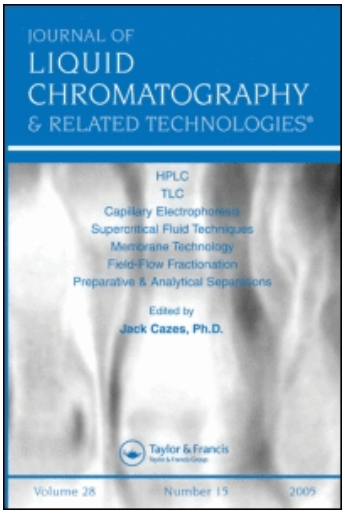

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Comparison of the Efficiency of Nucleotide Extraction by Several Procedures and the Analysis of Nucleotides from Extracts of Liver and Isolated Hepatocytes by HPLC

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To cite this Article Riss, T. L. , Zorich, N. L. , Williams, M. D. and Richardson, A.(1980) 'A Comparison of the Efficiency of Nucleotide Extraction by Several Procedures and the Analysis of Nucleotides from Extracts of Liver and Isolated Hepatocytes by HPLC', Journal of Liquid Chromatography & Related Technologies, 3: 1, 133 — 158
To link to this Article: DOI: 10.1080/01483918008060160
URL: http://dx.doi.org/10.1080/01483918008060160

JOURNAL OF LIQUID CHROMATOGRAPHY, 3(1), 133-158 (1980)

A COMPARISON OF THE EFFICIENCY OF NUCLEOTIDE EXTRACTION BY SEVERAL PROCEDURES AND THE ANALYSIS OF NUCLEOTIDES FROM EXTRACTS OF LIVER AND ISOLATED HEPATOCYTES BY HPLC

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ABSTRACT

A high pressure liquid chromatography (HPLC) system was developed, capable of resolving most 5'-nucleotides and nucleotide-sugars present in liver tissue. Using three different extraction procedures, the recovery of the twelve major 5'-ribonucleotides from solutions of nucleotide standards, or liver samples, or isolated hepatocytes was compared. Nucleotides were obtained from acid extracts for HPLC analysis by adsorbing the nucleotides on charcoal, or precipitating the acid (perchloric acid) by KOH, or extracting the acid with alamine/freon. The last two procedures were found to be superior to the charcoal adsorption procedure for recovering nucleotides from acid extracts. The recovery of nucleotides from either liver samples or isolated hepatocytes by these two procedures was similar; however, the recovery of nucleotides from standard solutions was slightly higher for the alamine/freon procedure than the KOH precipitation procedure.

INTRODUCTION

Because nucleotides play an essential role in a wide variety of metabolic processes in living organisms, investigators have been interested in determining what effect various parameters, e.g., disease state, nutritional status, etc., have on the levels of nucleotides. In the past, the measurement of nucleotide levels in biological samples has been tedious because it was difficult to separate the large number of nucleotides present in biological samples and to quantify the small

levels of nucleotides in the samples by a routine and reproducible procedure.

With the advent of HPLC, biochemists and molecular biologists have available a method capable of separating and quantifying nucleotides. Brown (1) was one of the first investigators to show the usefullness of HPLC for studying nucleotides. There are several reports in the literature describing the separation of mixtures of nucleotides by various HPLC systems (2-10), but most of these separations have been accomplished using nucleotide standards. We were interested in specifically developing an HPLC system capable of separating the twelve major 5'-ribonucleotides (AMP, CMP, GMP, UMP, ADP, CDP, GDP, UDP, ATP, CTP, GTP, and UTP) from other nucleotides in liver samples. Reports by Henry et al. (3) and Floridi et al. (6) have shown HPLC chromatograms of liver extracts; however, these investigators made no attempt to determine if the nucleotides from the liver extracts were completely resolved or to quantify the nucleotides extracted. Using HPLC, Jackson et al. (5) determined the content of the twelve 5'-nucleotides in liver and hepatoma 392A cells. However, it was necessary for Jackson et al. (5) to use three different systems to resolve all twelve 5'-nucleotides.

In addition to developing an HPLC system which would routinely and rapidly separate nucleotides from liver samples, we wanted to compare the efficiency of several procedures to extract nucleotides for analysis by the HPLC system developed in this study. Brown's laboratory (7, 11) has been the only research group to compare nucleotide extraction procedures for HPLC analysis. However, in these studies, the recovery of only a few nucleotides from solutions containing nucleotide standards was determined. In our study, the recoveries

of the twelve 5'-nucleotides from liver samples, isolated hepatocytes, and solutions containing nucleotide standards was determined using three extraction procedures. As a result of this study, a system for both routine and rapid extraction of nucleotides and HPLC analysis of nucleotides from liver tissue is available to investigators.

MATERIALS AND METHODS

Chromatographic Equipment

A multicomponent high pressure liquid chromatographic (HPLC) system was used in this study. This system consisted of a Spectra Physics pump (model 740B), a Valco model CV-6-UHPa-N60 sample injection valve, a Perkin Elmer model LC-55 variable wavelength spectrophotometer, and a Perkin Elmer Model 56 multirange recorder. In this study, strong anion exchange columns purchased from Whatman (Clifton, NJ) were used. A pellionex SAX guard column was used in conjunction with a prepacked Partisil 10-SAX (25 cm x 4.6 mm I.D.) column.

Solvent Preparation

Aqueous solvents were prepared from reagent grade KH_2PO_4 (Mallinckrodt, St. Louis, MO) and KCl (Fischer Scientific Co., Chicago, IL) using glass distilled water. It was not necessary to further purity the KH_2PO_4 and KCl purchased from these suppliers but we have had problems with uv-absorbing contamination in these reagents purchased from other suppliers. After filtering the solutions through a 0.45 µm membrane filter (Milliport, Bedford, MA), the pH of the various solutions was adjusted with either dilute H_3PO_4 or KOH. All solutions were degassed prior to use by mechanical stirring under reduced pressure.

Chemicals and Animals

All nucleotides and nucleotide containing compounds were purchased from the Sigma Chemical Co. (St. Louis, MO). Tricaprylyl tertiary

amine (Alamine 336) was purchased from McKerson Corp. (Minneapolis, MN). Activated charcoal was purchased from Pfansteil Laboratories, and Freon 113 was obtained locally. Female Sprague-Dawley rats were purchased from ARS Sprague Dawley (Madison, WI).

Chromatographic Procedure

The HPLC system empolyed in this study was developed to separate ribonucleotides from liver extracts. Separation of nucleotides was achieved in 50 min using a discontinuous buffer gradient system. After injection of 10 µl of sample, nucleotides were eluted initially with a dilute buffer (0.007 M KH_2PO_4, pH 4.2) at a flow rate of 1.6 ml/min. After 10 min, the flow rate was increased to 3 ml/min, and the gradient was initiated by adding 50 ml of concentrated buffer (0.007 M KH_2PO_4, 2.2 M KCl, pH 4.5) to 104 ml of the dilute buffer, with constant stirring, at a flow rate of 3 ml/min using a syringe pump.

Preparation of Standard Nucleotide Solutions

Standard solutions of nucleotides were prepared in 0.007 M KH_2PO_4 (pH 4.2) at concentrations ranging from 0.11 to 7.5 mM and were stored at -70°C. No breakdown of the nucleotides during storage was observed. The standard solutions contained a mixture of the nucleoside monophosphates, or nucleoside diphosphates, or nucleoside triphosphates. These three standard solutions were used so that the recovery of the nucleotides could be determined without cross contamination arising from the degradation of the nucleotides or from impurities in the purchased nucleotides.

Preparation of Liver Samples and Isolated Hepatocytes

To avoid anoxia when taking liver samples, the samples were obtained using the freeze clamp method described by Bucher and Swaffield (12). The frozen liver was ground to a powder under liquid nitrogen

with a mortar and pestle. The frozen liver powder was kept in liquid nitrogen until the nucleotides were extracted by the procedures outlined below. Hepatocytes were isolated from slices of liver tissue as described by Ricca et al. (13) and were suspended in Hanks' medium (14). The suspensions of hepatocytes were kept at 4°C for 5 to 15 min until nucleotides were extracted.

Perchloric Acid Extraction of Nucleotides

Nucleotides were extracted from standard solutions, or hepatocytes, or frozen liver powder using a modification of the perchloric acid (PCA) extraction procedure (PCA-KOH) described by Kraus and Reinhboth (15). Frozen liver powder (0.5 to 1.0 g), hepatocytes (20 to 30 million cells), or standard nucleotide solutions (1.0 ml aliquots) were homogenized in three volumes of precooled 0.4 M PCA using a Potter-Elvehjem homogenizer. The resulting suspension was centrifuged at 10,000 xg for 10 min at 4°C. The supernatant was saved, and the pellet was re-extracted twice more with 0.4 M PCA. The supernatants were combined and neutralized with 0.72 M KOH in 0.16 M $KHCO_3$. The precipitate was removed by low speed centrifugation, and the supernatant was filtered through a Millipore membrane filter (pore size 0.45 μm). The filtrate was frozen immediately in acetone-dry ice. The frozed sample was lyophilized to dryness and stored at -70°C. The extract was dissolved in 0.007 M KH_2PO_4 (pH 4.2) and analyzed immediately.

Charcoal Adsorption Extraction of Nucleotides

Nucleotides were extracted from standard solutions, or hepatocytes, or frozen liver powder using a modification of the charcoal adsorption procedure (C-A) described by Humphreys (16). Frozen liver powder (0.5 to 1.0 g), hepatocytes (20 to 30 million cells), or the standard solutions (1 ml aliquots) were extracted with PCA as described for the PCA-

KOH method, and the supernatants were combined and saved. Five mg of activated charcoal (Norit, 100 mg/ml water) was added per 0.9 ml of supernatant. The mixture was agitated at 4°C for 10 min and centrifuged at 10,000 xg for 5 min at 4°C. The supernatant was discarded and the Norit pellet, which contained the nucleotides, was washed three times with water at 4°C to removed salts and impurities. NH_4OH (0.1 M) in 50% ethanol (equal to the volume of the original acid-soluble extract) was added to the Norit pellet, and the resulting suspension was incubated at 37°C for 1 h with agitation. The charcoal suspension was centrifuged for 5 min at 3,000 xg. The supernatant was collected, filtered, lyophilized, and stored at -70°C. The extract was dissolved in 0.007 M KH_2PO_4 (pH 4.2) and was analyzed immediately.

Alamine/Freon Extraction of Nucleotides

The extraction of nucleotides from solutions of nucleotides standards, or hepatocytes, or frozen liver powder was accomplished by a modification of the alamine/freon (A-F) procedure described by Khym (17). Recently, the conditions used for nucleotide extraction described below have been shown to be optimum for nucleotide extraction by van Haverbeke and Brown (9). Frozen liver powder (0.5 to 1.0 g), hepatocytes (20 to 30 million cells), or nucleotide standard solutions (1 ml aliquots) were homogenized with a Potter-Elvehjem homogenized in three volumns of 10% trichloroacetic acid (TCA) at 4°C. The resulting homogenate was centrifuged at 10,000 xg for 10 min at 4°C. The supernatant was saved, and the resulting pellet was re-extracted twice with 10% TCA. The supernatants were combined and the TCA was removed with freshly prepared 0.5 M alamine in freon. Chen et al. (7) have reported that the optimum alamine concentration for recovery of nucleotides is 0.5 M and that a decreased recovery of nucleotides, especially the nucleotide triphosphates, occurs when the

alamine/freon solution has aged. The TCA is soluble in the organic alamine/freon solution while the nucleotides remain in the aqueous phase. Equal volumes of alamine/freon and the supernatant were mixed thoroughly by shaking for 3 to 4 min at 4°C. This mixture was centrifuged at low speed to aid in the separation of the organic and aqueous layers. The aqueous phase was removed, filtered, lyophilized and stored at -70°C. The extract was dissolved in 0.007 M KH_2PO_4 (pH 4.2) and was analyzed immediately.

Calculations

The nucleotide concentrations were determined by measuring the peak height of the 260 nm absorbing material eluting from the column. Hartwick and Brown (4) have shown that this method of quantifying nucleotide levels is reproducible and usually more accurate than peak area. Because nucleotides have different absorbance maxima and different shaped elution peaks, the levels of nucleotides were determined using calibration curves for each of the nucleotides studied. The recoveries of nucleotides from solutions of nucleotide standards was determined by measuring the peak heights before and after the extraction procedures.

RESULTS

In developing our HPLC system we modified the system described previously by Brown's laboratory (1, 4) to give maximum resolution of uv-absorbing material obtained from liver extracts in a mimimum amount of time. Our HPLC system uses a microparticulate strong anion exchange column as described by Hartwick and Brown (4); however, the buffer systems used to elute the nucleotides in our system were different from the buffer systems used by Hartwick and Brown (4). In addition, the

elution time for the nucleotides with our system was approximately 50 min; the system employed by Hartwick and Brown (4) required 90 min. McKeage and Brown (10) have recently described an HPLC system which separates nucleotides in 50 min; however, the buffer systems used by McKeage and Brown (10) are different from the buffer systems used for our HPLC system.

Figure 1 shows the ability of our HPLC system to resolve a variety of nucleotides. The total salt concentration of the gradient used to

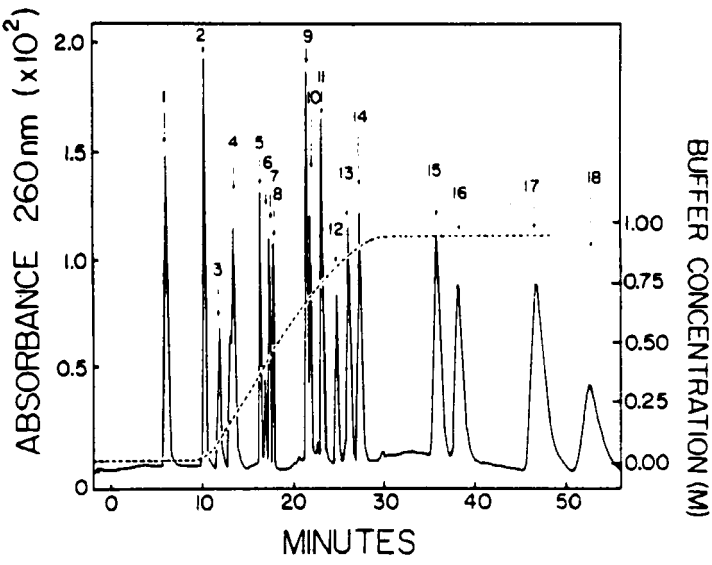

FIGURE 1

A chromatogram of a solution containing 19 nucleotide standards. The absorbance at 260 nm (——) and the anion concentration (---) of the buffer system used to elute the nucleotides are shown. The peaks correspond to the following nucleotides: CMP(1), AMP(2), UMP(3), 2'- and 3'-AMP(4), GMP(5), CDP-glucose(6), UDP-glucose(7), UDP-N-acetyl-glucosamine(8), NADP(9), UDP-glucuronic acid(10), UDP(11), CDP(12), ADP(13), GDP(14), UTP(15), CTP(16), ATP(17), and GTP(18).

elute the nucleotides is shown in Figure 1 also. This salt concentration was calculated mathmatically using the following equation:

$$C = C^* \left[1 - e^{-ft/60\ v_o}\right]$$

where C is equal to the salt concentration in g-mole/liter at any time t, C* is equal to the salt concentration of the more concentrated buffer, f is equal to the flow rate (ml/min) of the solvent entering and leaving the mixing-chamber and v_o is equal to the volume (ml) of the dilute buffer in the mixing chamber at time zero. The gradient generated under these conditions is slightly convex. Because the nucleosides and pyrimidine and purine bases are not negatively charged at pH 4.2, they elute rapidly from the column (within the first 3 to 5 min). The negatively charged nucleotides are retained on the column and elute on the basis of their overall charges, e.g., the more highly charged molecules (nucleoside triphosphates) elute after 30 min of chromatography while less charged molecules (nucleoside monophosphates) elute within the first 6 to 17 min.

To determine if the HPLC system used in this study could resolve the twelve 5'-ribonucleotides from other nucleotides that might be found in liver extracts, we measured the retention times of 40 nucleotides and orotic acid (Table 1). The retention times of the nucleotides varied from column to column; however, the resolution of the nucleotides and the order in which the nucleotides eluted from the column were constant. Although orotic acid is not a nucleotide, it elutes with the nucleoside monophosphates because of its negatively charged carboxyl. Orotic acid is a precursor of the pyrimidine nucleotides and we have found detectible levels of it in liver extracts. Because of the importance of nucleotide-sugars in a variety of biological reactions occurring in liver, the retention times of these

TABLE 1

Retention Times for Nucleotides

Nucleotide	Time (min)	Nucleotide	Time (min)
Adenosine-Containing			
2'AMP	13.0	NAD	10.5
3'AMP	13.0	FAD	21.5
5'AMP	10.5	NADP	21.5
ADP	26.0	dATP	41.25
ATP	42.5		
Cytidine-Containing			
2'CMP	11.0	CTP	36.2
3'CMP	11.0	CDP-glucose	17.25
5'CMP	6.0	CDP-ethanolamine	4.2
CDP	24.8	dCTP	35.5
Guanosine-Containing			
2'GMP	16.2	GTP	45.2
3'GMP	16.2	GDP-Mannose	18.5
5'GMP	16.5	dGTP	43.75
GDP	27.5		
Uridine-Containing			
2'UMP	15.5	UDP-glucose	17.8
3'UMP	15.5	UDP-N-acetyl-glucosamine	17.2
5'UMP	11.8	UDP-glucuronic acid	22.0
UDP	23.2	UDP-mannose	17.5
UTP	34.0	UDP-galactose	17.25
Miscellaneous Nucleotides			
Orotic acid	10.0	FMN	18.2
OMP	22.2	TMP	12.5
IMP	17.5	TTP	34.0

compounds were determined also. The elution of nucleotide-sugars has not been shown in the previous reports which have described the separation of nucleotides by HPLC (1-10). Our HPLC system is capable of

separating the 5'-nucleoside monophosphates from the 2'- or 3'-nucleoside monophosphates. However, as Figure 1 and Table 1 demonstrate, it is not possible to resolve the 2'- and 3'-nucleoside monophosphates. From the retention times, it is possible to predict which nucleotides would coelute with the twelve 5'-nucleotides. The deoxynucleoside triphosphates were poorly resolved from the ribonucleoside triphosphates; however, because the deoxynucleotide pool is only one percent of the ribonucleotide pool (18), contamination of the ribonucleoside triphosphates by the deoxynucleoside triphosphates would be minimal. Both 2'- and 3'-GMP coeluted with 5'-GMP, and 5'-AMP coeluted with NAD. HPLC analysis of liver extracts showed that the levels of the 2'- and 3'-analogues of AMP or UMP were insignificant compared to the amounts of the 5'-analogues of AMP or UMP. However, 5'-AMP and NAD are present in cells at similar concentrations (19). Although uv-absorbing material eluting at 10.5 min in liver extracts will be referred to as AMP, NAD would contribute to the absorbance of this peak. Except for AMP, it appears that our HPLC system can be used to quantify the levels of the major 5'-ribonucleotides in liver extracts.

Using the HPLC system described in the Methods, the concentration of the twelve 5'-nucleotides was determined by measuring the peak height. Figure 2 shows that the peak height response was linear for a wide range of adenine containing nucleotide concentrations. The correlation coefficients of linear regression analysis were 0.998 for AMP, 0.999 for ADP, and 0.988 for ATP. Similar results were obtained for the uracil, cytosine, and guanine containing nucleotides.

The comparison of the extraction of the twelve 5'-nucleotide standards is given in Table 2. The A-F extraction procedure was the

TABLE 2

Comparison of the Recovery of the Twelve 5'-Nucleotide Standards by Three Extraction Procedures[a]

Nucleotide	Extraction Procedure		
	C-A	A-F	PCA-KOH
Nucleoside Monophosphates			
AMP	35.3 ± 3.9	89.3 ± 1.9	73.0 ± 2.5
CMP	43.6 ± 7.7	99.3 ± 1.3	60.0 ± 1.2
GMP	40.1 ± 8.3	81.0 ± 5.9	86.8 ± 0.5
UMP	42.0 ± 7.4	88.8 ± 1.0	77.5 ± 1.6
Nucleoside Diphosphates			
ADP	34.1 ± 3.9	96.3 ± 0.4	76.9 ± 1.9
CDP	39.4 ± 2.0	88.9 ± 2.2	79.4 ± 0.5
GDP	30.0 ± 3.5	90.5 ± 3.5	80.0 ± 0.9
UDP	41.6 ± 3.0	94.0 ± 0.7	80.2 ± 1.3
Nucleoside Triphosphates			
ATP	49.1 ± 4.4	91.1 ± 1.7	80.0 ± 3.1
CTP	57.0 ± 4.2	90.6 ± 1.7	82.1 ± 3.5
GTP	44.9 ± 3.6	89.8 ± 2.5	82.1 ± 3.9
UTP	60.3 ± 2.4	89.8 ± 0.7	83.5 ± 2.6

[a]All values are expressed as the percent of the peak heights of unextracted standards and are given as the mean ± SEM of three different analyses.

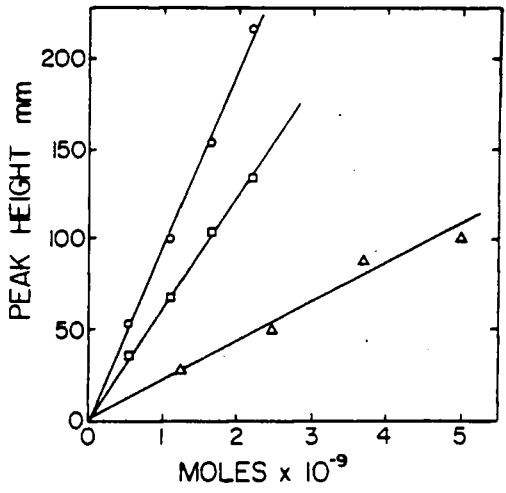

FIGURE 2

The peak height response of various concentrations of ATP (Δ), ADP (□), and AMP (O).

most efficient of the three nucleotide extraction procedures. The recovery of nucleotides for the A-F procedure ranged from 81 to 99% with a mean recovery of 91% for the twelve 5'-nucleotides. The recovery of nucleotides by the PCA-KOH procedure ranged from 68 to 87% with a mean recovery of 78%. The C-A procedure gave the lowest recovery of nucleotides; recoveries ranged from 30 to 60% with a mean recovery of 41%. The differential extraction of purine and pyrimidine containing nucleotides was pronounced in the C-A extraction procedure and the difference was statistically significant. The mean recovery of the purine and pyrimidine containing nucleotides was 39% and 49%, respectively. No statistically significant difference in the extraction of purine and pyrimidine containing nucleotides was observed with the A-F and PCA-KOH procedures.

The degradation of nucleotides during the extraction of standard nucleotide solutions was determined also. There was negligible degradation of nucleoside monophosphates by the three extraction procedures; however, there was some degradation of nucleoside diphosphates and nucleoside triphosphates to nucleoside monophosphates and nucleoside diphosphates, respectively. Figure 3 shows chromatograms of solutions containing the nucleoside triphosphates before and after extraction by the A-F procedure. It is apparent that the nucleoside triphosphate standards were contaminated initially with samll amounts of the nucleoside diphosphates. Brenton et al. (8) have reported that commercially available nucleotides are not pure. Upon extraction, an increase in the nucleoside diphosphates was observed with a concomitant decrease in the nucleoside triphosphates. There was no detectable appearance of the nucleoside monophosphates after extraction of the nucleoside triphosphates.

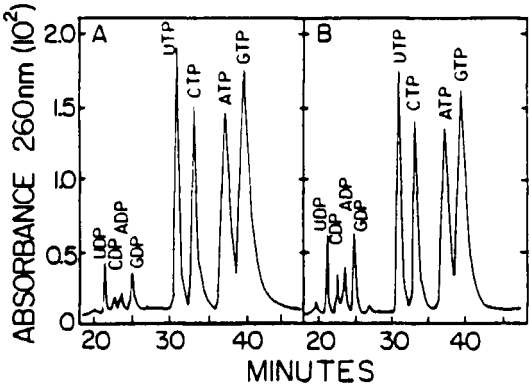

FIGURE 3

Chromatograms of nucleoside triphosphate standards before (Graph A) and after (Graph B) extraction by the A-F procedure.

From Table 3 it is possible to compare the degradation of the four nucleoside triphosphates during the three nucleotide extraction procedures. Two to 4% of the nucleoside triphosphates were degraded to nucleotide diphosphates during extraction. Statistical analysis of the data in Table 3 by analysis of variance demonstrated that the degradation of the nucleoside triphosphates to diphosphates by the three extraction procedures was significant at the $P > 0.05$ level. The amount of nucleoside triphosphate degradation by each of the three extraction procedures was not significantly different.

Table 4 shows the amount of each of the nucleoside diphosphate standards degraded to nucleoside monophosphates by the three extraction procedures. Statistical analysis of the data by analysis of variance showed that the amount of AMP, CMP, or UMP did not increase significantly during extraction by the PCA-KOH procedure. However,

TABLE 3

The Percent Nucleoside Diphosphate Appearing in Nucleoside Triphosphate Standards Before and After Extraction by the Three Procedures[a]

	Unextracted Triphosphate Standard	C-A	A-F	PCA-KOH
ADP	2.41 ± 0.32	5.77 ± 0.80	4.37 ± 0.76	4.49 ± 0.82
CDP	1.73 ± 0.18	3.36 ± 0.36	4.88 ± 0.83	4.69 ± 1.07
GDP	4.88 ± 0.18	9.23 ± 0.36	9.23 ± 1.24	9.36 ± 1.12
UDP	4.64 ± 0.10	5.77 ± 0.08	7.11 ± 0.76	6.71 ± 0.71

[a]All values are expressed as molar percent diphosphate appearing in triphosphate chromatograms and are given as the mean ± SEM of three trials.

TABLE 4

The Percent Nucleoside Monophosphates Appearing in Nucleoside Diphosphate Standards Before and After Extraction by the Three Procedures[a]

	Unextracted Diphosphate Standard	C-A	A-F	PCA-KOH
AMP	14.33 ± 1.10	16.00 ± 0.38	19.83 ± 2.42	15.23 ± 0.70
CMP	5.30 ± 0.42	7.79 ± 0.38	20.83 ± 2.67	5.74 ± 0.41
GMP	12.83 ± 0.23	19.03 ± 1.69	22.00 ± 3.10	16.63 ± 0.83
UMP	6.80 ± 0.38	7.31 ± 1.15	15.96 ± 3.30	8.12 ± 0.81

[a]All values are expressed as molar percent monophosphate appearing in diphosphate chromatograms and are given as the mean ± SEM of three trials.

the increase in GMP was significant ($P > 0.05$). The increase in the four nucleoside monophosphates after the A-F or C-A extraction procedures was significant at the $P > 0.05$ level. The degradation of the nucleoside diphosphates to the nucleoside monophosphates was significantly greater ($P > 0.05$ level) for the A-F procedure than for either the C-A or PCA-KOH procedures.

The extraction of nucleotides from liver samples and isolated hepatocytes by the three procedures was compared next. Figure 4 shows chromatograms of liver extracts obtained by the three procedures. Two observations were made by comparing the three chromatograms. First, slight but consistent changes in the retention times of the nucleotides occurred from procedure to procedure. The change in retention times was most apparent for the nucleoside triphosphates, e.g., ATP (the major peak eluting around 40 min) eluted slightly

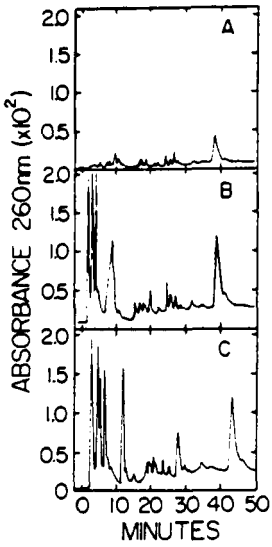

FIGURE 4

Chromatograms of nucleotide extracts obtained from rat liver by the C-A (A), A-F (B), and PCA-KOH (C) procedures are shown. Each chromatogram represents a sample size of 10 µl corresponding to 2.5 mg of frozen liver powder.

before 40 min in liver extracts obtained by the C-A or A-F procedures; however, in liver extracts obtained by the PCA-KOH procedure, ATP eluted after 40 min. Therefore, the elution times of the twelve 5'-nucleotides obtained with each of the extraction procedures was determined using the standard addition method. Secondly, the recovery of uv-absorbing material from frozen liver powder varied depending upon the extraction procedure used. Because the chromatograms in Figure 4 were obtained using the same amount of frozen liver powder, a comparison of the peak heights of the uv-absorbing material is an indication of the extraction efficiency of each procedure. The peak heights of uv-absorbing material obtained from liver extracts prepared by the C-A procedure were reduced greatly compared to the peak heights

obtained by the PCA-KOH and A-F procedures. It is interesting to note that the C-A chromatogram was essentially devoid of uv-absorbing material which eluted during the first 9 min. Similar observations were made when chromatograms of extracts prepared by the three procedures from isolated hepatocytes were compared.

Table 5 gives the concentration of the twelve 5'-nucleotides extracted from frozen rat liver powder by the three procedures. The amounts of nucleotides recovered from liver by the C-A procedure were consistently lower than by either the PCA-KOH or A-F procedures. The concentrations of nucleotides obtained by the PCA-KOH and A-F procedures were similar. There was essentially no difference in the amount of any of the four nucleoside triphosphates recovered from rat liver by the PCA-KOH or A-F procedures. However, in general, the quantities of the nucleoside diphosphates recovered by the A-F procedure were lower than those from the PCA-KOH procedure. On the other hand, the quantities of the nucleoside monophosphates recovered by the PCA-KOH procedure were less than by the A-F procedure. This difference in recovery of the nucleoside monophosphates and diphosphates by the PCA-KOH and A-F procedures could arise because the nucleoside diphosphates are degraded to nucleoside monophosphates during the A-F extraction procedure to a greater extent than during the PCA-KOH extraction procedure (Table 4). This possibility is borne out to some extent because the sum of the monophosphate and diphosphate concentrations for the adenosine-, guanine-, and uridine-containing nucleotides is similar for the A-F and PCA-KOH procedures. Similar results were obtained when the concentrations of the twelve 5'-nucleotides recovered from isolated hepatocytes by the C-A, A-F, and PCA-KOH procedures were compared (Table 6). The recovery of nucleotides

TABLE 5

The Concentration of the Twelve 5'-Nucleotides Recovered from Rat Liver by the Three Extraction Procedures[a]

Nucleotide	Extraction Procedure		
	C-A	A-F	PCA-KOH
Nucleoside Monophosphates			
AMP[b]	85 ± 13	402 ± 94	228 ± 64
CMP	17 ± 1	40 ± 2	32 ± 7
GMP	17 ± 4	75 ± 7	47 ± 7
UMP	51 ± 11	113 ± 15	76 ± 16
Nucleoside Diphosphates			
ADP	301 ± 80	537 ± 5	637 ± 83
CDP	84 ± 0	408 ± 8	127 ± 5
GDP	68 ± 8	69 ± 3	96 ± 28
UDP	76 ± 4	141 ± 1	165 ± 8
Nucleoside Triphosphates			
ATP	843 ± 67	1398 ± 5	1407 ± 98
CTP	132 ± 8	108 ± 8	124 ± 14
GTP	208 ± 8	253 ± 9	263 ± 9
UTP	181 ± 9	256 ± 4	265 ± 8

[a]All values are expressed as nmoles of nucleotide/g frozen liver powder and are given as the mean ± SEM of three separate extractions from three different animals.
[b]The values represent both AMP and NAD because NAD coelutes with AMP (Table 1).

TABLE 6

The Concentration of the Twelve 5'-Nucleotides Recovered from Isolated Hepatocytes by the Three Extraction Procedures[a]

Nucleotide	Extraction Procedure		
	C-A	A-F	PCA-KOH
Nucleoside Monophosphates			
AMP[b]	88 ± 4	490 ± 13	345 ± 44
CMP	55 ± 4	160 ± 27	199 ± 10
GMP	47 ± 1	274 ± 7	210 ± 13
UMP	68 ± 2	376 ± 12	276 ± 10
Nucleoside Diphosphates			
ADP	343 ± 7	742 ± 69	822 ± 55
CDP	109 ± 9	520 ± 30	567 ± 28
GDP	98 ± 14	620 ± 20	621 ± 16
UDP	155 ± 5	724 ± 113	978 ± 154
Nucleoside Triphosphates			
ATP	2017 ± 64	8740 ± 225	8706 ± 111
CTP	393 ± 10	935 ± 209	860 ± 49
GTP	513 ± 10	2075 ± 128	2802 ± 110
UTP	354 ± 60	557 ± 138	663 ± 73

[a]All concentrations are expressed as nmoles/10^9 hepatocytes and are given as mean ± SEM of three separate experiments with three individual animals.
[b]These values represent both AMP and NAD because NAD coelutes with AMP (Table 1).

from isolated hepatocytes by the C-A procedure was lower than by the PCA-KOH or A-F procedures, and the recoveries of nucleotides by the PCA-KOH and A-F procedures were similar.

DISCUSSION

This is the first report to describe an HPLC system developed specifically to study nucleotides from liver extracts. This system is capable of separating a wide variety of nucleotides and nucleotide-sugars in less than 50 min. Although this system was developed independently in our laboratory, the separation and retention times of nucleotides appear to be similar to the HPLC system recently described by McKeag and Brown (10). To determine the ability of our system to resolve a wide variety of nucleotides that would be found in liver extracts, the retention times of 40 nucleotides were determined (Table 1). Except for AMP, the other eleven major 5'-ribonucleotides were resolved from other nucleotides. Therefore, this system can be used to routinely separate and quantify the major 5'-ribonucleotides in liver extracts.

Because the extraction of nucleotides for analysis by HPLC has been studied to a very limited extent (7,11) a comprehensive study was conducted to compare the extraction of the twelve 5-nucleotides from solutions of nucleotide standards, liver samples, and isolated hepatocytes by three commonly used extraction procedures. Nucleotides were extracted either by the precipitation of PCA by KOH neutralization, or the removal of TCA by alamine/freon as described by Khyme (17) and optimized by van Haverbeke and Brown (9), or the removal of nucleotides from a PCA solution by absorption on activated charcoal. The recoveries of nucleotides by charcoal absorption was

less than one-half that obtained by the other two procedures. The recoveries of nucleotides from solutions containing nucleotide standards ranged from 81 to 99% for the TCA-alamine/freon procedure and 68 to 87% for the PCA-KOH neutralization procedure. These recoveries of nucleotides are slightly lower than that reported by Chen et al. (7). However, Chen et al. (7) performed only a minimum number of manipulations in their extraction procedures. Recently, van Haverbeke and Brown (9) found that the recoveries of nucleotides from solutions containing nucleotide standards was reduced 10% when a filtration step was added to the TCA-alamine/freon procedure described by Chen et al. (7). The recoveries of nucleotides by TCA-alamine/freon procedure that we obtained (Table 2) are slightly higher than the recoveries reported by van Haverbeke and Brown (9).

Chen et al. (7) also reported that pyrimidine containing nucleotides were extracted by the TCA-alamine/freon procedure more efficiently than purine containing nucleotides. However, no statistical analysis of their data was shown and the only pyrimidine containing nucleotide extracted from solution was CTP. In our study, when the extractions of the six pyrimidine containing and six purine containing compared by either the PCA-KOH neutralization or TCA-alamine/freon procedures, no statistically significant different in the extraction of these two types of nucleotides was observed.

Although the efficiency of nucleotide extraction from solutions containing standard nucleotides was greater for the TCA-alamine/freon procedure than for the PCA-KOH neutralization procedure, the recovery of the twelve 5'-nucleotides from liver samples or isolated hepatocytes was similar for these two extraction procedures. Chen et al. (7) did not recommend the PCA-KOH neutralization procedure when

nucleotides were analyzed by HPLC because of potential problems that might arise from the neutralization procedure. We have encountered no serious problems with the PCA-KOH neutralization procedure when nucleotides were analyzed by HPLC because of potential problems that might arise from the neutralization procedure. We have encountered no serious problems with the PCA-KOH neutralization procedure. Initially, we occasionally observed problems with the resolution of nucleotides extracted by this procedure. This problem appears to have arisen because it was not always possible to remove all of the $KClO_4$ precipitate by low speed centrifugation. However, after the addition of the filtration step in the extraction procedure, all of the $KClO_4$ precipitate was removed and no problem was observed with the resolution of nucleotides from the PCA-KOH neutralization extraction procedure.

In addition to comparing the efficiency of nucleotide extraction by the three procedures, we also determined whether any significant degradation of nucleotides occurred during the extraction procedures. van Haverbeke and Brown (9) reported that their results indirectly indicated that no degradation of the nucleoside triphosphates occurred during the TCA-alamine/freon extraction procedure. We specifically examined whether degradation of nucleoside monophosphates, diphosphates, or triphosphates occurred with the three extraction procedures. No detectable degradation of nucleoside monophosphates was observed with any of the procedures. The degradation of nucleoside triphosphates to nucleoside diphosphates by the three procedures was small (2 to 4%); however, it was statistically significant. The degradation of nucleoside diphosphates to nucleoside monophosphates also occurred with the three extraction procedures, and this degrada-

tion was significantly greater for the TCA-alamine/freon procedure than the other two procedures.

ACKNOWLEDGEMENTS

We would like to thank Dr. Daniel Paschal for his helpful assistance in developing the HPLC system used in this study. This work was supported in part by NIH grant 1 RO1 AG 00344-02.

APPENDIX

List of Abbreviations

Abbreviations of nucleotides: AMP or 5'AMP (adenosine 5'-monophosphate), 2'AMP (adenosine 2'-monophosphate), 3'AMP (adenosine 3'-monophosphate), ADP (adenosine 5'-diphosphate), ATP (adenosine 5'-triphosphate), NAD (nicotinamide adenine dinucleotide) FAD (flavin adenine dinucleotide), NADP (nicotinamide adenine dinucleotide phosphate), dATP (deoxyadenosine 5'-triphosphate), CMP or 5'CMP (cytidine 5'-monophosphate), 2'CMP (cytidine 2'monophosphate), 3'CMP (cytidine 3'-monophosphate), CDP (cytidine 5'-diphosphate), CTP (cytidine 5'-triphosphate), dCTP (deoxycytidine 5'-triphosphate), GMP or 5'GMP (guanosine 5'-monophosphate), 2'GMP (guanosine 2'-monophosphate), 3'GMP (guanosine 3'-monophosphate), GDP (guanosine 5'-diphosphate), GTP (guanosine 5'-triphosphate), dGTP (deoxyguanosine 5'-triphosphate), UMP or 5'UMP (uridine 5'-monophosphate), 2'UMP (uridine 2'-monophosphate), 3'UMP (uridine 3'-monophosphate), UDP (uridine 5'-monophosphate), UTP (uridine 5'-triphosphate), TTP (thymidine 5'-triphosphate), TMP (thymidine 5'-monophosphate), FMN (flavin mononucleotide), IMP (inosinic acid), and OMP (orotidine 5'-monophosphate).

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