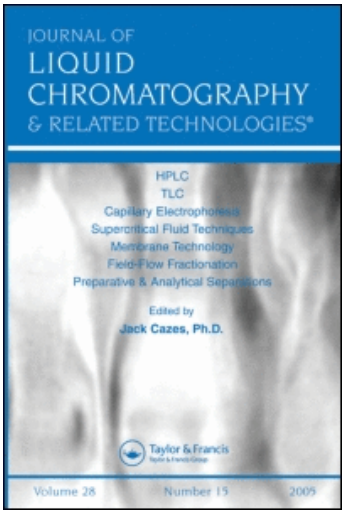

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INVESTIGATION OF THE BEHAVIOR OF OXIDIZED PTERINS IN LIQUID CHROMATOGRAPHIC SYSTEMS

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ABSTRACT

Cation-exchange, reverse-phase, and "ion-pair" reverse-phase chromatography were evaluated for the separation of several oxidized pterins. Chromatographic parameters effecting the retention of the pterins in these chromatographic systems were investigated. "Ion-pair" reverse-phase chromatography was found to be the most satisfactory system and a succesful separation using this system was developed.

INTRODUCTION

The pterins are a family of compounds of biological interest. A reduced pterin is a required cofactor for the enzymatic hydroxylation of phenylalanine, tyrosine, and tryptophan (1). Abnormal pterin concentrations have been associated with several diseases, including; phenylketonuria (2,3), rheumatoid arthritis (4), kidney dysfunction (4), Parkinson's

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disease (5), and senile dementia (6). Elevated concentrations of the pterin neopterin have been proposed as a diagnostic marker for neoplasia (7) and Aquired Immunodeficiency Syndrome, AIDS (8).

For these reasons, determination of pterins in biological samples has recieved much attention recently. The most popular techniques for determination of pterins involve liquid chromatography with either electrochemical detection (9-11) or fluorescence detection (12-15). In the development of separation methods for the pterins, reverse-phase (13-15), "ion-pair" reverse-phase (9-11), and cation-exchange chromatography (12) have all been employed. In the course of developing an analytical methodology based on liquid chromatography/electrochemistry (LCEC), these approaches were evaluated for the separation of several oxidized pterins.

Several chromatographic parameters effecting the retention of pterins in these chromatographic systems were investigated. These parameters included the mobile phase pH, ionic strength, and organic modifier concentration. The effect of varying the chromatography column temperature was also studied. For the "ion-pair" reverse-phase system, the effect of the mobile phase ion-pairing reagent concentration was investigated. From these investigations a separation of several oxidized pterins was achieved.

MATERIALS AND METHODS

Reagents. Pterin, pterin-6-carboxylic acid, and xanthopterin were purchased from Sigma Chemical Co. (St. Louis, MO). Biopterin was obtained from Calbiochem-Behring (La Jolla, CA). Neopterin was obtained from Fluka (Basle, Switzerland). 6-Hydroxymethylpterin was prepared by the method of Thijssen (18). Octyl sodium sulfate was purchased from Eastman Kodak (Rochester, NY). All other chemicals were reagent grade or better and used without purification.

Apparatus. The chromatographic system consisted of an Altex 110 pump with a Rheodyne 7125 injection valve with a 20 uL sample loop. A Biophase ODS 5u column (2.5 cm x 4.6 mm) was used in the reverse-phase and "ion-pair" reverse-phase experiments. For the cation-exchange experiments, a Zorbax 300 SCX 10u column (2.5 cm x 4.6 mm) was employed. The column temperature was controlled by an LC-23 column heater and an LC-22 temperature controller (Bioanalytical Systems, Inc., West Lafayette, IN). Detection was with a Bioanalytical Systems LC-4B amperometric detector using a glassy carbon working electrode and a Ag/AgCl reference electrode. The electrochemical detector was operated at -700 mV versus the Ag/AgCl reference.

Mobile Phase Preparation. All mobile phases were prepared from distilled, deionized water and glass distilled methanol. Each mobile phase was filtered through a 0.22 um filter (Millipore, Milford, MA) prior to use. Oxygen was removed by continuous purging with nitrogen and maintaining the mobile phase reservoir at 40°C. A flow rate of 1.0 mL/minute was used in all experiments.

RESULTS AND DISCUSSION

Reverse-phase Chromatography. Reverse-phase chromatography has been the most widely used method to separate the pterins using fluorescence detection (12-15). Electrochemical detection puts two constraints on the system not experienced with fluorescence detection. First, the mobile phase must contain an electrolyte, preferably a buffer. In order to minimize solution resistance, the ionic strength should be approximately 0.1 molar, although lower concentrations have been employed successfully. Second, for optimal electrochemical detection, the mobile phase should be acidic in order to lower the reduction potentials of the oxidized pterins.

To investigate the behavior of the pterins in a reverse-phase system, the pH of the mobile phase was varied from 2.0 to 3.6 (Figure 1). It has previously been shown that above pH 5.0 the retention of the pterins is relatively independent of pH (15). For all of the pterins studied, except pterin-6-carboxylic acid, retention increased as the mobile phase pH increased. This is as expected since the pterins are protonated at lower pH (pKa's 1-2) and therefore have little affinity for the hydrophobic stationary phase. Pterin-6-carboxylic acid is zwitterionic, having both an acidic (carboxyl) and basic (amine) functionality. As the pH is increased the carboxyl group dissociates and thereby decreases retention.

Another parameter which was investigated was the mobile phase ionic strength. Figure 2 illustrates the strong

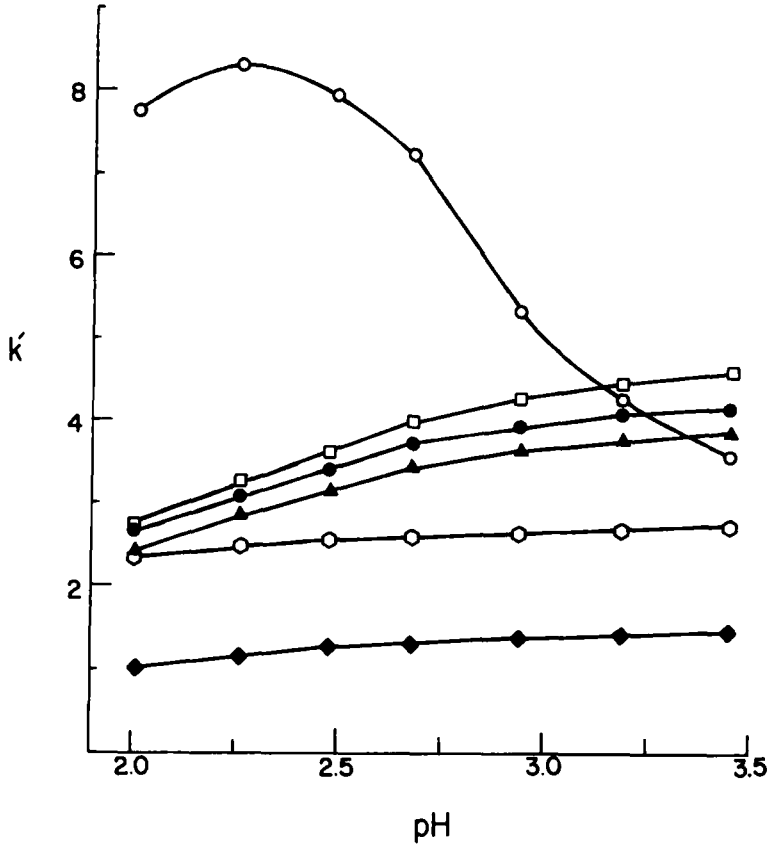

Figure 1.

Effect of pH on reverse-phase retention. ▲ = Biopterin, □ = 6-Hydroxmethylpterin, ● = Neopterin, ● = Pterin, ○ = Pterin-6-carboxylic acid, ⬡ = Xanthopterin.

effect increased ionic strength has on the retention of the pterins. From this result it is not surprising that the majority of reports of reverse-phase separations of the pterins have been achieved in unbuffered mobile phases (13,14).

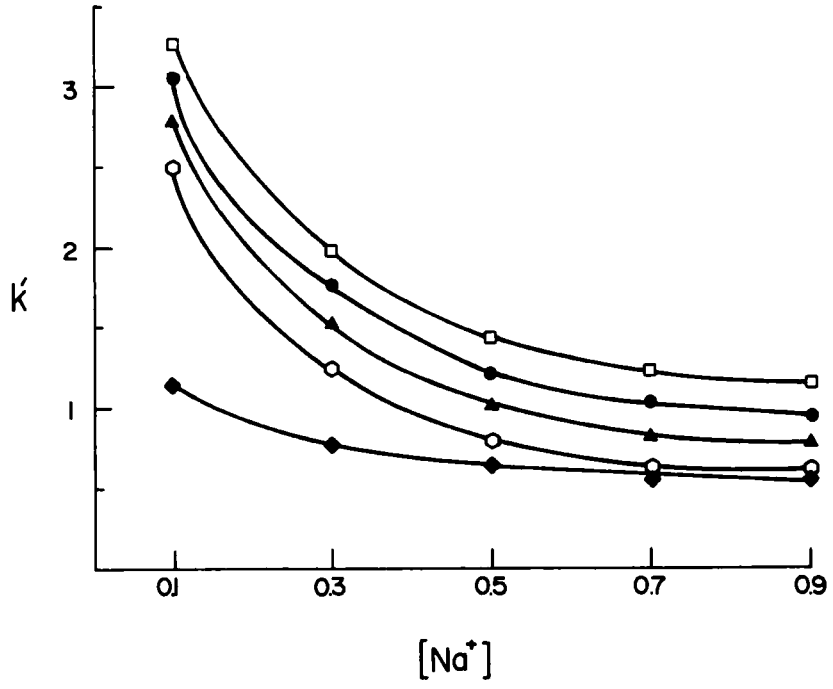

Figure 2.

Effect of ionic strength on reverse-phase retention. Symbols as in Fig. 1.

Overall, the largest capacity factor achieved for any pterin, other than pterin-6-carboxylic acid, was less than five. Therefore, the reverse-phase system lacks the flexibilty for adjusting the separation as needed for a complex sample. Figure 3 illustrates the optimum separation achieved with this system. It can be seen that xanthopterin elutes as a broad peak, most likely due to a mixed retention mechanism involving silanol groups on the packing material. It appears that a reverse-phase system is unsatisfactory for the separation of all of the pterins

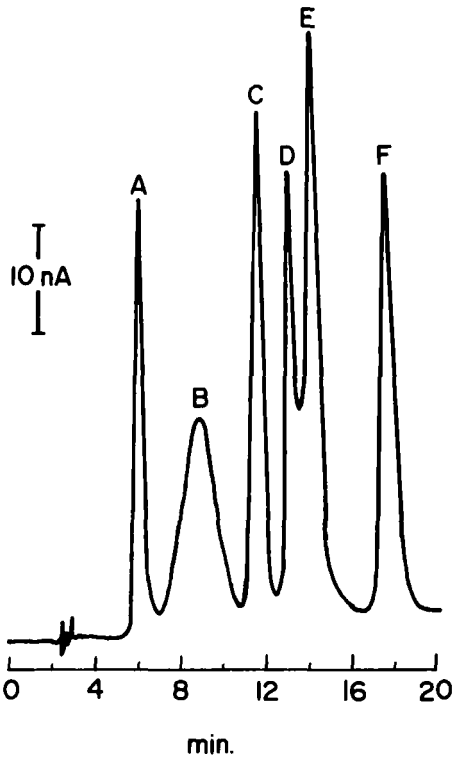

Figure 3.

Reverse-phase separation of pterins. Mobile phase; 0.1 M sodium phosphate buffer, pH 3.0. Peak identities: A, Neopterin; B, Xanthopterin; C, Biopter D, Pterin; E, 6-Hydroxymethylpterin; F, Pterin-6-Carboxylic Acid.

studied. The conditions could, however, be optimized to provide satisfactory separation of a sample containing only one or two pterins.

Cation-exchange Chromatography. At low pH the pterins are protonated and therefore amenable to cation-exchange chromatography. Figure 4 illustrates the effect of mobile phase pH on the retention of the pterins on a cation-

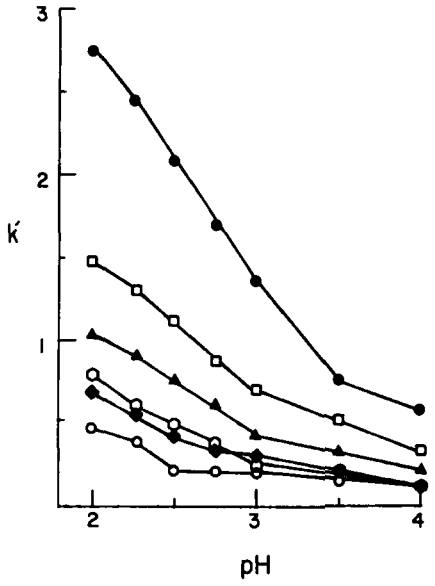

Figure 4.
Effect of pH on cation-exchange retention. Symbols as in Fig. 1.

exchange column. In contrast to reverse-phase chromatography, for cation-exchange chromatography an increase in mobile phase pH causes a decrease in the retention of the pterins. In this system, xanthopterin elutes as a symmetric peak. However, as for the reverse-phase system, the largest capacity factor is less than five. Indeed, at no pH studied was pterin-6-carboxylic acid removed from the void response. As can be seen from Figure 5, even with the optimal conditions, xanthopterin and neopterin are not well resolved. This small retention and poor resolution makes cation-exchange chromatography unsuitable for the separation of a mixture of several pterins.

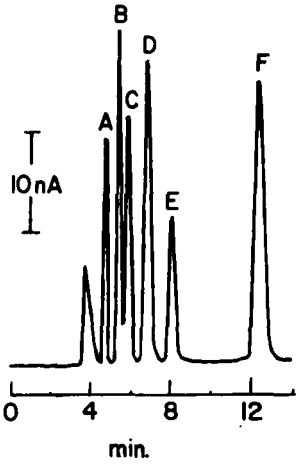

Figure 5.

Cation-exchange separation of pterins. Mobile phase; 0.1 M sodium phosphat buffer, pH 2.0. Peak identities: A, Pterin-6-Carboxylic Acid; B, Neopterin C, Xanthopterin; D, Biopterin; E, 6-Hydroxymethylpterin; F, Pterin.

"Ion-pair" Reverse-phase Chromatography. The advantages of both reverse-phase chromatography and cation-exchange chromatography can be realized through the addition of an ion-pairing reagent, octyl sodium sulfate, to the mobile phase. This gives the hydrophobic column cation-exchange properties. This can be seen in Figure 6 where the effect of mobile phase pH on the retention of the pterins is shown. As in the case of cation-exchange chromatography, retention decreases as the mobile phase pH increases. However, in the limit of high pH the retention is more similar to that for reverse-phase chromatography.

"Ion-pair" reverse-phase chromatography provides an additional chromatographic parameter, namely the ion-pairing

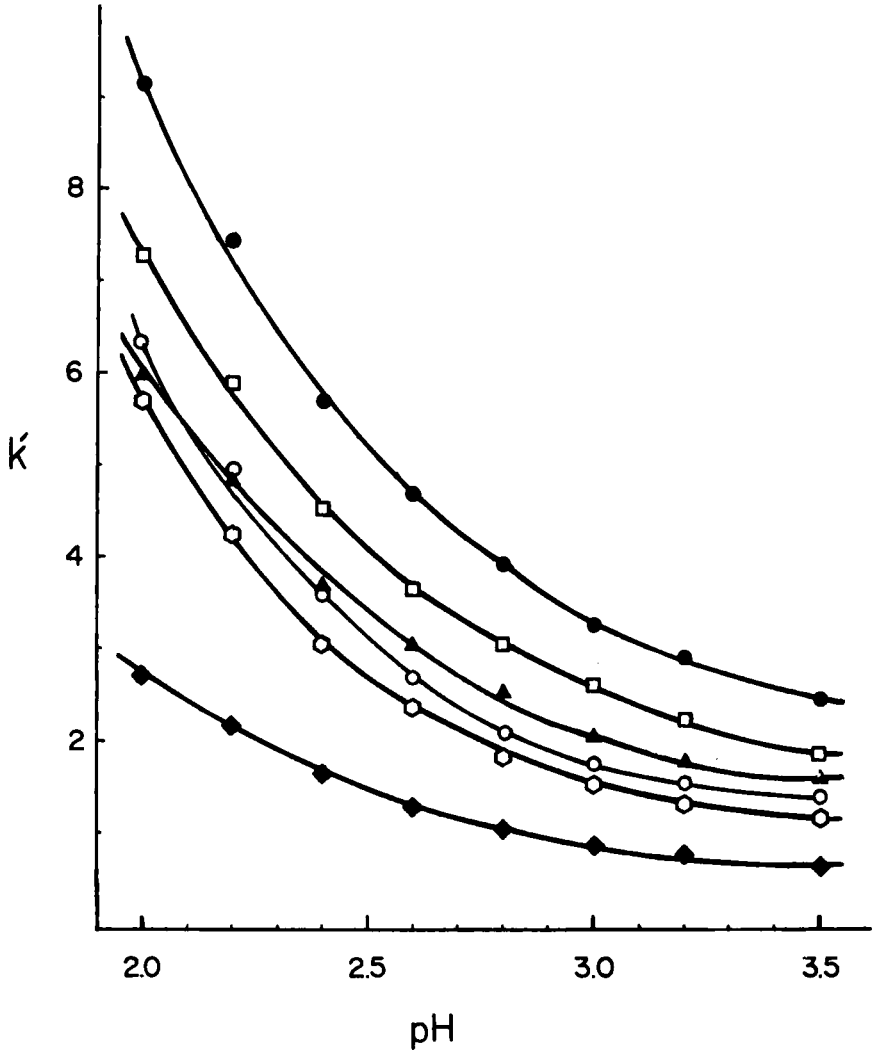

Figure 6.

Effect of pH on reverse-phase "ion-pair" retention. Symbols as in Fig. 1.

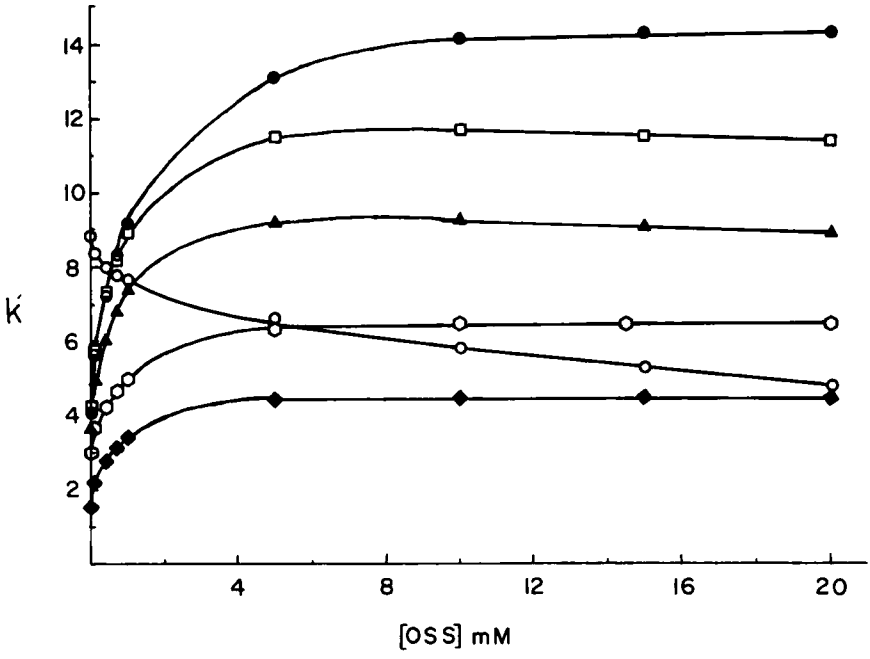

Figure 7.

Effect of ion-pairing reagent concentration on retention. Symbols as in Fig. 1. [OSS] = octyl sodium sulfate concentration.

reagent concentration in the mobile phase. Figure 7 shows the effect of ion-pairing reagent concentration on retention. Retention increases as the concentration of ion-pairing reagent increases, however, pterin-6-carboxylic acid is an exception. Pterin-6-carboxylic acid's behavior is likely due to charge repulsion between the ion-pairing reagent's sulfate group and the carboxyl group of this pterin.

Retention is a function of the surface concentration of adsorbed ion-pairing reagent, which in turn is related to

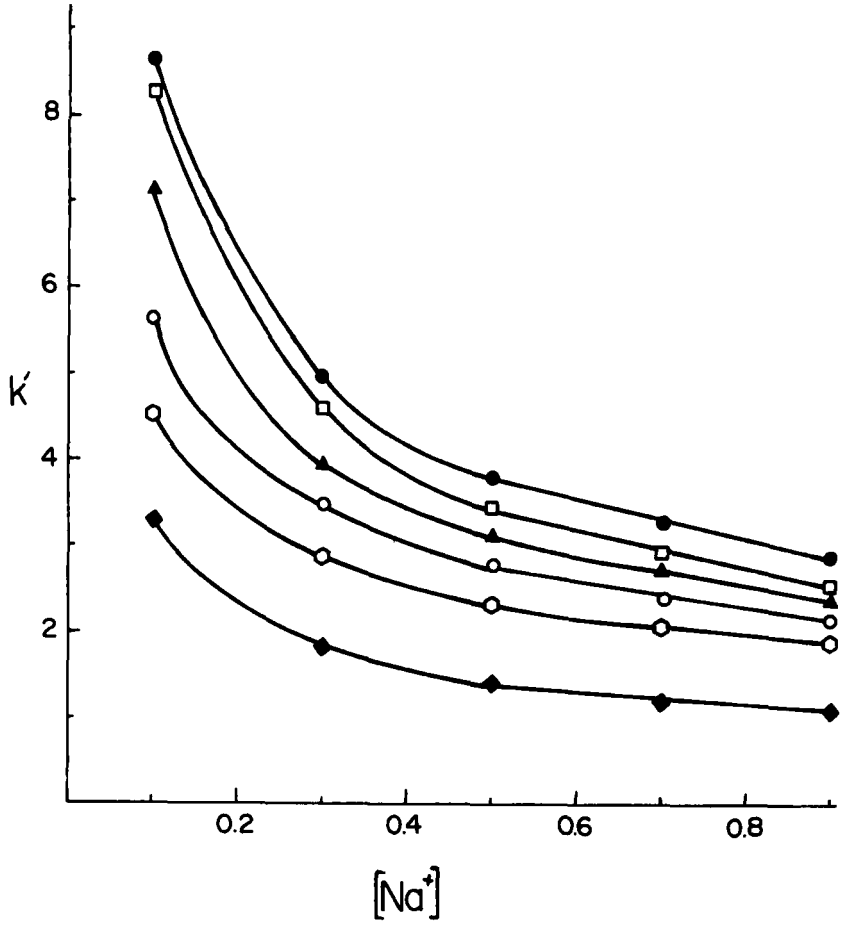

Figure 8.

Effect of ionic strength on reverse-phase "ion-pair" retention. Symbols as in Fig. 1.

the mobile phase ion-pairing reagent concentration by a Langmuir adsorption isotherm (16). The curves of Figure 6 do show the shape expected of a Langmuir isotherm. It should be noted that ion-pairing reagent concentrations above 5 mM have little effect on retention. This is due to saturation of the stationary phase with ion-pairing reagent.

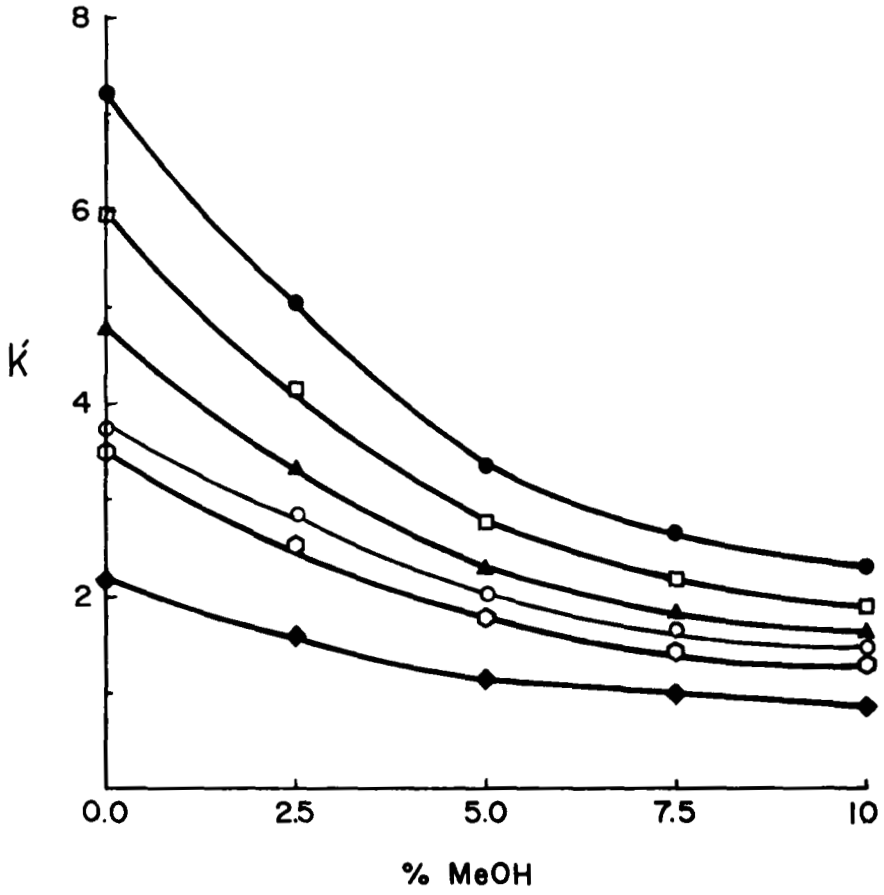

Figure 9.

Effect of methanol concentration on reverse-phase "ion-pair" retention. Symbols as in Fig. 1.

Two other parameters which effect the retention of pterins in this ion-pairing system are the mobile phase ionic strength and organic modifier (methanol) concentration. Increases in both of these parameters decrease retention with little effect on resolution (Figures

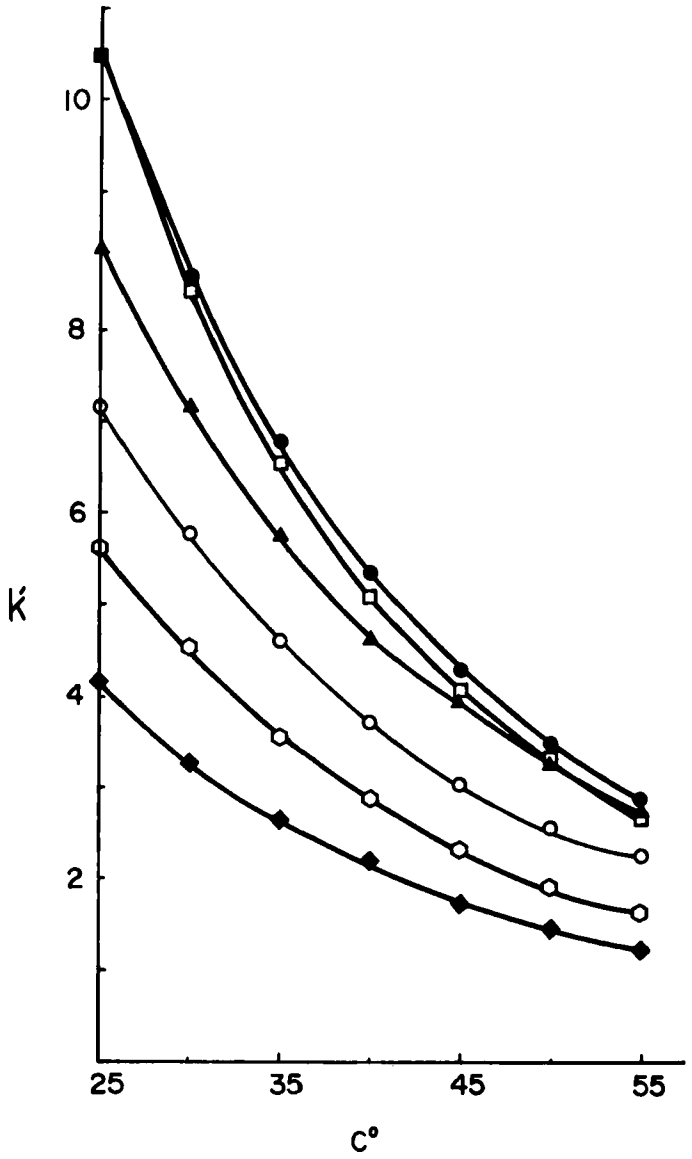

Figure 10.

Effect of column temperature on reverse-phase "ion-pair" retention. Symbols as in Fig. 1.

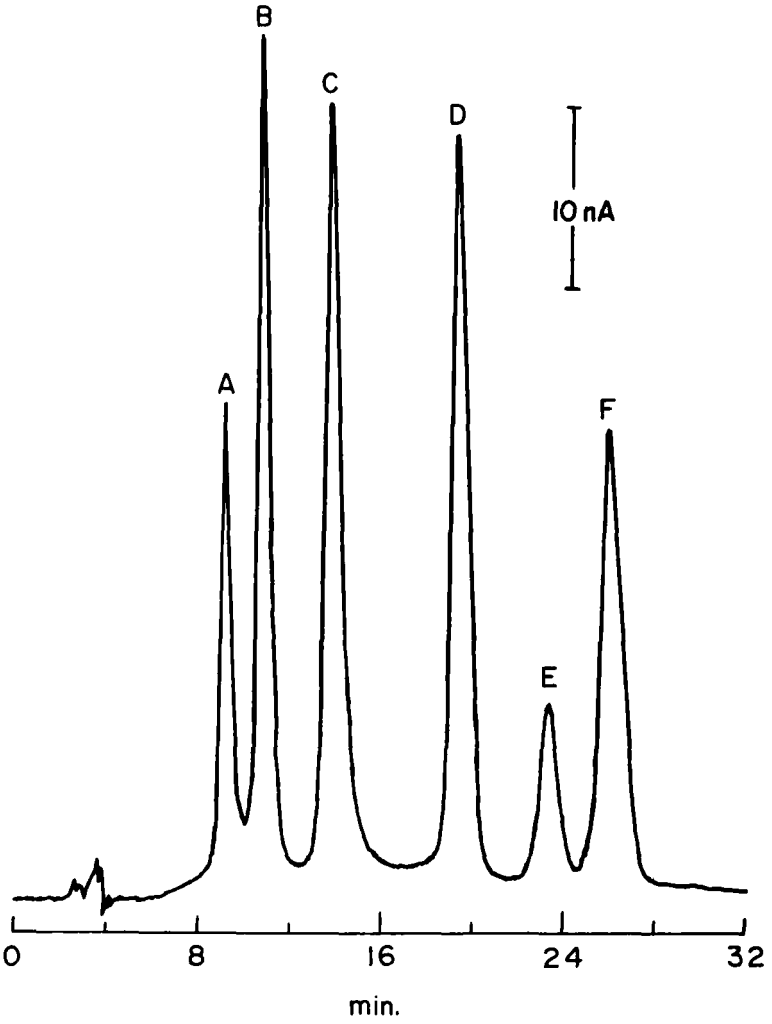

Figure 11.

Reverse-phase "ion-pair" separation of pterins. Mobile phase: 3 mM octyl sodium sulfate in 0.1 M sodium phosphate buffer, pH 2.5, at 30° Peak identities: A, Neopterin; B, Xanthopterin; C, Pterin-6-Carboxylic Acid; D, Biopterin; E, 6-Hydroxymethylpterin; F, Pterin.

8 and 9 respectively). Variation of either mobile phase ionic strength or methanol concentration is an excellent method of controlling the solvent strength without effecting the separation.

A final parameter which effects the "ion-pair" reverse-phase separation of pterins is the column temperature. The effect of column temperature on the retention of pterins is shown in Figure 10. For most pterins, as temperature increases retention decreases with little change in relative retention. Howevr, temperature has a much more pronounced effect on 6-hydroxymethylpterin than on the other pterins studied. Changes in column temperature can be used to "fine-tune" the retention of 6-hydroxymethylpterin to achieve the optimal resolution. Using "ion-pair" reverse-phase chromatography the separation shown in Figure 11 was achieved.

CONCLUSION

Neither reverse-phase chromatography or cation-exchange chromatography proved adequate to separate a mixture of several oxidized pterins. "Ion-pair" reverse-phase chromatography was found to combine the advantages of the other two techniques to give a satisfactory separtion. In addition, the "ion-pair" reverse-phase system offers the flexibility to readily modify the separation for specific analytical requirements.

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