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REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF 5-FLUOROURACIL, 5'-DEOXY-5-FLUOROURIDINE, THEIR NUCLEOSIDES, MONO-, DI-, AND TRIPHOSPHATE NUCLEOTIDES WITH A MIXTURE OF QUATERNARY AMMONIUM IONS

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SUMMARY

Several quaternary ammonium ions were evaluated in the development of a reversed-phase ion-pair high-performance liquid chromatographic assay for the separation of the known nucleosides and nucleotides of 5-fluorouracil (FU) and its analogue, 5'-deoxy-5-fluorouridine (5'-dFUR). The capacity factors of FU, 5'-dFUR, and their eight anabolites including 5-fluorouridine and its mono-, di-, triphosphate and diphosphoglucose, 5-fluorodeoxyuridine and its mono- and diphosphate nucleotides, were dependent on the chain length and concentration of the counter ions, pH, type of buffer, as well as the type of bonded stationary phase. Separation of FU, 5'-dFUR, their nucleosides and monophosphate nucleotides was readily achieved using tetrabutylammonium ion alone as the counter ion. However, under these conditions, the di- and triphosphate nucleotides were eluted from the column only after lengthy gradient elution and with poor reproducibility. Optimal conditions for a simultaneous separation of the ten fore-mentioned compounds were achieved using a two-step elution with a mixture of tetraethylammonium (C_8) and tetrabutylammonium (C_{16}) ions. The first eluent consisted of C_8 and C_{16} ions in a mixture of acetate—phosphate buffer and methanol, the second eluent contained an additional 30 m*M* phosphate. FU, 5'-dFUR, their nucleosides and monophosphates and diphosphoglucose were separated by isocratic elution from a μBondapak C_{18} reversed-phase column using the first eluent; and the di- and triphosphate nucleotides were subsequently eluted, isocratically with the second eluent. This assay does not require gradient elution and can be completed within 50 min with good reproducibility.

INTRODUCTION

5-Fluorouracil (FU) is one of the most widely used agents for palliative treatment of solid tumors [1]. FU may undergo anabolism to form ribose

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and deoxyribose nucleosides, and their mono-, di-, and triphosphates. Among these, 5-fluorodeoxyuridine monophosphate (FdUMP) which inhibits thymidylate synthetase and DNA synthesis, and 5-fluorouridine triphosphate (FUTP) which is incorporated to form fraudulent RNA, are believed to be responsible for the cytotoxicity of FU [1]. An additional metabolite, 5-fluorouridine diphosphoglucose (FUDPG), has been observed in bacteria [2] and in some rodent tumor cells [3]. 5'-Deoxy-5-fluorouridine (5'-dFUR), an FU analogue recently introduced by Cook et al. [4], has shown improved therapeutic index over that of FU in some animal tumor models [5—8]. This compound is presumably an FU prodrug [4—7] and should follow the same metabolic scheme to form FdUMP and FUTP. The formation and breakdown of FdUMP and FUTP may depend on the pool sizes of the other metabolites, i.e. 5-fluorouridine (FUR), 5-fluorodeoxyuridine (FUdR), 5-fluorouridine mono- (FUMP), and diphosphate (FUDP), and 5-fluorodeoxyuridine diphosphate (FdUDP). Due to the complexity of the metabolic network, it is desirable to quantitate the intracellular pool sizes of all of these metabolites in the study of biochemical determinants of tumor sensitivity or resistance to FU and 5'-dFUR. In addition, since the size of biological samples is usually minute, a simultaneous assay is preferred.

The separation of FdUMP and FUTP has been achieved by thin-layer chromatography and anion-exchange chromatography [3, 9]. More recently, several reversed-phase ion-pair high-performance liquid chromatographic (HPLC) assays using exclusively tetrabutylammonium ion as the counter ion have been described for the separation of FU, FUR, FUdR, FUMP, and FdUMP [10, 11]. The only available HPLC assay for FUDP and FUTP is by gradient elution from an anion-exchange column where FdUDP coeluted with FUDP [12], and none of the existing assays allows simultaneous separation of FU and its eight known metabolites. For economic and practical reasons, the ideal separation of these ten compounds should be by isocratic elution.

In this investigation, several quaternary ammonium ions were evaluated in the development of a reversed-phase ion-pair HPLC assay for the anabolites of FU and 5'-dFUR. The effects of pH, buffer concentration, stationary phase, and chain length of counter ions were studied. Optimal separation of mono-, di-, and triphosphate nucleotides was obtained using a mixture of tetraethyl- and tetrabutylammonium ions. This rapid, simultaneous assay for all of the known anabolites of FU and 5'-dFUR using a two-step elution is reported here.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents used are of analytical reagent or spectroquality grade. Tetrabutylammonium hydrogen sulfate (C_{16}) as purchased from Aldrich (Milwaukee, WI, U.S.A.), triethylammonium hydroxide (C_6) and tetraethylammonium bromide (C_8) from Sigma (St. Louis, MO, U.S.A.), tetrapropylammonium hydroxide (C_{12}) and triethylamine from Eastman Kodak (Rochester, NY, U.S.A.). 5'-dFUR was generously supplied by Hoffmann-LaRoche (Nutley, NJ, U.S.A.). FU, FUR, FUdR, FUMP, FdUMP, uridine diphosphate

(UDP), uridine diphosphoglucose (UDPG), deoxyuridine diphosphate (dUDP), and uridine triphosphate (UTP) were obtained from Sigma. Standard chemicals were dissolved in methanol. The retention volume of solvent front is taken as the void volume of the column. The pH of buffers was adjusted with acetic acid.

In all HPLC systems used in this investigation, the chromatographic behavior of uracil, uridine, deoxyuridine, uridine monophosphate, and deoxyuridine monophosphate were very similar to their fluorinated counter part; their corresponding capacity factors differed from each other only by 10 to 20%. Therefore, UDPG, dUDP, UDP, and UTP were used in place of FUDPG, DdUDP, FUDP, and FUTP in the assay development.

Apparatus

Assay development was done on an HPLC unit consisting of a Model 6000A solvent delivery system which provides a three-solvent selection valve, an U6K injector and a Model 440 dual-wavelength UV detector at 254 and 280 nm (Waters Assoc., Milford, MA, U.S.A.). The analytical HPLC columns used were μBondapak C_{18} (30 cm × 4 mm I.D., 10 μm), radiocompression module with C_{18} reversed-phase column (RCM-C_{18}) (10 cm × 8 mm I.D., 10 μm) (Waters Assoc.), Zorbax TMS (25 cm × 4.6 mm I.D., 7—8 μm) and Zorbax C_8 columns (25 cm × 4.6 mm I.D., 7—8 μm) (DuPont Instruments, Wilmington, DE, U.S.A.).

RESULTS AND DISCUSSION

The mechanism of reversed-phase ion-pair chromatography has been reviewed by Snyder and Kirkland [13]; the capacity factor of a sample anion is dependent on the pH, concentration of secondary ions, chain length and concentration of counter ions. The results obtained in this investigation are in general agreement with previous studies.

Effect of stationary phase

Among the different types of stationary phase tested, the μBondapak C_{18} provides the best peak symmetry and more importantly, the optimal separation of FU, FUR, and FUdR. These compounds have pK_a values of about 8 [14, 15] and are mainly unionized in the pH range of 3—6. Their k' values were least affected by the type and concentration of counter ions used or by a change of pH of the mobile phase from 3 to 7 (Fig. 1). This is consistent with the observation of Gelijkens and De Leenheer [11]. The retention mechanism of this type of compounds is by partitioning into the stationary phase and is governed by the usual reversed-phase chromatographic processes; their k' values are dependent on the ionic strength, and strength of the mobile phase such as the composition of the organic solvent [13]. The capacity factors of these compounds in four commercially available reversed-phase columns with different bonded stationary phases are compared in Table I. FU, FUR, FUdR were not retained on the Zorbax TMS and were only weakly retained on Zorbax C_8. The retentions of FU, FUR, FUdR on RCM-C_{18} were stronger than that on the μBondapak C_{18}, but the retention of nucleotides

TABLE I

EFFECT OF STATIONARY PHASE ON THE CAPACITY FACTORS, k', OF FU, FUR AND FUdR

	Stationary phase			
	Zorbax TMS	Zorbax C_8	RCM-C_{18}	μBondapak C_{18}
	Eluent			
	2.5 m*M* C_6	50.0 m*M* C_6 10.0 m*M* C_{16} 10.0 m*M* sodium acetate 1.0 m*M* sodium/potassium phosphate	0.1 m*M* C_{16} 10.0 m*M* sodium acetate	0.5 m*M* C_{16} 20.0 m*M* sodium acetate
	pH 4.3	pH 6.2	pH 6.3	pH 4.0
FU	1.8	1.7	2.2	1.2
FUR	1.8	3.2	5.0	2.4
FUdR	1.8	3.6	6.4	3.3

ion-paired with ammonium ions in the RCM-C_{18} was increased unproportionally which makes RCM-C_{18} an undesirable choice for a rapid simultaneous assay. Unless specified otherwise, the results in the remaining portion of this report were obtained using μBondapak C_{18} columns. Due to the small differences in the k' values of FU, FUR, and FUdR, the counter ion concentration, buffer concentration, and strength of the mobile phase were kept minimal to optimize the separation of these three compounds.

Effect of pH

The nucleotides are relatively strong acids with the first acidic protons with pK_{a1} of about 1 [14, 15]. In addition, there are one, two, and three second acidic protons of pK_{a2} of about 6 for the mono-, di-, and triphosphate nucleotides, respectively [14, 15]. At the pH range of 2—5, the first acidic proton of these nucleotides is mainly ionized and can form ion pairs with ammonium ions. When the pH is near or above the pK_{a2}, the degree of ionization of the second acidic proton becomes significant and these compounds can ion-pair with the second ammonium ion resulting in an increase in their retention. The retention of FUMP and FdUMP depends on the pH of the mobile phase (Fig. 1). The optimal pH range for the separation of FUMP and FdUMP from their nucleosides is 4—6 where their k' values varied linearly with the pH. At pH above 6, FUMP and FdUMP exist primarily as divalent anions and the elution of their ion pairs becomes lengthy and impractical.

In the previously described assays, FUMP and FdUMP were eluted at pH 7 using C_{16} ion as the counter ion [10, 11]. In an attempt to reproduce these assays, it was discovered that FdUMP and UDPG coeluted. The ratios of k' values of FdUMP and UDPG were pH-dependent with an optimum at pH 6

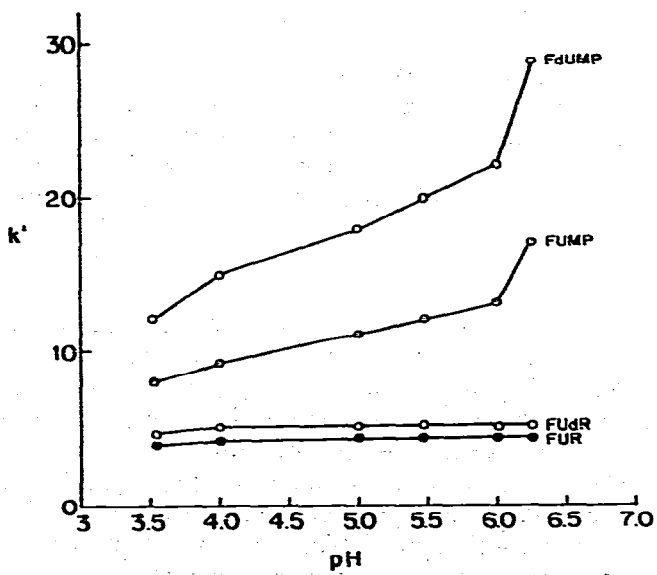

Fig. 1. Effect of pH on capacity factors, k', of FUR, FUdR, FUMP and FdUMP. The stationary phase was μBondapak C_{18}. The eluting buffer was 0.1 m*M* C_{16} in 10 m*M* sodium acetate buffer.

(Fig. 2). In UDPG, the proton with pK_a of 1 is replaced by the glucose moiety, the two remaining most acidic protons have pK_a values of about 6 [14, 15]. The decrease in the ratio of k' (FdUMP) to k' (UDPG) at higher pH may be partially explained by the fact that the degree of ionization of UDPG is relatively insensitive to an increase in pH up to 6 when compared to that of FdUMP.

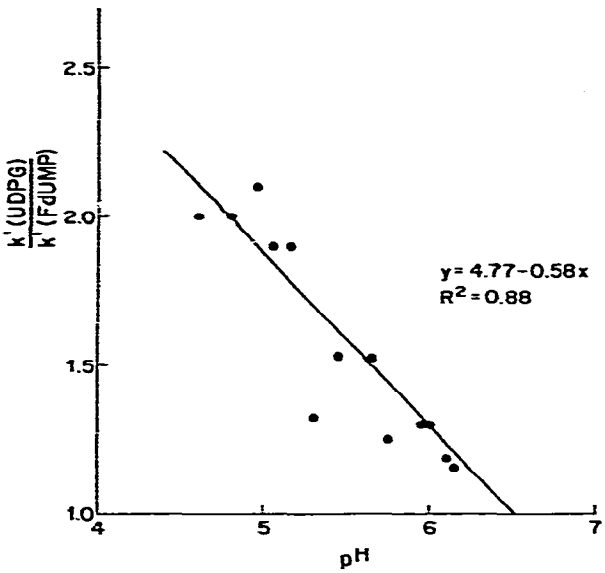

Fig. 2. Effect of pH on the ratios of capacity factors, k', of FdUMP and UDPG. The eluting buffers were 2.5—5.0 m*M* C_2, 0.1 m*M* C_{16}, 0.5—2.0% methanol in 0.5—5.0 m*M* sodium acetate buffer. The stationary phase was μBondapak C_{18}.

The change in retention of UDP and UTP by altering the pH of the mobile phase from 3 to 6 is similar to that of FUMP and FdUMP (data not shown). But unlike FUMP and FdUMP, their peak symmetry is pH-dependent with severe band tailing at pH 3. The optimal pH for the separation of FU, FUR, FUdR, 5'-dFUR, FUMP, FdUMP, UDPG, UDP, and UTP is, therefore, between 5 and 6.

Effect of buffer concentration

Two buffer systems were used to maximize the buffering capacity at pH 6. The dependence of the k' values of mono-, di-, and triphosphates on the composition of acetate and phosphate buffer is illustrated in Fig. 3. Optimal separation was obtained when the acetate and phosphate concentrations were at about 1:1 ratio. The effect of acetate and phosphate concentrations on the relative capacity factors of different compounds are compared in Fig. 4. The retentions of UDPG, UDP, and UTP were decreased when either acetate or phosphate concentrations were increased. The difference in the k' values of UDP and UDPG diminished in the presence of elevated phosphate concentration. Phosphate concentration has a more pronounced effect on the k' values of FUMP and FdUMP than has acetate, probably due to the 4-fold difference

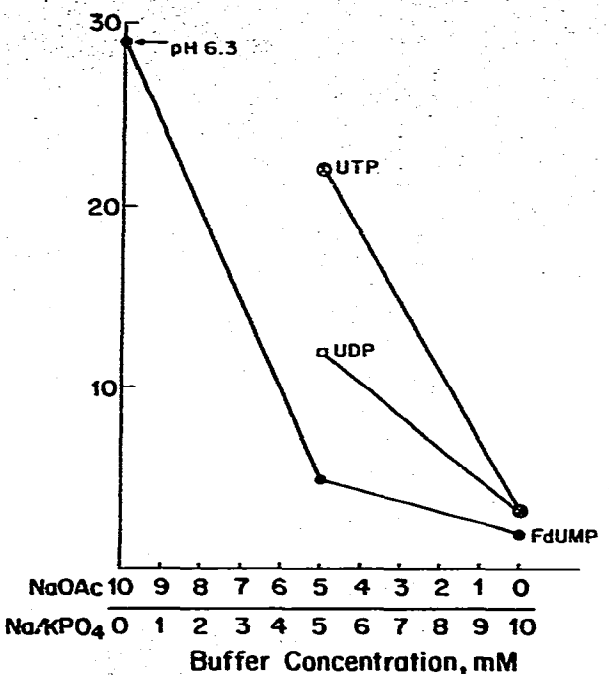

Fig. 3. Effect of altering the composition of sodium/potassium phosphate and sodium acetate buffers on capacity factors, k', of the mono-, di-, and triphosphate nucleotides of uracil of FU. The eluting buffer was 0.1 m*M* C_{16}. The pH was 7 except where noted. The stationary phase was RCM-C_{18}.

in the ionic strength of the two buffers. The phosphate ion may also be more effective in competing with the anionic phosphate portion of the nucleotides from forming ion pairs with the ammonium ions. From Fig. 4, the optimal buffer concentrations for separating the nine compounds are 2—2.5 m*M* for acetate and 1—1.5 m*M* for phosphate.

Effect of chain length of the counter ion

As summarized by Snyder and Kirkland [13], for monovalent anions, R^-_{aq}, which form ion pairs with equal number of moles of counter ions, i.e. $R^-_{aq} + Amm^+_{aq} \rightleftharpoons R^-amm^+_{org}$, their k' values are proportional to the concentration of the counter ion, (Amm^+). Theoretically, up to two, three, and four moles of ammonium ions may be associated with the mono-, di-, and triphosphate nucleotides, respectively. In this case, the interaction between the divalent monophosphate nucleotides can be expressed as $R^{2-}_{aq} + 2\ Amm^+_{aq} \rightleftharpoons R^{2-}Amm^{2+}_{org}$, and their k' values would be proportional to $(Amm^+)^2$. Likewise, k' values of the di- and triphosphate nucleotides would be proportional to $(Amm^+)^3$ and $(Amm^+)^4$, respectively. Thus, the effect of concentration and chain length of counter ion would be most pronounced on the di- and triphosphate nucleotides. C_{16} ions have been used to separate the monophosphates from their nucleosides [10, 11]. By using 0.5 m*M* C_{16} in 10 m*M* sodium acetate buffer (pH 4), a separation of FU, FUR, FUdR, FUMP, 5'-dFUR, and FdUMP was obtained. Their corresponding k' values were 2.4, 4.8, 6.6,

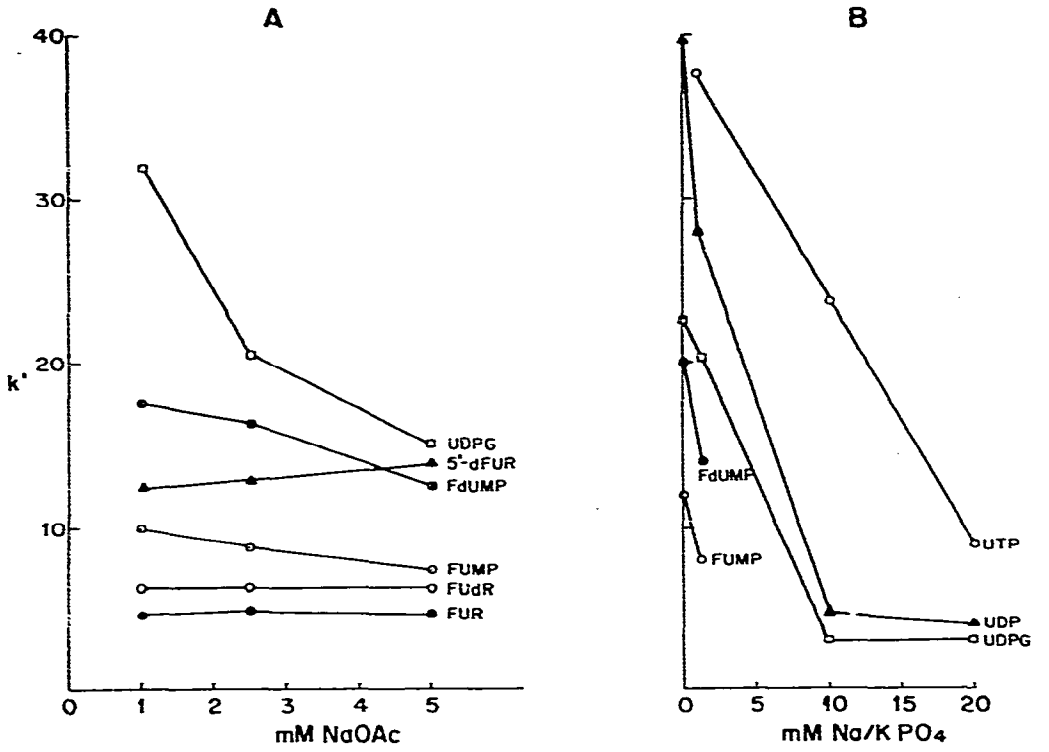

Fig. 4. (A) Effect of sodium acetate concentration on capacity factor, k'. Elution with 0.1 m*M* C_{16} and 2.5 m*M* C_8 in 0.5% methanol. pH 5.3—5.7. (B) Effect of sodium/potassium phosphate concentration on k'. Elution with 0.1 m*M* C_{16} and 2.5 m*M* C_8 in 1% methanol and 2.5 m*M* sodium acetate. pH 6.0—6.4. The stationary phase was μBondapak C_{18}.

10.0, 13.3 and 16.0, respectively. However, the ion pairs of UDPG, UDP, and UTP with C_{16} were strongly retained on the stationary phase. Results of gradient elution of these compounds by increasing the strength of the mobile phase to 50% acetonitrile were irreproducible with large intra-day variation in their k' values, the reason for which is unclear at present. The other disadvantage of gradient elution with increasing mobile phase strength is the lengthy reequilibration, probably due to the wash-off of the C_{16} ions from their adsorption sites on the stationary phase [13].

Three other ammonium ions, C_6, C_8, and C_{12} were evaluated. Since the addition of one —CH_2-group to the counter ion molecule may result in an increase in the k' value of up to 2.5 [13], the retentions of the fluorinated pyrimidine base, nucleosides, and nucleotides by C_6, C_8, and C_{12} ions of appropriate concentrations were investigated and the results are presented in Table II. The nucleotides were only weakly retained by the C_6 ions and were eluted prior to the nucleosides. C_8 and C_{12} were more efficient in retaining the nucleotides with an elution pattern of FU→FUR→FUMP→FUdR→FdUMP →UDPG, as compared to the pattern of FU→FUR→FUdR→FUMP→FdUMP→ UDPG by C_{16}.

Two mechanisms of ion-pair chromatography have been proposed and

TABLE II

EFFECT OF CHAIN LENGTH OF THE AMMONIUM IONS ON CAPACITY FACTORS, k'

The stationary phase was μBondapak C_{18}.

	C_6, 2 m*M* pH 4.2	C_8, 2.5 m*M* pH 3.8	C_{12}, 0.1 m*M* pH 4.9
FU	2	2.2	2.1
FUR	5.3	5.7	4.9
FUMP	4.2	6.6	4.9
FUdR	8.5	8.4	7.3
FdUMP	5.6	11.0	7.3
UDPG	5.0	18.6	not available

reviewed by Knox and Hartwick [16]. One, ion-pair formation may take place between sample ion in the mobile phase and counter ion adsorbed onto the stationary phase; and two, ion-pair formation may take place in the mobile phase and the ion-pair complex partitions into the non-polar stationary phase [16]. These two mechanisms have been studied and discussed using a single counter ion in the mobile phase [13, 16]. In this study, we have used combinations of two ions such as C_6 plus C_{16}, and C_8 plus C_{16}. According to the first mechanism, as long as the adsorption sites on stationary phase are not saturated, an increase in total counter ion concentration in the mobile phase will result in a greater amount of counter ion being adsorbed onto the stationary phase. Consequently, retention of a sample ion will be increased regardless of the chain length of counter ion used in the combination; however, as shown below, this is not the case. Alternatively, according to the second mechanism, the two different counter ions in the mobile phase will be competing to form an ion pair with the sample ion. Consequently, assuming the association of sample anion with ammonium ion is a random process and is not affected by the size of ammonium ion, one would expect that by using a mixture of counter ions of different chain length, the net k' would be an intermediate value of the k' values resulting from using either counter ion alone.

The effect of varying the composition of mixtures of C_6 and C_{16}, and of C_8 and C_{16} are illustrated in Figs. 5 and 6, respectively. The two systems used in studying C_6/C_{16} combination employ two different stationary phases, i.e., μBondapak C_{18} and RCM-C_{18}, and varying C_6 and C_{16} concentrations. Nevertheless, a general trend is observed in that the retention of nucleotides as ion pairs with C_6/C_{16} ions increased with the concentration of the stronger counter ion, C_{16}; the effect is more pronounced on UDP and UDPG than on the monophosphate nucleotides (Fig. 5). When the ratio of C_{16} concentration to C_6 concentration was above 0.2, the k' values of UDP and UTP were greater than 50 whereas FUMP and FdUMP were not resolved from their nucleosides. Thus, the C_6/C_{16} combination was not desirable. In the case of the C_8/C_{16} combination, the composition of eluents varied only in the C_8 concentration, the C_{16} and buffer concentrations as well as the pH were kept constant. As shown in Fig. 6, the retention of FUMP and FdUMP decreased when the C_8 concentration was increased. Melin et al. [17] have shown that the site cover-

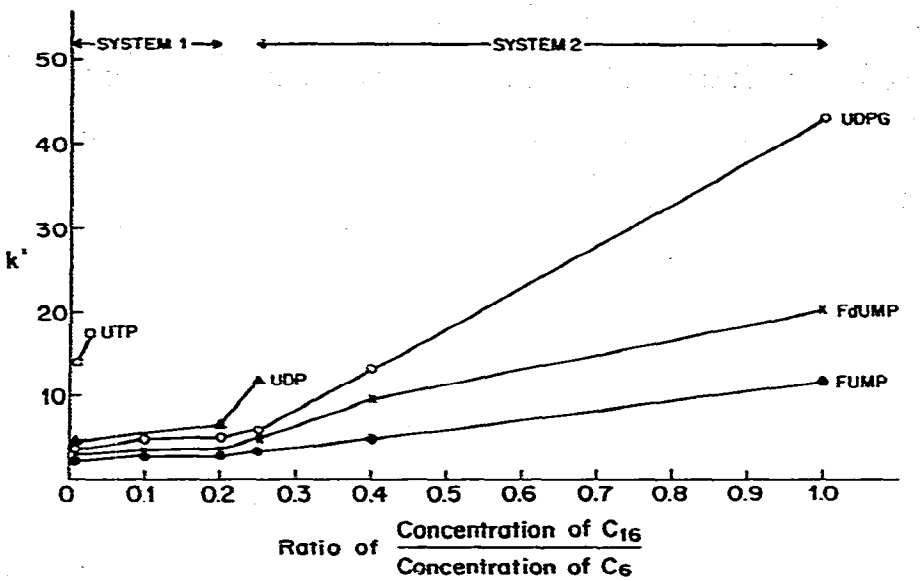

Fig. 5. Effect of composition of mixtures of C_{16}/C_6 ammonium ions on the capacity factors, k'. System 1: 50.0 mM C_6, 0.5—10.0 mM C_{16}, 0.5—1.0 mM phosphate, 10.0 mM sodium acetate, pH 5.5—6.0; System 2: 0.25—0.4 mM C_6, 0.15—0.25 mM C_{16}, 10.0 mM sodium acetate (pH 5.0). The stationary phases were μBondapak C_{18} for system 1 and RCM-C_{18} for system 2.

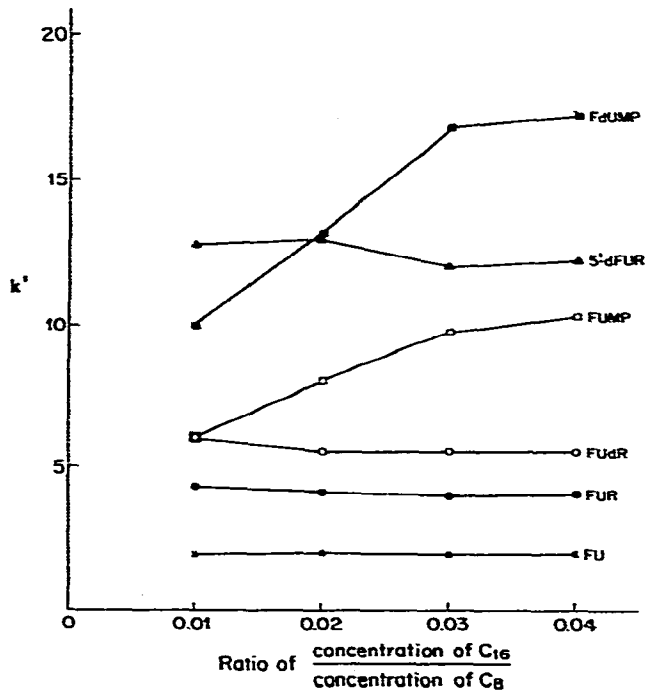

Fig. 6. Effect of composition of mixtures of C_{16}/C_8 ammonium ions on capacity factors, k'. Elution with 0.1 mM C_{16} and 2.5—10.0 mM C_8 in 2.0% methanol and 2.5 mM sodium acetate. pH 5.0. The stationary phase was μBondapak C_{18}.

age of a μBondapak C_{18} stationary phase by 5 m*M* C_{16} was 75%. The C_{16} concentration used here was 0.1 m*M* and would not have saturated the adsorption sites. These data argue against the first mechanism of ion-pair formation between sample ion in mobile phase and the adsorbed counter ion in stationary phase; and are in favor of the second mechanism which describes the partition of already formed ion pair in mobile phase to stationary phase. The resolution of FUMP and FdUMP by C_{16}/C_8 in a ratio of 1:25 was sufficient (Fig. 6) while the retentions of UDPG and UDP were kept minimal.

Optimal conditions

The optimal separation of FU, FUR, FUdR, FUMP, 5′-dFUR, FdUMP, and UDPG is by isocratic elution with 0.1 m*M* C_{16}, 2.5 m*M* C_8, and 2% methanol in 2 m*M* sodium acetate—1.5 m*M* phosphate buffer (pH 6.0); whereas UDP, dUDP, and UTP can be rapidly eluted using a second buffer with an additional 30 m*M* phosphate. This assay can be completed in 40—50 min, the one-step gradient going from the first buffer to the second buffer alleviates the need of second solvent pump. A representative chromatogram is illustrated in Fig. 7. This assay has been applied in comparative metabolism studies of FU and 5′-dFUR in cultured human lymphocytes, mouse leukemia L1210 cells, rat bone marrow cells and transplanted dimethylhydrazine-induced rat colon tumor cells [18].

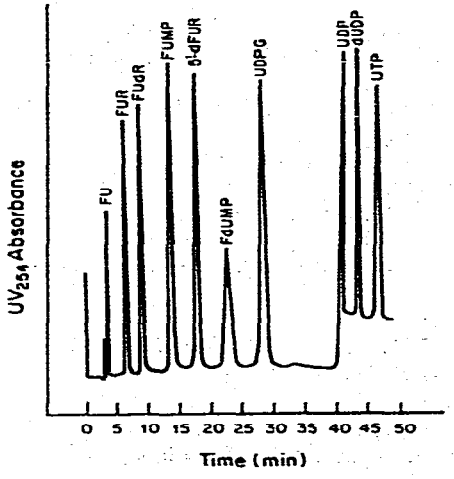

Fig. 7. Separation of authentic chemicals of FU, 5′-dFUR, FU nucleosides and nucleotides, and uracil nucleotides. Eluting buffer from 0 to 30 min: 0.1 m*M* C_{16}, 2.5 m*M* C_8, and 2% methanol in 2 m*M* sodium acetate—1.5 m*M* phosphate buffer (pH 6.0) (A); from 30 to 50 min: A + 30 m*M* phosphate.

CONCLUSIONS

A rapid reversed-phase ion-pair HPLC assay is described here. This assay permits simultaneous separation of the fluoropyrimidine base, nucleosides and nucleotides and is useful in pharmacological studies of biochemical determinants of cytotoxicity of FU and 5′-dFUR. This investigation is not intended

to distinguish the mechanism of ion-pair chromatography, however, data presented in this report suggest the following. First, ion pairs of fluoropyrimidine nucleotides and ammonium ions are formed in mobile phase and are retained by partitioning into stationary phase. Second, in addition to factors such as pH, buffer concentration and solvent strength, retention of sample ion by ion-pair chromatography can be further modified by using mixtures of counter ions of different chain length; this is particularly useful in separation of compounds with similar pK_a values and different number of ionizable sites.

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