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ION-PAIR CHROMATOGRAPHY OF SELECTED NUCLEOSIDES, BASES AND OTHER LOW-MOLECULAR-WEIGHT ULTRAVIOLET ABSORBING COMPOUNDS

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SUMMARY

Chromatographic conditions used in the profiling of nucleosides and bases in physiological fluids by ion-pair chromatography were invstigated. The parameters examined included type, concentration and size of counter-ion, pH of the mobile phase, temperature, percent organic modifier in the mobile phase and the alkyl chain length of the stationary phase. The compounds studied were creatinine, hypoxanthine, orotidine, L-tyrosine, uracil, uric acid, uridine monophosphate and xanthine. It was found that the quaternary amines were better pairing agents than the sulfonic acids for varying the retention of these compounds and improving the resolution of the chromatogram over the first several minutes. The optimum set of conditions for retention was a mobile phase of 1 m*M* tetrabutylammonium phosphate in 20 m*M* phosphate buffer, adjusted to pH 5.7, at a temperature 40°C and with a C_8 stationary phase. Under these conditions the first segment of the chromatogram of plasma samples was simplified because the retention time of uric acid was increased; thus, it is now possible to determine the compounds present in the first part of the chromatogram which are normally masked by the uric acid peak. Conditions are also presented for the improved separation of hypoxanthine, xanthine and uridine. Finally, evidence is given to support the ion-exchange mechanism for retention of the solutes.

INTRODUCTION

In earlier research conducted in our laboratory on the high-performance liquid chromatographic (HPLC) profiling of the nucleosides, bases and amino acids in serum and/or plasma, noticeable differences were detected between the profiles of normal and diseased individuals [1, 2]. These differences were both qualitative and quantitative in nature. For example, breast cancer patients could be differentiated from the normal subjects and patients with benign fibrocystic changes by the presence of 1-methylinosine and N-2-methyl-

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guanosine [1]; adenosine and elevated levels of several other nucleosides were determined in the plasma of acute lymphocytic leukemics [2]. In a chemometric approach to classifying the chromatographic profiles of acute and chronic leukemic and normal patients by multivariant linear analysis [3] and pattern recognition techniques [4], it was further demonstrated that the earliest time segment was the most important in discriminating between the diseased and normal groups. This time interval is composed of weakly retained compounds (i.e. L-tyrosine and uric acid) or ionic, polar or other compounds which are not retained under reversed-phase conditions. In another area of research in which the authors have been active, the nucleoside and base profiling of Australian marsupials for taxonomical and phylogenetic purposes [5], this quantitative difference in the beginning portion of the chromatogram again surfaced as an important piece of data in determining the individual groups.

Ion-pair chromatography (IPC) is a technique which by modification of the partitioning process permits the simultaneous separation of ionic as well as non-ionic compounds [6, 7]. By the addition of a pairing agent to the mobile phase, ionic sample components combine with the counter-ion of the pairing agent to form neutral ion-pairs which are retained by the chromatographic system. Theoretically neutral sample components are unaffected by the pairing agent. It is believed that if IPC can be applied to the chromatographic systems utilized above, the components of the earliest time segment may be separated and further identified, providing a better understanding of the biochemistry and physiology involved.

In this report, results are presented on the reversed-phase, ion-pair investigation of a series of standards, some of which are known to be present in the beginning segment of the chromatograms. The variables of IPC examined include the type of pairing agent (i.e. chain length), concentration of the pairing agent, pH of the mobile phase, percent organic modifier, hydrophobic chain length of stationary phase and temperature.

EXPERIMENTAL

Instrumentation

A Series 3B liquid chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a Rheodyne Model 7125 injector, LC-75 spectrophotometric detector, LC-15 fixed-wavelength (254 nm) detector and LC-100 column oven was used. A dual-channel Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.) was used to record the 280- and 254-nm detector signals. Retention times and peak areas were obtained in earlier work using the Sigma 10 Data Station (Perkin-Elmer) and later with the Model 3600 Chromatographics Intelligent Terminal (Perkin-Elmer).

Columns and chromatographic conditions

An analytical C_{18} (Perkin-Elmer) chemically bonded, 10-μm column (25 cm $\times$ 4.6 mm) was used. A pre-column (5 cm $\times$ 4.6 mm) tap-packed with pellicular, 30—38 μm, reversed-phase material (Whatman, Clifton, NJ, U.S.A.) was used to guard and prolong the life of the main column. The dead time of

the system was determined by the injection of 5 μl of a 3 *M* potassium chloride solution with double-distilled, deionized water as the mobile phase. For ten replicate injections an average value of 2.17 min with a standard deviation of zero was measured for the dead time. In order to study the effect of stationary-phase chain length, a Partisil PXS 10/25 C_8 column (25 cm × 4.6 mm, Whatman) was used for one data set.

The mobile phase in all of the experiments was comprised of 0.02 *M* potassium dihydrogen phosphate, HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.), plus some concentration of pairing agent, prepared by dissolving the anhydrous powder in double-distilled, deionized water and adjusting the pH either with phosphoric acid or potassium hydroxide. Prior to use, the eluent was filtered by passage through 0.45 μm filters (Millipore, Bedford, MA, U.S.A.) and helium-degassed. Column equilibration with the desired mobile phase occurred overnight at low flow-rates. All analyses were performed at a flow-rate of 1.5 ml/min and unless specified, at ambient temperature.

Chemicals

Pairing agents were purchased from various vendors. 1-Hexanesulfonic acid, 1-octanesulfonic acid and tetrabutylammonium phosphate were obtained from Eastman-Kodak (Rochester, NY, U.S.A.); tetramethylammonium hydroxide and tetraethylammonium hydroxide were from Aldrich (Milwaukee, WI, U.S.A.); 1-dodecanesulfonate was bought from Altex, a division of Beckman Instruments (Berkeley, CA, U.S.A.) and 1-heptanesulfonic acid from Fisher Scientific. All standard reference compounds were purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions were prepared in double-distilled, deionized water.

TABLE I

COMPOUNDS INVESTIGATED, THEIR ABBREVIATIONS AND THEIR ACID DISSOCIATION (p*K*) CONSTANTS*

Compound	p*K*
Creatinine (Cre)	3.6
Hypoxanthine (Hyp)	8.9, 12.1
Orotidine (Ort)	—
L-Tyrosine (Tyr)	2.20, 9.11, 10.13
Uracil (Ura)	9.5, >13
Uric acid (UA)**	5.57, 10.3
Uridine (Urd)	9.2, 12.5
Uridine monophosphate (UMP)	1, 6, 9.5
Xanthine (Xan)	7.5, 11.1

*Values from H.A. Sober (Editor), CRC Handbook of Biochemistry, The Chemical Rubber Co., Cleveland, OH, 2nd ed. 1970.

**Values from D.S. Newcombe, Inherited Biochemical Disorders and Uric Acid Metabolism, University Park Press, Baltimore, MD, 1975.

RESULTS

Since the identity of the constituents of the early portion of the chromatogram are unknown, we have selected for this investigation compounds encompassing negatively charged, neutral and positively charged species which are thought to be present in blood fluids. The standard compounds selected include creatinine (Cre), hypoxanthine (Hyp), orotidine (Ort), L-tyrosine (Tyr), uracil (Ura), uric acid (UA), uridine (Urd), uridine monophosphate (UMP) and xanthine (Xan). Table I lists these compounds and their acid dissociation constants; Fig. 1 depicts their structures. UA, Tyr and Cre are known to be constitutents in the early time segment of the chromatogram; Ura and Ort are components which might be present. Hyp, Urd and Xan were included

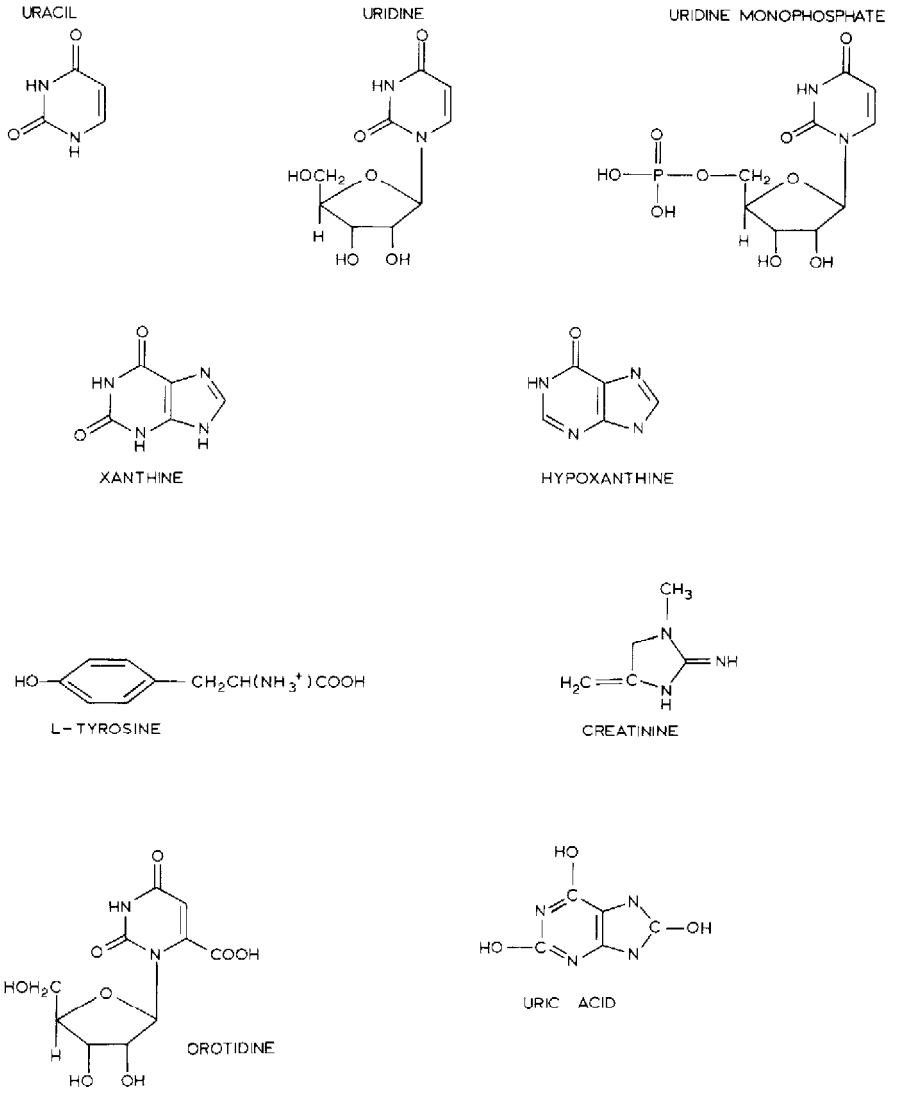

Fig. 1. Structures of the compounds investigated.

because of their importance in clinical manifestations and the inability to resolve readily all three of these components using the present chromatographic conditions. UMP was chosen as a model for any nucleotides which might exist in the physiological fluids.

Generally for negatively charged entities, quarternary amines are used as the pairing agent, while for positively charged components, alkyl sulfonates are employed. Because of the uncertainty as to the chemical composition of the early time segment, both classes of pairing agents have been investigated. Table II lists the pairing agents, their molecular formulae, and the abbreviations we shall use.

TABLE II

ION-PAIR REAGENTS INVESTIGATED

Quaternary amines	
Tetramethylammonium hydroxide pentahydrate (TMA)	$(CH_3)_4NOH\ 5H_2O$
Tetraethylammonium hydroxide (TEA)	$(C_2H_5)_4NOH$
Tetrabutylammonium phosphate (TBA)	$(C_4H_9)_4NH_2PO_4$
Sulfonates	
1-Hexanesulfonic acid, sodium salt (HxSA)	$CH_3(CH_2)_5SO_3Na$
1-Heptanesulfonic acid, sodium salt (HpSA)	$CH_3(CH_2)_6SO_3Na$
1-Octanesulfonic acid, sodium salt (OSA)	$CH_3(CH_2)_7SO_3Na$
1-Dodecanesulfonate, sodium salt (DDS)	$CH_3(CH_2)_{11}SO_3Na$

Effect of the pairing agent

In order to determine the effects of the variables in IPC, we have selected a mobile phase of 0.02 *M* phosphate buffer adjusted to pH 5.7 as our reference point. In an investigation of the pairing agent, there are three important parameters: the type of pairing agent itself (acidic or basic), its chain length, and its concentration. Each of the pairing agents listed in Table II was investigated over a concentration range of several orders of magnitude, typically 0.01 to 10 m*M*. Experimentally, the acidic pairing agent, tetrabutylammonium phosphate (TBA), produced the most significant changes (Fig. 2). The chain length of the alkyl portion of the pairing agent was found to be very critical. For example, the retention times are virtually unaffected by the addition of the quaternary amines, tetramethylammonium hydroxide pentahydrate (TMA) and tetraethylammonium hydroxide (TEA); however, large shifts occur when TBA is used for UMP, Ort and UA which have negative charges at pH 5.7 (Table I). Cre, on the other hand, which has a slight positive charge, is repelled by TBA leading to a decrease in retention. It is interesting to note that neutral species also decrease in retention at the higher concentrations of TBA. For the majority of standard compounds in this study, the sulfonates are not particularly useful. With the sulfonates the retention is longest with 1-heptanesulfonic acid sodium salt (HpSA) and decreases with acids of chain length to either side of it. Ort and UMP have retention times in the presence of the sulfonates shorter than the dead time (2.17 min) of the system. This observation indicates ionic repulsion within the column. Since these compounds have negative charges under the conditions employed, this

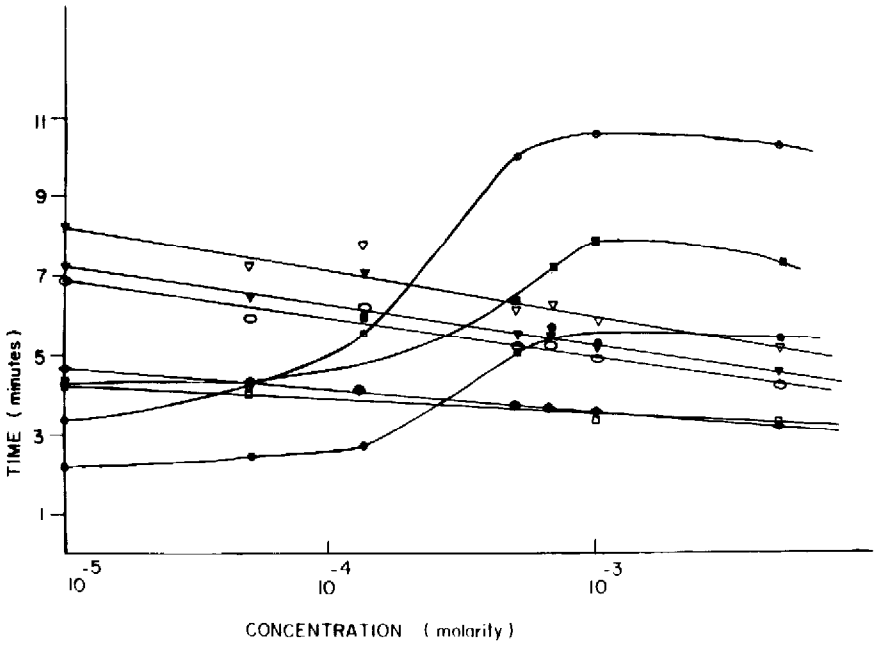

Fig. 2. Effect of concentration of TBA on retention time. Detection, 254 nm; column, analytical C_{18}; guard column, Co-Pell ODS; program, isocratic; eluent, 0.02 *M* monobasic phosphate buffer + pairing agent, adjusted to pH 5.7; flow-rate, 1.5 ml/min; temperature, ambient. Symbols: (♦) creatinine; (▾) hypoxanthine; (●) orotidine; (◇) L-tyrosine; (□) uracil; (■) uric acid; (○) uridine; (○) uridine monophosphate; (▽) xanthine.

type of evidence has been used in support of the ion-exchange mechanism to explain the retention behavior in ion-pairing [8—10]. In this hypothesis the ion-pair reagent coats the stationary phase forming a dynamic ion-exchanger. As a result, the stationary phase now has a charge associated with it; components of similar charge to the stationary phase are repelled by the stationary phase and hence elute from the column faster. The observed decrease in retention of the neutral species can also be explained by this coating action, which would cover some of the sites normally available for partitioning of these compounds [11]. Further evidence for alteration of the stationary phase is seen by the very rapid decrease in retention of all components with the increasing concentration of the longest alkyl chain pairing agent, dodecanesulfonic acid. From an exprimental standpoint, the large volume of mobile phase (typically several hundred milliliters) necessary before equilibration was obtained and the large volume of water necessary to remove the pairing agent and restore the column to its previous conditions, also supports the dynamic ion-exchange mechanism.

Brown et al. [12] reported a reversed-phase IPC separation of Hyp, Xan and UA using heptanesulfonic acid; however, Urd was not investigated. Similarly, Popovich [13] separated Ura and Xan with a mobile phase containing HpSA, but did not examine the possibility of interferences from Urd or Hyp. During our investigation of the sulfonic acids, improved resolution of Hyp, Xan and Urd was noted. With a mobile phase of 0.02 *M* phosphate buffer, pH 5.7, these three compounds were not resolved; however, upon the addition of either

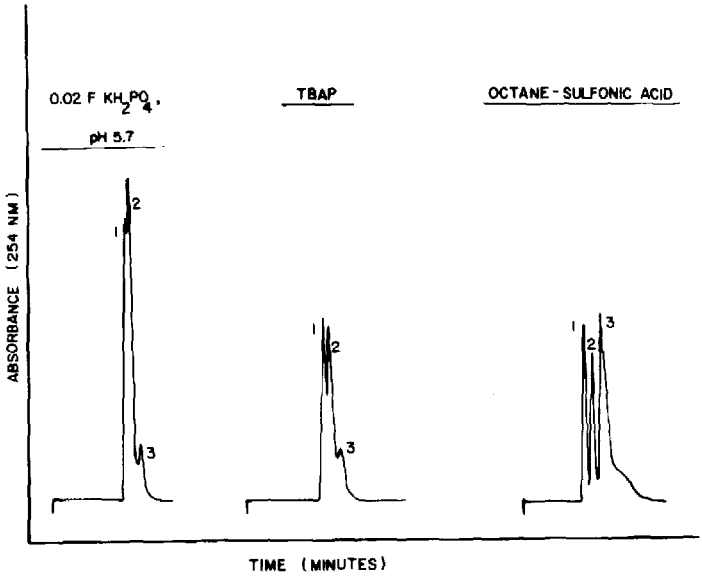

Fig. 3. Separation of uridine (1), hypoxanthine (2) and xanthine (3) with different mobile phases adjusted to pH 5.7: 0.02 *M* monobasic phosphate buffer (left), 0.02 *M* monobasic phosphate buffer + 0.001 *M* TBA (middle), and 0.02 *M* monobasic phosphate buffer + 0.05 m*M* OSA (right). Remaining chromatographic conditions are as in Fig. 2.

hexane-, heptane- or octanesulfonic acid, baseline resolution was achieved (Fig. 3).

Although the retention times of Hyp, Xan and Urd either do not change or change by less than 0.5 min, baseline resolution is obtained. Of the three pairing agents, the heptane- and octanesulfonic acids reduced the peak tailing of xanthine. Since lower concentrations of octanesulfonic acid are necessary to improve the separation as compared to the other sulfonic acids, it is the preferred pairing agent. This improved separation does not occur with the quarternary amines. It is difficult to understand why improved resolution of Hyp, Urd and Xan is obtained with the sulfonic acids. Hyp and Xan are purines differing by a carbonyl group at the 2-position (Fig. 1), while Urd is a dioxopyrimidine with a ribose group at the 9-position. Xan has a lower acid dissociation constant than Hyp and Urd; however, at the pH of the mobile phase, 5.7, this is not a significant factor. It is possible that these compounds are undergoing lactam—lactim tautomerism and that the lactim has some affinity for the pairing agent [14]. Another explanation though, may be that the pairing agent is disrupting the base stacking between the Hyp, Xan and Urd [14]. Because of the high concentrations of these compounds in the standard solutions, it is possible that heterogeneous or homogeneous base stacking occurs.

Effect of pH

Since it was determined that a 1 m*M* solution of TBA in 0.02 *M* phosphate buffer, pH 5.7, produced the most significant changes in retention times, in all subsequent discussions, this mobile phase will be used as the basis for comparison. Table III summarizes the changes in retention times with the mobile phase from pH 5.7 to 3.0, pH values higher than 7 were not investigated

TABLE III

EFFECT OF pH OF THE MOBILE PHASE ON RETENTION TIME (min)

Values listed are the average of three injections.

Compound	pH	
	3.0	5.7
Creatinine	1.92	3.44
Hypoxanthine	6.20	5.12
Orotidine	14.43	5.26
L-Tyrosine	5.00	4.38
Uracil	4.09	3.47
Uric acid	6.16	7.76
Uridine	6.74	4.78
Uridine monophosphate	22.40	10.48
Xanthine	7.24	5.72

because of silica-based column limitations. Cre has a pK of 3.6; UA has a pK value for the 9-position of 5.57. The decreased retention of Cre at pH 3 is thought to be caused by charge repulsion between Cre and TBA. However, at a mobile-phase pH of 5.7, Cre is a neutral molecule, therefore this charge repulsion is no longer a factor and increased retention is observed. The effect of pH on UA is difficult to understand. UA has a greater negative-charge density at pH 3 than at pH 5.7; hence ion-pairing should occur more efficiently at pH 3 and increased retention should result. This is not observed. At pH 5.7 UA is predominately non-ionic and it is thought that the hydrophobic interactions of reversed-phase chromatography (RPC) may dominate. A marked decrease in retention is observed for Ort and UMP from pH 5.7 to pH 3. For UMP at a pH of 3 the phosphate moiety undergoes primary dissociation; at pH 5.7 secondary dissociation of this phosphate group occurs yielding a more negatively charged molecule which should exhibit greater affinity for the cationic TBA. Thus the retention time should increase; however, this was not observed. Gelijkens and De Leenheer [15] have also reported a decrease in the capacity factor of UMP with increasing pH of the mobile phase. The retention times of Hyp, Tyr, Ura, Urd and Xan are slightly longer with a mobile phase of pH 5.7. This trend has been reported in the literature for these compounds for the pH range 2.2—6.3 [16]; however, we do not have an explanation for this change in retention.

Effect of temperature

The effect of temperature on retention time is plotted in Fig. 4. For Ort, UA and UMP, the compounds which ion-pair with the TBA, a maximum in retention is observed at 40°C followed by a decrease with further increase in temperature. For the remaining six compounds a slow decrease is observed over the entire temperature range investigated which is typical behavior for a reversed-phase system. Although Cre and Urd were not included in Fig. 4, the trends observed are similar to those for Hyp, Tyr, Ura and Xan. Therefore, it is evident that control of temperature may aid in specific ion-pair separations.

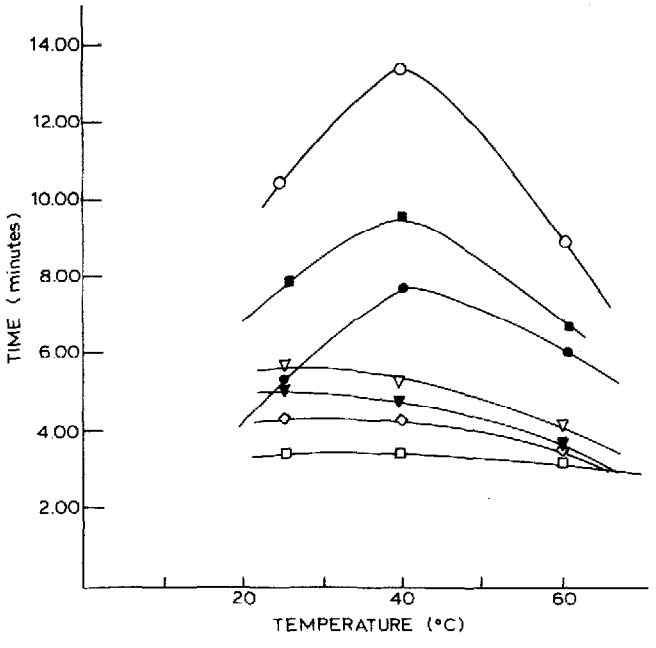

Fig. 4. Effect of temperature on retention time (min). Eluent, 0.001 *M* TBA in 0.02 *M* monobasic phosphate buffer adjusted to pH 5.7. Remaining chromatographic conditions and symbols are as in Fig. 2.

Effect of percent organic modifier

Generally, the addition of an organic modifier, such as methanol or acetonitrile, to a reversed-phase system decreases the retention of the solutes. In order to determine the effects of an organic modifier on ion-pairing, a mobile phase of 0.001 *M* TBA in 0.02 *M* potassium phosphate, pH 5.7, in which there was 5% methanol, was investigated. The retention times of solutes using a mobile phase with the methanol were compared to the retention times obtained without methanol (Table IV). It was observed that the retention times

TABLE IV

EFFECT OF AN ORGANIC MODIFIER IN THE MOBILE PHASE ON RETENTION TIME (min)

Values are the average of three injections.

Compound	No methanol	5% Methanol
Creatinine	3.44	3.88
Hypoxanthine	5.12	4.54
Orotidine	5.26	5.03
L-Tyrosine	4.38	3.94
Uracil	3.47	3.38
Uric acid	7.76	5.56
Uridine	4.78	4.17
Uridine monophosphate	10.48	7.61
Xanthine	5.72	4.98

of all the compounds evaluated decreased in the presence of methanol. This effect was more dramatic with the compounds UA and UMP, which ion-pair to the greatest extent. This effect has been noted in the literature for a variety of compounds [11, 17—20] and has been attributed to a decrease of the surface concentration of the counter-ion because of the competition by the cosolvent.

Effect of the stationary phase

The alkyl chain length of the stationary phase can be an important parameter in IPC optimization. Retention data were obtained for a C_8 and a C_{18} column (Table V). Retention for the neutral compounds is not significantly different between the two columns; however, the retention of Ort, UA and UMP is much longer with the C_8 versus the C_{18} column. This observation is difficult to interpret. The retention behavior of the compounds examined suggests an ion-exchange mechanism. Thus, adsorption of the counter-ion onto the stationary phase prior to ion-pair formation is postulated. This adsorption can occur at two sites, either onto the hydrophobic portion or any unreacted silanol groups present. If the adsorption were on the hydrophobic portion, one would expect to see little difference between the C_8 and C_{18} columns. However, if the adsorption is at the unreacted silanols more counter-ion could be adsorbed on the C_8 column since the C_8 chains are less bulky than the C_{18} chains; hence, increased retention would be expected. This line of reasoning though, must be applied cautiously. Two columns from two different manufacturers were used in this investigation. Therefore, the extent of capping of the unreacted silanols and the percent carbon loading of the C_8 or C_{18} onto the silica backbone may vary, which may contribute to the large change in retention times.

TABLE V

EFFECT OF THE STATIONARY PHASE ON RETENTION TIME (min)

Values are the average of three injections.

Compound	Column	
	C_8	C_{18}
Creatinine	3.38	3.44
Hypoxanthine	5.82	5.12
Orotidine	10.92	5.26
L-Tyrosine	4.94	4.38
Uracil	3.92	3.47
Uric acid	11.25	7.76
Uridine	6.15	4.78
Uridine monophosphate	21.09	10.48
Xanthine	6.63	5.72

Application

The optimum conditions for improving the separation of the early eluting compounds in plasma chromatograms are achieved by a reversed-phase system with a mobile phase of 1 m*M* TBA and 20 m*M* phosphate buffer, adjusted to

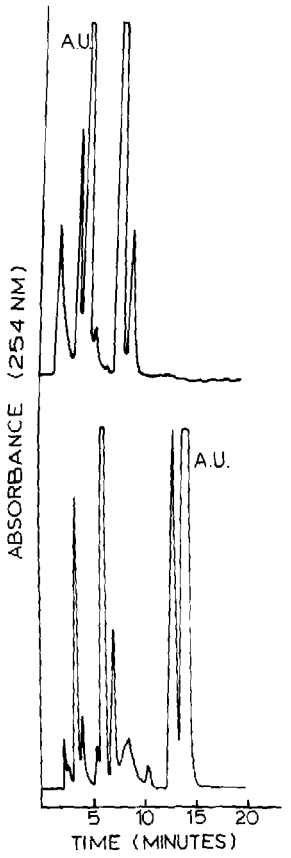

Fig. 5. A 40-μl sample of plasma from a normal individual chromatographed using a mobile phase, 0.02 *M* monobasic phosphate buffer, pH 5.7, without (top) and with (bottom) a pairing agent, 0.001 *M* TBA. Remaining chromatographic conditions are as in Fig. 2.

pH 5.7. The profiles of the plasma of a normal individual under chromatographic conditions with and without the pairing agent are shown in Fig. 5. Two changes are evident. First, the retention of uric acid and some other component not yet identified, increased. The removal of uric acid, which is present in such large amounts, greatly simplified the early part of the chromatogram. Second, decreased retention of the neutral compounds, Hyp, Urd and Xan, was observed. Both these observations were anticipated from the study on the standards. No other components were shifted; therefore, the components still present in this segment must be polar rather than ionic.

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