Journal of Chromatography, 194 (1980) 305–314
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,765

REVERSED-PHASE LIQUID CHROMATOGRAPHY OF 5-FLUOROURACIL NUCLEOSIDES AND NUCLEOTIDES IN THE PRESENCE OF QUATERNARY AMMONIUM IONS

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(First received November 22nd, 1979; revised manuscript received February 18th, 1980)

SUMMARY

Reversed-phase liquid chromatography is used for the separation of 5-fluorouracil, its deoxyribo- and ribonucleosides and nucleotides. The bases and nucleosides are easily separated from their naturally occurring analogues on an octadecyl silica column eluted with $2 \cdot 10^{-2}$ *M* KH_2PO_4 (pH 5.0) containing 5% (v/v) of methanol. This system can be applied to the measurement of 5-fluoro-2′-deoxyuridine serum levels down to 0.1 μg/ml. Addition of small amounts (10^{-3} *M*) of tetrabutylammonium phosphate to the eluent results in a large retention increase for the nucleotides, while the capacity ratios of the bases and nucleosides remain unchanged. The influence of the tetrabutylammonium phosphate and ammonium phosphate concentrations and pH of the eluent on the various k' values was investigated. Evidence is presented indicating that the quaternary ammonium compound is adsorbed onto the octadecyl silica surface; nucleotides are probably retained as adsorbed tetrabutylammonium ion-pairs.

INTRODUCTION

The biochemical importance of FU* and its nucleosides and nucleotides has been demonstrated and reviewed by Heidelberger[1]. High-performance liquid chromatography has been extensively used for the separation of nucleic acid constituents. Bases and nucleosides have been chromatographed on reversed-phase[2–5] and ion-exchange[6–11] columns, while nucleotide separations are performed mostly by anion-exchange chromatography[12–17]. More recently, the reversed-phase mode has also been applied in this field[18–20], but few systems are available for the simultaneous determination of pyrimidine bases, nucleosides and nucleotides. The introduction of

* The abbreviations used are: FU = 5-fluorouracil; FUDR = 5-fluoro-2′-deoxyuridine; FURD = 5-fluorouridine; 5-FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; URD = uridine; UDR = 2′-deoxyuridine; dUMP = 2′-deoxyuridine 5′-monophosphate; UMP = uridine 5′-monophosphate; THYM = thymidine; TBA = tetrabutylammonium.

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an ion-pairing agent into the reversed-phase eluent provides a possible approach to this problem[19,21–23]. However, the exact retention mechanism of the nucleotides under these chromatographic conditions is not entirely clear[24]. Undoubtedly, this is a complex process and several mechanisms, including ion exchange, ion-pair formation and partition are involved. In this paper, we consider several parameters from a practical point of view, in order to obtain optimal separation of the nucleic acid constituents related to FU when using a reversed-phase chromatographic system.

EXPERIMENTAL

Bases, nucleosides and nucleotides

Pure FU, FUDR, UDR, URD, dUMP, UMP and THYM were obtained from Sigma (St. Louis, MO, U.S.A.); FdUMP was purchased from Collaborative Research Inc. (Waltham, MA, U.S.A.). 1-(2-Deoxy-β-D-lyxofuranosyl)-5-fluorouracil was prepared according to the method of Horwitz *et al.*[25]; FURD was a gift from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.).

Chemicals

Analytical grade KH_2PO_4, $NaHCO_3$, Na_2CO_3, H_3PO_4, KOH, $(NH_4)_2HPO_4$, CH_2Cl_2, CCl_4, acetonitrile, picric acid, acetic acid and methanol were obtained from E. Merck (Darmstadt, G.F.R.). Tetrabutylammonium hydroxide (40%, w/v, aqueous solution) came from Aldrich Europe (Beerse, Belgium) and was converted into the phosphate salt by addition of H_3PO_4. Its TBA concentration was measured by the picrate method[26]. The AG 1-X4 (Cl^-) resin (100–200 mesh) was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Carbonate buffer (0.025 *M*, pH 10) contained 2.1 g $NaHCO_3$ and 2.7 g Na_2CO_3 per litre. Glass-distilled deionized water was used for the preparation of reagents, eluents and standard solutions.

Column

All analyses were performed on a 15 × 0.32 cm column packed with 5-μm RSIL C_{18} HL (octadecyl silica with 18% bonded organic material). The column tubing (Lichroma SS) and reversed-phase packing material were obtained from RSL (St. Martens-Latem, Belgium). A slurry packing method[27] was used under the following conditions: slurry liquid, CCl_4–methanol (9:1, v/v); slurry concentration, 10% (w/v); packing pressure, 200 bar; pressurizing liquid, acetonitrile; pump, Haskel gas-driven Model DSTV-122 (Haskel Engineering and Supply Co., Burbank, CA, U.S.A.).

Apparatus

A Pye Unicam (Cambridge, Great Britain) liquid chromatograph was used, consisting of a Model LC3-XP pump, a LC3 variable-wavelength detector, a PM 8251 100 mV-range recorder and a Valvo CV-6-UHPa-N60 injection valve (Valco Instrument Co., Houston, TX, U.S.A.) with a 50-μl loop. The column was eluted at a flow-rate of 0.8 ml/min at ambient temperature. The detector was operated at 270 nm.

Chromatographic conditions

Standard eluent. $2 \cdot 10^{-2}$ *M* KH_2PO_4 (pH 5.0) containing 5% (v/v) of methanol.

Influence of the eluent TBA concentration. Different amounts of a concentrated $TBAH_2PO_4$ solution (pH 5.0) were added to the standard eluent so as to obtain the following TBA concentrations: $2.5 \cdot 10^{-4}$ *M*, $5.0 \cdot 10^{-4}$ *M*, $10.0 \cdot 10^{-4}$ *M*, $15.0 \cdot 10^{-4}$ *M* and $20.0 \cdot 10^{-4}$ *M*.

Influence of the eluent $NH_4H_2PO_4$ concentration. Different solutions of the standard eluent were prepared containing $20 \cdot 10^{-4}$ *M* of $TBAH_2PO_4$ and following concentrations of $NH_4H_2PO_4$: $10.0 \cdot 10^{-4}$ *M*, $20.0 \cdot 10^{-4}$ *M*, $40.0 \cdot 10^{-4}$ *M*, $60.0 \cdot 10^{-4}$ *M* and $80.0 \cdot 10^{-4}$ *M*.

Influence of the eluent pH. The standard eluent, containing $5.0 \cdot 10^{-4}$ *M* $TBAH_2PO_4$ was adjusted to pH values of 5.0, 6.0, 7.0 and 8.0 by addition of concentrated KOH solution. Bases, nucleosides and nucleotides were then chromatographed using the different eluents described and their respective capacity ratios (k') were calculated.

Adsorption of TBA on the packing material. The standard eluent, containing $20 \cdot 10^{-4}$ *M* $TBAH_2PO_4$, was adjusted to pH values of 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 (prior to the addition of methanol). The reversed-phase column was equilibrated by the passage of 150 ml of solvent at a flow-rate of 1 ml/min. The equilibrated column was then eluted with 100 ml of methanol at the same flow-rate; the effluent was collected and evaporated to dryness on a rotary evaporator. The residue was dissolved in 100.0 ml of phosphate buffer[26] and the TBA concentration was determined by the picrate method. The experiment was repeated for each of the eluent pH values. From these measurements, the amount of TBA adsorbed on the reversed-phase packing can be calculated after subtraction of the amount of TBA present in the column void volume.

Analysis of serum samples supplemented with FUDR

Aliquots of a blank serum pool of human origin were supplemented with FUDR so as to obtain concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 μg/ml. Samples (1 ml) were mixed with 100 μl of internal standard (IS) solution [containing 1.0 mg of 1-(2-deoxy-β-D-lyxofuranosyl)-5-fluorouracil per 100 ml of distilled water], diluted with 5 ml of carbonate buffer (0.025 *M*, pH 10) and applied to a column (2 cm × 6 mm I.D.) of AG 1-X4 (Cl^-) resin. After adsorption of the diluted sample, the column was washed with 10 ml of water and 10 ml of methanol; elution is performed with 10 ml of 0.3 *M* acetic acid in methanol. The collected eluate is evaporated under a stream of nitrogen at 50°C and the residue is dissolved in 0.5 ml of distilled water. A 50-μl aliquot is injected into the chromatographic system described; standard eluent is used. A calibration curve is constructed by plotting the peak height ratios of FUDR to IS *versus* the serum concentration.

RESULTS AND DISCUSSION

Pyrimidine bases and nucleosides related to FU are easily separated on a reversed-phase column using the standard eluent described, as shown in Fig. 1. Structural data for the compounds studied are given in Table I. This chromatographic system also allows the measurement of FUDR serum levels in the 0–1 μg/ml

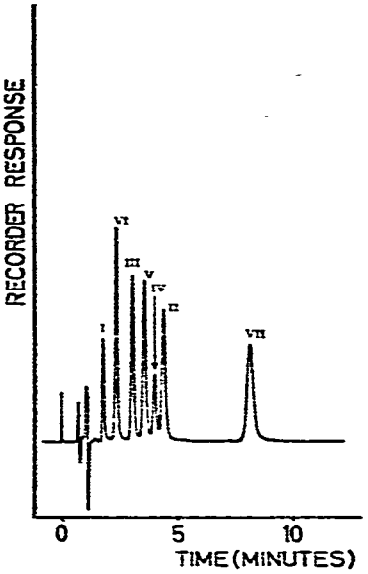
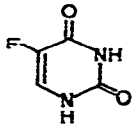


Fig. 1. Separation of FU (I), URD (VI), FURD (III), UDR (V), 1-(2-deoxy-β-D-lyxofuranosyl)-5-fluorouracil (IV), FUDR (II) and THYM (VII). Column: 5 μm RSIL C_{18} HL (15 × 0.32 cm). Eluent: $2 \cdot 10^{-2}$ *M* KH_2PO_4 (pH 5.0) with 5% (v/v) methanol; flow-rate 0.8 ml/min. Sensitivity: 0.040 a.u.f.s.

TABLE I

STRUCTURES OF THE COMPOUNDS STUDIED



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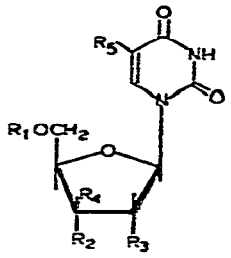
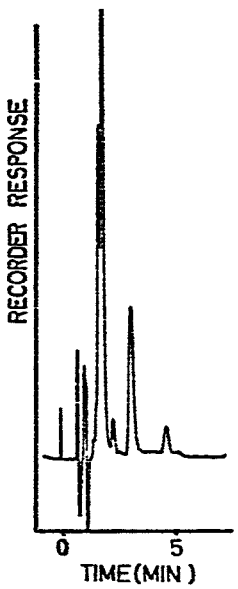


II–X

Compound	R_1	R_2	R_3	R_4	R_5
I 5-Fluorouracil	—	—	—	—	—
II 5-Fluoro-2′-deoxyuridine	H	OH	H	H	F
III 5-Fluorouridine	H	OH	OH	H	F
IV 1(2-Deoxy-β-D-lyxofuranosyl)-5-fluorouracil	H	H	H	OH	F
V 2′-Deoxyuridine	H	OH	H	H	H
VI Uridine	H	OH	OH	H	H
VII Thymidine	H	OH	H	H	CH_3
VIII 5-Fluoro-2′-deoxyuridine 5′-monophosphate	HPO_3Na	OH	H	H	F
IX 2′-Deoxyuridine 5′-monophosphate	HPO_3Na	OH	H	H	H
X Uridine 5′-monophosphate	HPO_3Na	OH	OH	H	H

range, using 1-(2-deoxy-β-D-lyxofuranosyl)-5-fluorouracil, a close structural analogue, as the internal standard (IS) (Figs. 2 and 3). A linear calibration curve is obtained when plotting the peak height ratios of FUDR to IS *versus* the serum concentration ($y = 0.990\,x + 0.014$, $r = 0.999$). The detection limit is 0.1 μg FUDR/ml. More details on the serum extraction procedure are given elsewhere[28].



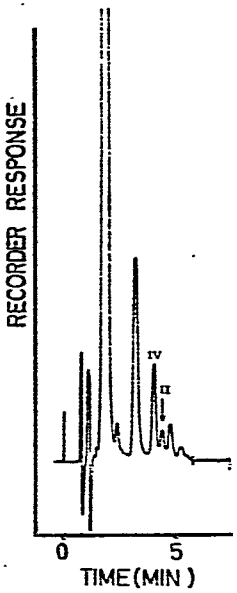


Fig. 2. Chromatogram of a blank serum extract. Conditions as in Fig. 1.

Fig. 3. Chromatogram of an extract of serum supplemented with 1 μg 1-(2-deoxy-β-D-lyxofuranosyl)-5-fluorouracil (IV) and 0.25 μg FUDR (II) per ml. Conditions as in Fig. 1.

Under these chromatographic conditions, the pyrimidine nucleotides elute near the void volume. In order to estimate these products, suitable retention times should be obtained. The addition of tetrabutylammonium phosphate ($TBAH_2PO_4$) to the eluent produces the desired retention increase, as can be seen in Fig. 4. While the k' values for the bases and the nucleosides are independent of the TBA concentration of the eluent, the k' values for the nucleotides seem to reach a maximum. Indeed, at pH 5.0, the bases and nucleosides examined here are present in an undissociated form[29,30] and cannot form ion-pairs with TBA; the 5'-phosphate group of the nucleotides, however, is ionized and can undergo interaction with the quaternary ammonium surfactant. A chromatogram obtained under these conditions illustrates this (Fig. 5). Fig. 6 shows the effect on k' values of adding different concentrations of ammonium phosphate to the eluent. Again, k' values for the nucleosides are unaffected; this allows regulation of the retention of the nucleotides without changing that of the other nucleic acid constituents. The influence of the eluent pH on the retention is illustrated in Fig. 7; a drastic decrease in the k' values for both nucleotides is observed.

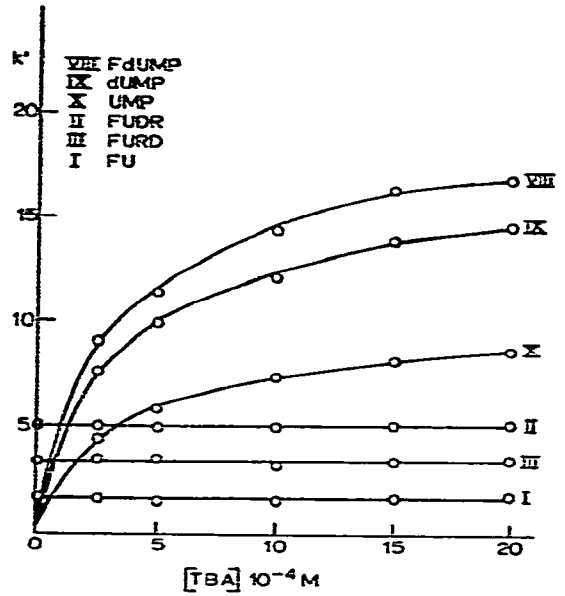


Fig. 4. Influence of the eluent TBA concentration on k' values.

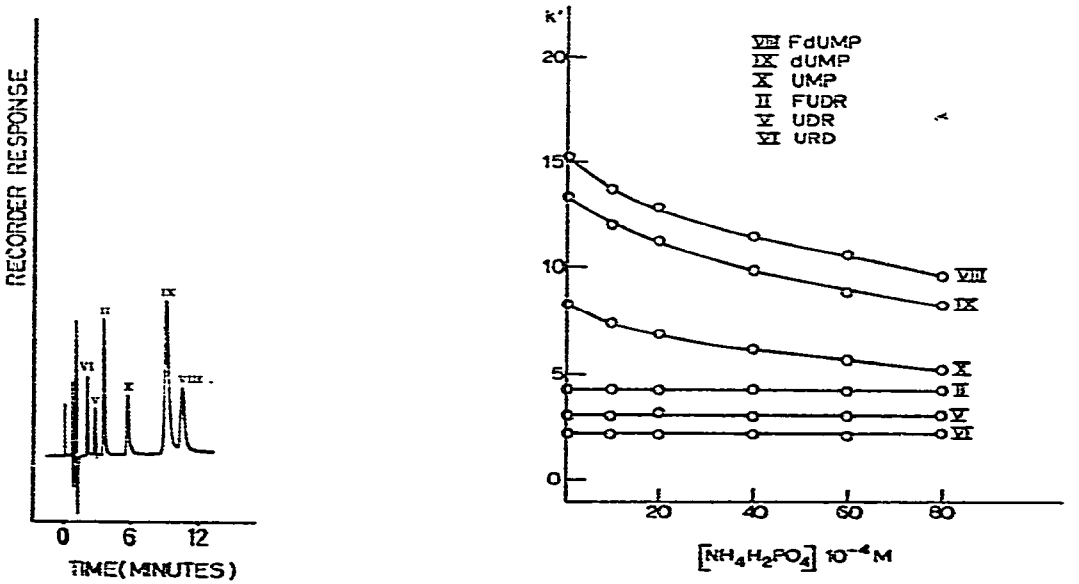


Fig. 5. Separation of URD (VI), UDR (V), FUDR (II), UMP (X), dUMP (IX) and FdUMP (VIII). Column: 5 μm RSIL C_{18} HL (15 × 0.32 cm). Eluent: $2 \cdot 10^{-2}$ *M* KH_2PO_4, $15 \cdot 10^{-4}$ *M* $TBAH_2PO_4$ (pH 5.0) with 5% (v/v) methanol; flow-rate 0.8 ml/min. Sensitivity: 0.040 a.u.f.s.

Fig. 6. Influence of the eluent $NH_4H_2PO_4$ concentration on k' values.

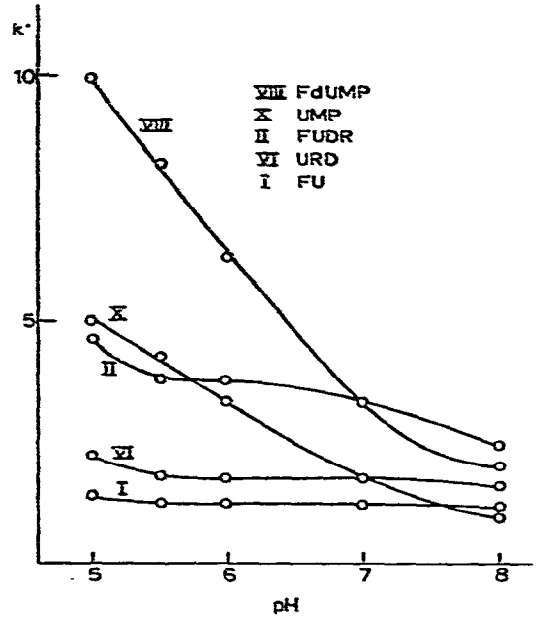


Fig. 7. Influence of the eluent pH on k' values.

From our experiments, it is clear that TBA is adsorbed onto the octadecyl silica surface; this phenomenon has been observed by several workers with surfactant ions in reversed-phase chromatography[19,24,31,32]. Moreover, a linear relationship seems to exist between the amount of TBA adsorbed (mmol $\times 10^{-2}$, y) and the pH of the eluent (x) : $y = 0.81x + 0.86$; $r = 0.997$ (Fig. 8). Thus, at pH 5.0, $5 \cdot 10^{-2}$ mmol of TBA were found to be adsorbed. This is in agreement with the experimental results obtained by Melin *et al.*[33] and suggests the presence of an ionic interaction between the reversed-phase packing material and the quaternary ammonium compound. This effect might be due to the fact that the solid phase contains residual silanol groups with different acidic properties, which can dissociate at higher pH[34,35] and thus provide an increased adsorbing capacity for quaternary ammonium ions. Indeed, for these hydroxyl groups, pK_a values as high as 7.1[36] have been reported; thus the total cation-adsorbing capacity would only be available at higher pH values ($\geqslant 9$).

Several chromatographic models[31,32,37,38] have been proposed, based upon the hypothesis that the octadecyl silica-adsorbed TBA acts as a quaternary ammonium-type anion exchanger, together with the possibility of interactions between the solute and surfactant micelles[31,37,39] in the mobile phase.

However, our experimental results seem to fit well into the retention model proposed by Melin *et al.*[33], assuming the distribution of ion-pairs between the mobile and stationary phases. These considerations have led to the following equation[33] for the capacity ratio, k'_X, of X^-

$$k'_X = \frac{q \cdot K_0 \cdot K_{QX} \cdot [Q^+]_m}{1 + K_{QZ} \cdot [Q^+]_m \cdot [Z^-]_m} \quad (1)$$

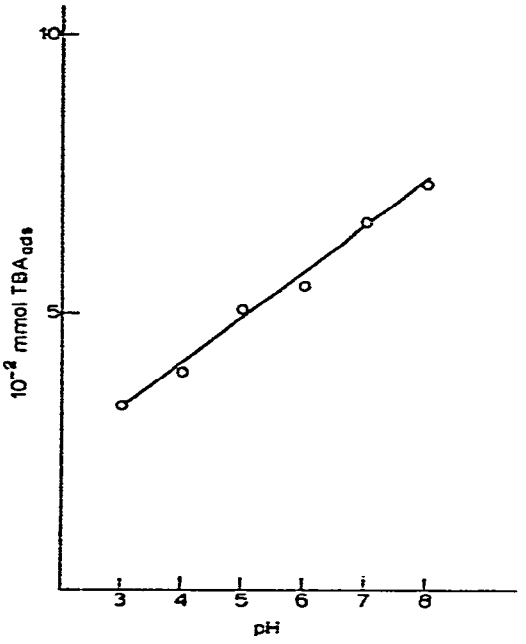


Fig. 8. Adsorption of TBA on the RSIL C_{18} HL material as a function of pH.

wherein q = the ratio of solid to mobile phase in the column, K_0 = maximum number of ion-pairs that can be adsorbed per gram of packing material (monolayer capacity), $[Q^+]_m$, $[Z^-]_m$ = tetrabutylammonium and total anion concentrations in the mobile phase, X^- = solute (nucleotide) anion and K_{QX}, K_{QZ} = equilibrium constants for the ion-pair distribution of X^- and Z^- (buffer anions) between the mobile phase and the adsorption site, A_s:

$$Q^+_m + X^-_m + A_s \rightleftarrows QXA_s$$

$$Q^+_m + Z^-_m + A_s \rightleftarrows QZA_s$$

Provided that QZ and QX occupy the same area on the solid phase surface, K_0 can be written as:

$$K_0 = [A]_s + [QXA]_s + [QZA]_s$$

and is a function of the pH and methanol concentration of the eluent[33]. Eqn. 1 can be rearranged to:

$$\frac{1}{k' \, [Z^-]_m} = \frac{1}{q \cdot K_0 \cdot K_{QX} \cdot [Q^+]_m \cdot [Z^-]_m} + \frac{K_{QZ}}{q \cdot K_0 \cdot K_{QX}} \qquad (2)$$

Thus, a plot of $1/k'_X \cdot [Z^-]_m$ *vs.* $1/[Q^+]_m \cdot [Z^-]_m$ should be linear, as illustrated in Fig. 9. Analogously, straight lines were obtained when plotting $1/k'_X$ *vs.* $[Z^-]_m$ at constant $[Q^+]_m$ values.

These plots confirm the validity of the assumption made[33]. The retention of

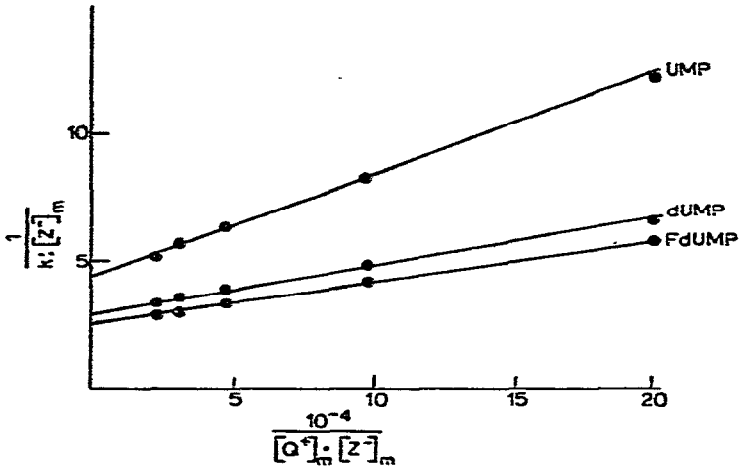


Fig. 9. Retention of UMP (X), dUMP (IX) and FdUMP (VIII) as tetrabutylammonium ion-pairs. Eluent: $TBAH_2PO_4$ in $2 \cdot 10^{-2}$ *M* KH_2PO_4 (pH 5.0) with 5% (v/v) methanol.

the nucleotides can be regulated by adjusting the mobile phase concentrations of Q^+ or an ion of the same charge, Z^- (phosphate in the present experiments).

The influence of the pH of the eluent on the different k' values is more difficult to explain and illustrates the complexity of the system. However, the following mechanism may be considered. As is evident from Fig. 8, TBA adsorption onto the octadecyl silica surface increases with increasing eluent pH. Due to this extensive adsorption of TBA ion-pairs of the eluent buffer, the capacity of the retaining phase to take up sample (nucleotide) ion-pairs decreases[33], since the total number of adsorption sites (A_s) is limited. This effect is also noticeable in Fig. 4, as the curves show a tendency to flatten out at higher TBA concentrations. Additionally, the 5'-phosphate group of the nucleotides is further ionized at higher pH values, which makes retention of the molecule by a normal reversed-phase mechanism unlikely. These combined effects may at least partially account for the low k' values of the nucleotides at higher eluent pH values. In order to clarify this matter, more thorough experimental work would be needed.

CONCLUSION

Good separation of pyrimidine bases, nucleosides and nucleotides related to FU is obtained, using a reversed-phase column eluted with phosphate buffer containing tetrabutylammonium phosphate; the selectivity of the system can be adjusted in various ways. The retention mechanism is probably based on the adsorption of the different ion-pairs on the octadecyl silica material.

ACKNOWLEDGEMENTS

The authors are indebted to Professor Dr. G. Schill and Dr. K. G. Wahlund for useful discussions. This work was supported in part by the FGWO through grants Nos. 20452 and 3.004.76.

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