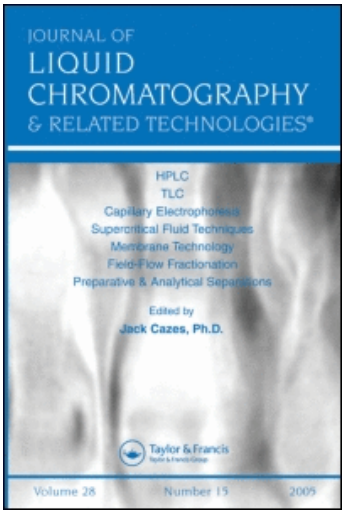


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# EFFECT OF pH, TEMPERATURE, AND IONIC STRENGTH ON REVERSED-PHASE ION-PAIR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PURINE NUCLEOTIDE MONOPHOSPHATE

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ABSTRACT

Ion-pair reversed phase high performance liquid chromatography on Zorbax ODS was applied to the separation of the purine nucleotide monophosphates, GMP, IMP, XMP, AMP and ZMP. Three parameters (pH, ionic strength and temperature) which greatly effect ionic equilibrium were studied as to their effect on retention and resolution of the purine nucleotide monophosphates (PuMPs). GMP and IMP essentially co-elute at pH 4 - 6.25 but GMP elutes earlier than IMP as the pH is lowered from 4.0 to 2.25. Lowering the pH 6.25 to 2.25 lowers k′ of those PuMPs (GMP, XMP, AMP and ZMP) with a pKa between 2 and 4 but not the k′ of IMP which does not have a pKa in the region.

Column temperature was varied from 15° to 45° in 5° increments for several pH values from 2.25 to 6. The effect of column temperature was similar for all pHs checked. As temperature was elevated above 25°, there was a decrease in k′ which resulted in a deterioration in resolution and as the temperature was decreased to 15° there was an increase in k′ and a concomitant improvement in resolution, but there was no resolvement of peaks which co-elute at 25°. Similarly, an increase in ionic strength decreased k′ and decreasing ionic strength increased k′. Good resolution of the selected PuMPs is obtained between pH 2.25 and 3 with 20 mM $KPO_4$, 5mM tetrabutylammonium hydroxide and 3.5% acetonitrite. Some improvement in resolution is observed below 25° but there is an increase in backpressure. Lowering the phosphate concentration below 20 mM leads to large k′ with very broad peaks.





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## INTRODUCTION

The metabolic pathway for synthesis of purine nucleotides and recycling purine bases and nucleosides to nucleotides is subject to alteration as a normal cell goes through the steps which ultimately yield a cancerous cell. Several enzymes involved in the synthesis of purine nucleotides have been found to have increased activity in the preneoplastic and cancerous tissue (1). The activity of most enzymes involved in the purine bases and nucleosides salvage pathway is lowered in the preneoplastic and cancerous cell. However, the effect of chemical carcinogens on the cellular concentration of purine nucleotides is not well studied. A major reason for this scarcity of information on the purine nucleotides and in particular the purine monophosphates (PuMP) is the absence of techniques to routinely quantitate the PuMPs. The emergence of HPLC as an analytical tool has resulted in the application of the ion-exchange HPLC to quantitate the nucleotides. However, ion-exchange HPLC dictates the availability of gradient chromatograph and usually is a lengthy procedure as a column re-equilibration is required. Reverse HPLC has been applied to nucleotide analysis but is more suitable to the nucleosides than to the more polar nucleotides. Ion-pair reversed phase HPLC is well suited to quantitation of nucleotides and has been applied by several investigators. However, the emphasis of such reports has not been on the PuMPs and thus have not included all of the PuMP involved in the synthesis of the purine nucleotides. We have thus investigated the behavior of the PuMPs on ion-pair HPLC to develop a system that will separate the PuMPs and permit quantitation at the levels expected in rat liver. The initial conditions employed were chosen from the

several available in the literature utilized to separate various mixtures of nucleotides (reviewed in ref. 2). However, these conditions caused GMP and IMP to co-elute. Therefore, we investigated the effect that varying pH, ionic strength and temperature have on the elution of the PuMPs in an ion-pair reversed phase application.

## MATERIALS AND METHODS

### APPARATUS

The chromatograph consisted of a Shimadzu LC-5A pump, a Rheodyne model 7120 valve with 100 ul sample loop, a Shimadzu SPD-2 UV spectrophotometer equipped with 8 ul flow through cell and a Shimadzu Chromatopac C-R2AX data processor. The column was enclosed in a water jacket with the column temperature regulated by a Lauda K/RD refrigerated circulating water bath. A 0.46 cm x 25 cm column filled with 37 - 53 micron silia was placed in front of the injection valve. A 0.46 cm x 5 cm column filled with Chromosorb LC-4 (Johns - Manville) was between the sample valve and the analytical column.

### REAGENTS

Acetonitrile (HPLC grade) was obtained from Fisher Scientific. The nucleotides and tetrabutylammonium hydroxide were obtained from Sigma Chemical Company. All other chemicals were of reagent grade. Deionized water was passed over Whatman LRP-1 prior to use. The phosphate buffer was made by adding 20 ml of 1M phosphoric acid and 3.5 ml tetrabulylammonium hydroxide in a 1 l. volumetric flask and filling to volume with water after which

the pH was adjusted to the desired pH (± .005) with solid KOH. The pH meter was calibrated with a pH 4.0 standard buffer. The mobile phase was prepared by adding 35 ml of acetonitrite to 1 l. volumetric flask and filling to the mark with the appropriate phosphate - TBAP buffer. After mixing, the mobile phase was filtered through a 0.47 μ nylon filter under vacuum.

## CHROMATOGRAPHY

A prepared Zorbax ODS column with a 5 micron particle size and dimensions of 4.6 mm x 25 cm was obtained from DuPont. The flow rate was 2 ml/min. Column temperature was 25). Detection was at 260 mm. The column was equilbrated with mobile phase for at least 1 hour prior to use. Two Zorbax ODS columns were used for the experiments described in this paper with no essential difference noted in the two columns.

## RESULTS AND DISCUSSION

In ion pair chromatography the ion pair reagent has ionic attraction with an ionized solute so that the stronger the ionic attraction to the solute, the longer the solute is retained by the column. The pKa of the phosphate on the PuMPs is 6.1 to 6.3 (3). The interaction of the TBAP with the PuMPs should be stronger in comparison to the corresponding nucleoside and thus lead to long k′ at pH $\leq$ 6.2. Thus the ionization of the PuMPs which leads to short k′ on reversed phase HPLC at a pH close to the $pK_{apo4}$ (4) will result in large k′ under ion pair HPLC.

Ion pair HPLC of the non-succinyl PuMPs was first tested at pH5. Essential coelution of GMP and IMP was observed under these chromatographic conditions which make quantitation of GMP and IMP

from rat liver by these conditions impractical as GMP and IMP are present in similar quantities (5). Therefore, various changes in the chromatographic conditions were investigated. Changing the concentration of acetonitrite or replacement with methanol, while changing k´ of the PuMPs, does not alter the resolution of GMP and IMP. Since changes in the mobile phase composition which would affect hydrophobic - hydrophyilic interactions more than ionic interactions did not resolve GMP, we turned our attention to those chromatography conditions which affect ionic equilibrium. Three parameters (pH, ionic strength and temperature) that affect ionic equilibrium where investigated for their effect on retention and resolution of the PuMPs.



The effect of pH on elution of the PuMPs is shown in figure 1. The pyrimidine monophosphates CMP and UMP were included as they are present in rat liver and coisolate with the PuMPs. BAMP (8-bromo AMP) was included in this study to determine if it were suitable as a internal standard. 8-Bromo GMP and 6-Bromo purine monophosphate were also tested but are retained on the column under conditions, i.e. acetonitrite concentration, which eluted the non succinyl-PuMPs and were not further used. The succinyl PuMPs, S-ZMP and S-AMP acid groups with two carboxylic are retained on the ODS column with 3.5% acetonitrile. Elution of S-ZMP and S-AMP is accomplished by 20% acetonitrile in the mobile phase which places this elution in the same region as the di and tri phosphates of the purine nucleotides. The succinyl-PuMPs were not included in this study on the purine monophosphates due to their large k´ in relation to non-succinyl PuMPs. ZMP was included in this investigation after the experiments in figure 1 were concluded. A less detailed study on the effect of pH on ZMP

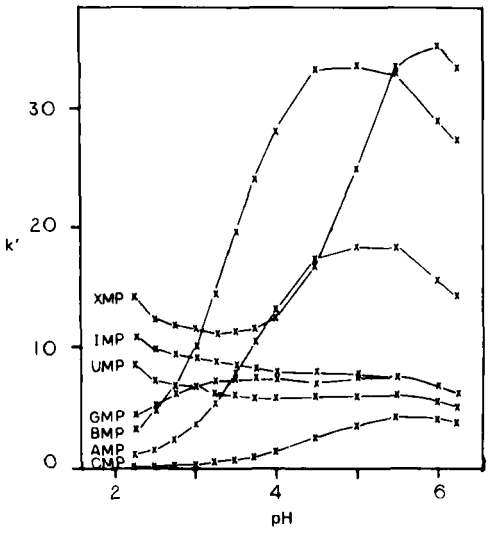



Figure 1 Effect of pH on k´ of PuMPs



is shown in figure 2 with GMP and IMP shown for comparison. The inflection point in the sinnodal curves for AMP and CMP corresponds well with the respective pKas (3.7 vs.3.74, 4.3 vs.4.5). The uniform decrease in k´ as the pH is raised above 5.5 is not explained by accompanying increase in ionization of the phosphate group. The apparent anamolous behavior is due to an increase in ionic strength as more KOH is needed to raise the pH. The ionic equilibrium is very sensitive to ionic strength and as described later, the k´ does decrease with increasing ionic strength. The increase in k´ of XMP and UMP as pH is decreased below 3 is principally due to the concomitant decrease in ionic strength. IMP which does not have a $pK_a$ below 6.2 is not affected to a great extent by pH while GMP which has a $pK_a$ = 2.4 is affected as the pH is lowered below pH 3.5. Therefore, there is sufficient resolution between GMP - IMP at pH $\leq$ 3.5 to

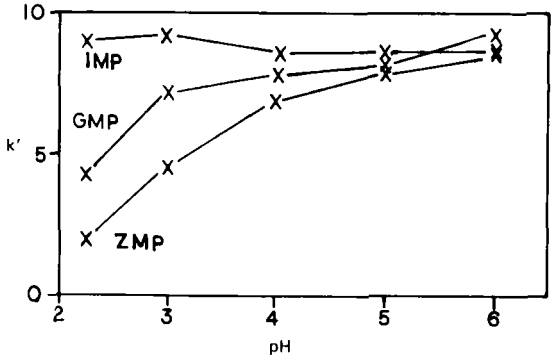


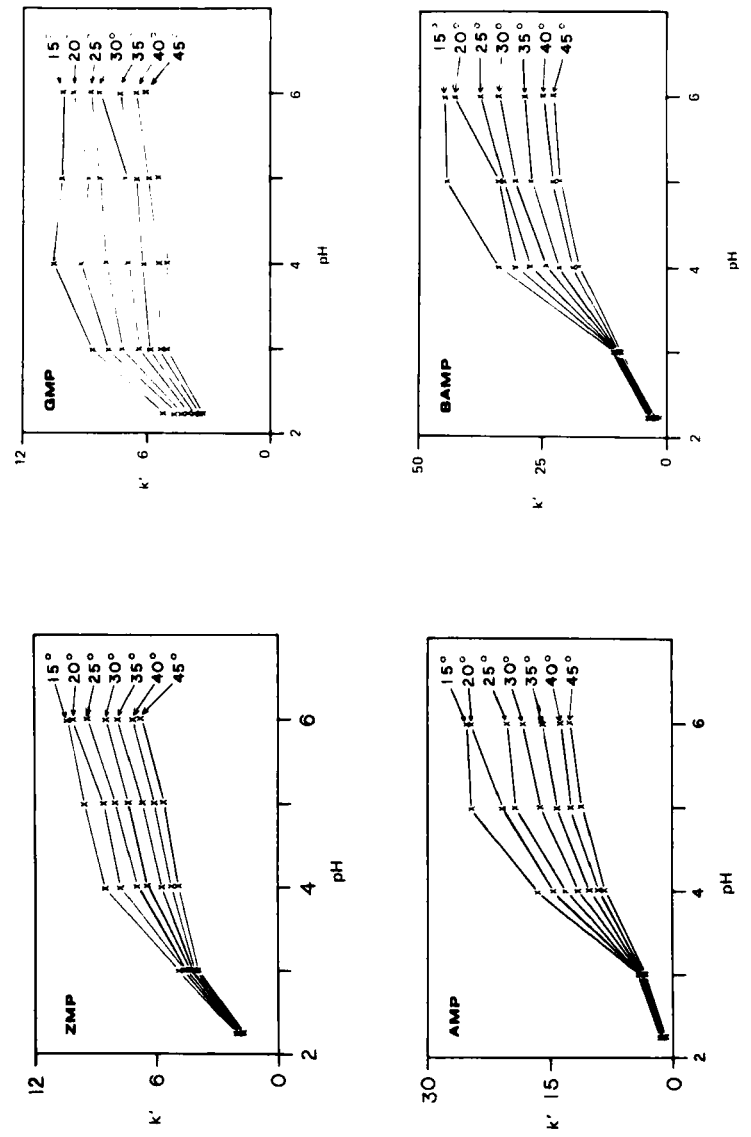

Figure 2 Effect of pH on k´ of ZMP



permit quantitaion. The great effect of pH on AMP causes AMP to elute along with another PuMP from pH 5.25 to 3. Thus, the more acidic pH s yield more suitable chromatogram.

Ionic equilibrium can be very sensitive to temperature. In our initial experiments with ion-pair HPLC of the PuMPs, the column temperature was not controlled. Though our interest at the time was in resolution of the PuMPs and not in k´, it soon became apparent that the variation in k´, and even resolution between similar chromatographic runs were greater than warranted. These variations disappeared when the column temperature was regulated. Due to the noticed variation in resolution of several PuMPs with just a few degree differences in ambient temperature, the effect of temperature on the chromatographic behavior of the PuMPs was determined at selected pHs (figure 3).

The effect of increasing column temperature from 25 $^{0}$ to 45 $^{0}$ is to decrease retention of the PuMPs. Though not obvious from fig 3, the resolution of the PuMPs is similarly decreased. Thus,



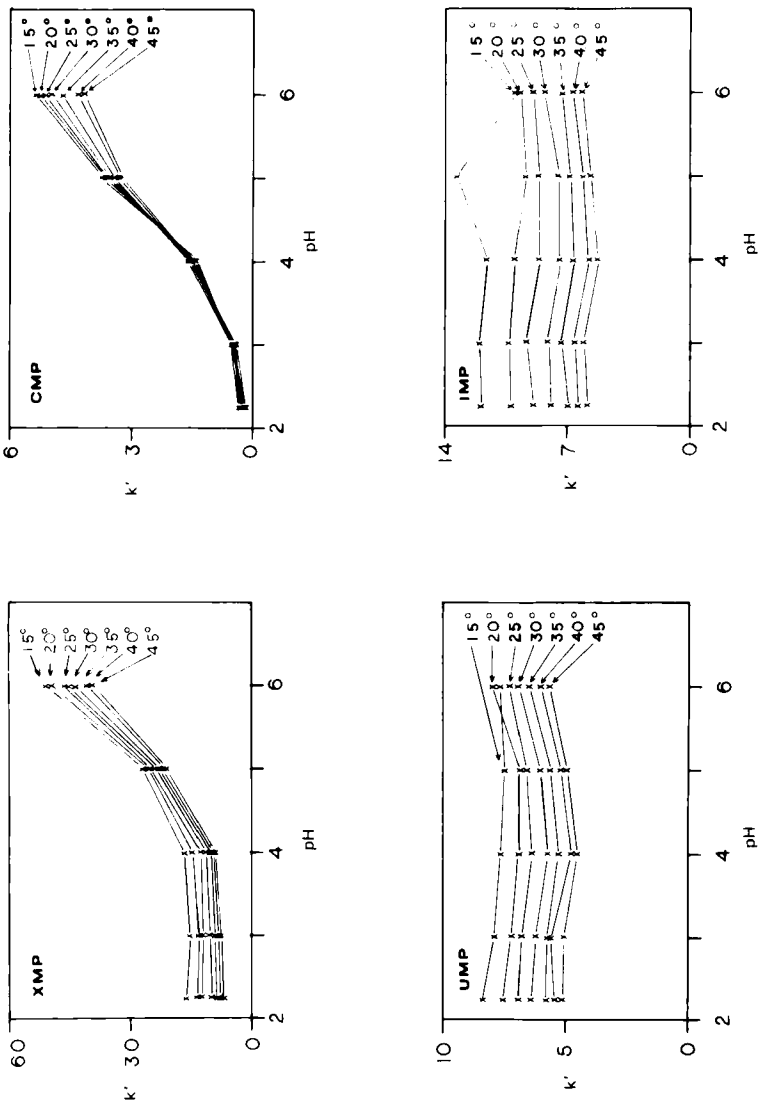



Figure 3 Effect of temperature of k′ of PuMPs

decreasing the organic portion of the mobile phase to increase retention would not be expected to enhance resolution. Decreasing column temperature from 25° to 15° increases retention and does increase resolution of some PuMPs, notably GMP/IMP at pH $\leq$ 3. This behavior may be exploited for the analysis of GMP and IMP. However, for XMP, which is retained the longest and which is present in the liver in the least amount of the common non-succinyl PuMPs, any increase in k′ leads to a broadening of the peak and subsequently to lower sensitivity.

The effect of ionic strength was checked at pH 6 (figure 4). The effect of increasing phosphate concentration from 20 mM to 60 is to lower k′ and to decrease resolution. A decrease in phosphate to 10 mM results in a drastic increase in k′ and significant in resolution of peaks resolved at 20 mM but not in



TABLE 1

Effect of 10 mM phosphate on k′(1) of nucleotide monophosphates on Zorbax ODS

| Nucleotide | pH | | | | |
|---|---|---|---|---|---|
| | 2.25(2) | 3 | 4 | 5 | 6 |
| CMP | 1.54 | 1.35 | 1.50 | 1.43 | 1.68 |
| UMP | 1.18 | 1.31 | 1.25 | 1.32 | 1.65 |
| GMP | 1.23 | 1.30 | 1.27 | 1.22 | 1.61 |
| IMP | 1.20 | 1.39 | 1.17 | 1.20 | 1.61 |
| ZMP | 1.33 | 1.31 | 1.31 | 1.14 | 1.68 |
| AMP | 1.19 | 1.29 | 1.24 | 1.27 | 1.54 |
| BAMP | 1.25 | 1.25 | 1.17 | 1.20 | 1.58 |
| XMP | 1.16 | 1.25 | 1.31 | 1.34 | 2.09 |

(1) Expressed as ratio to k′ at 20 mM phosphate.

(2) Actual phosphate concentration is 14.6 mM which is the amount of phosphoric acid required to adjust 5 mM tetrabutylammonium hydroxide to pH 2.25.

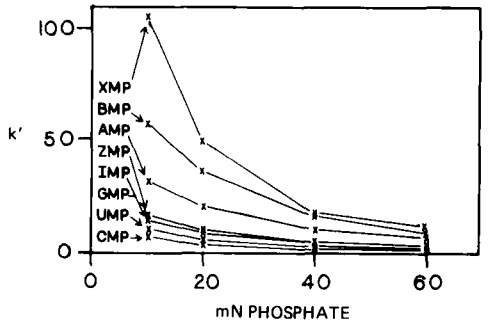



Figure 4 Effect of phosphate concentration on k′ of PuMPs at pH 6



peaks unresolved at 20 mM. The effect of decreasing the phosphate concentration to 10 mM phosphate at pHs 2,3,4 and 5 and the minimum phosphate (15mM) at pH 2.25 is shown in table 1. The decreased phosphate gives an increased k′ and generally better resolution for resolved peaks at a given pH. Peaks that are unresolved at 20 mM are not resolved at the lower concentration. The effect of ionic strength indicates that retention of all PuMPs is due to mainly ionic interaction and not just to hydrophobic interactions which are not greatly affected by ionic strength. It should be noted that the nucleosides are eluted prior to CMP irrespective of pH.

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