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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS AND PEPTIDES ON SILICA COATED WITH AMMONIUM TUNGSTOPHOSPHATE

I. CHARACTERISTICS OF THE STATIONARY PHASE

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SUMMARY

Silica coated with ammonium tungstophosphate is proposed as a stationary phase for high-performance liquid chromatography. The chromatographic characteristics of columns packed with this material were investigated. The retention of compounds containing an NH_4^+ group, such as amino acids and peptides, is determined by an ion-exchange mechanism; the silica coated with ammonium tungstophosphate behaves as a reversed phase in the case of aliphatic and aromatic organic acids when eluting with aqueous buffer solutions. The new stationary phase shows a high selectivity towards glycine and tyrosine oligomers.

INTRODUCTION

Recent progress in high-performance liquid chromatography (HPLC) is due to continuous improvements in the equipment[1,2] and to the use of new stationary phases[3–5]. Today, research into new stationary phases for selective chromatographic separations of organic compounds is one of the primary goals in this field[6]. The production of supports with polar functional groups is of particular interest[7,8]. We propose here the use of ammonium tungstophosphate (AWP), a synthetic inorganic ion exchanger that has already been widely used in the thin-layer chromatography (TLC) of many nitrogen-containing organic compounds[9–16], as a stationary phase for use in HPLC.

EXPERIMENTAL

Apparatus

HPLC separations were performed on a Perkin-Elmer Series 3B liquid chromatograph. The samples were monitored at 210 nm with a Perkin-Elmer LC-75 variable-wavelength detector. The columns were 25 × 0.46 cm I.D. stainless-steel tubes with stainless-steel frits at the ends, packed with a Shandon apparatus. The chro-

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matographic data were stored and processed with a Shimadzu computer system.

Thin layers for TLC (10 × 10 cm, thickness 300 μm) were prepared with a Chemetron automatic apparatus.

Reagents

Amino acids, peptides and organic acids were obtained from Sigma. Standard solutions were prepared by dissolving different amounts of the compounds in phosphate buffer (pH 2.1) to give concentrations in the range 0.5–2 mg/ml, depending on the detector response. The injection volumes varied between 1 and 5 μl of solution. In all separations elution was performed with equimolecular solutions of phosphoric acid and ammonium phosphate at different concentrations, and with solutions of phosphate buffer and acetonitrile.

Preparation of the stationary phase

A 50-g amount of Lichrosorb Si 60 (10 μm) (Merck) was suspended in 150 ml of 2 *M* nitric acid containing different amounts of tungstophosphoric acid (HAWP) to obtain silica with different degrees of coating (see Fig. 1a). A 150-ml volume of 2 *M* ammonium nitrate solution was added dropwise to the suspension with stirring. After 10 min the precipitate was collected in a glass filter funnel (Jena-Duran, porosity 3), washed with acetone and air dried. The resulting product was purified and fractionated according to the particle size using the following method: the material was slurried in isopropyl alcohol and then slowly transferred into a glass column (100 × 2.5 cm I.D.) filled with the same solvent, avoiding turbulence. After 10 min the first sediment was removed, and the solid sedimented after a further 20 min was recovered and the suspended particles were eliminated. The resulting fraction showed a narrow size range and was able to be packed.

HPLC column packing

The material was slurried in isopropyl alcohol and then packed into the column with 200 ml of phosphate buffer solution (pH 2.1) using both "up-flow" and "down-flow" methods successively. The packing pressure was about 8000 p.s.i.

Preparation of thin-layers

A 4-g amount of stationary phase and 1 g of $CaSO_4 \cdot 1/2H_2O$ were suspended in 50 ml of water and stirred for 10 min.

Determination of AWP content on the silica

A spectrophotometric method, based on the formation of yellow complexes of thiocyanate with tungsten, was applied[17]. The transmittance was measured at 420 nm after 20 min.

RESULTS AND DISCUSSION

The ammonium tungstophosphate obtained by precipitation from HAWP solution has a wide size range (1–5 μm), but there are also smaller particles (1 μm). In addition, AWP easily forms large agglomerates. Although the particles of AWP are strong enough to resist high packing pressures, the presence of the smallest particles,

which are difficult to separate and may obstruct the porous frits of the columns, makes this material unsuitable for packing. For this reason, silica coated with ion exchanger was prepared and used to pack the HPLC columns, as with ammonium molybdophosphate[18].

Characteristics of the stationary phase

The degree of coating of the silica depends on the concentration of tungstophosphoric acid in the solution from which AWP is precipitated. The relationship between these two parameters is shown in Fig. 1a, where the percentage of AWP on silica is plotted against the percentage of HAWP (calculated with respect to the weight of silica) in the solution. The resulting graph is similar to an adsorption isotherm. In fact, the degree of coating increases with increasing amount of tungstophosphoric acid in the solution up to the maximum value of 25%. When the percentage of HAWP increases above this value, the content of AWP on the silica remains almost unchanged.

In order to examine, by another method, the correlation of the degree of coating with the amount of HAWP in the solution and to show at the same time the retention capacity of this new stationary phase, thin-layer chromatographic data for

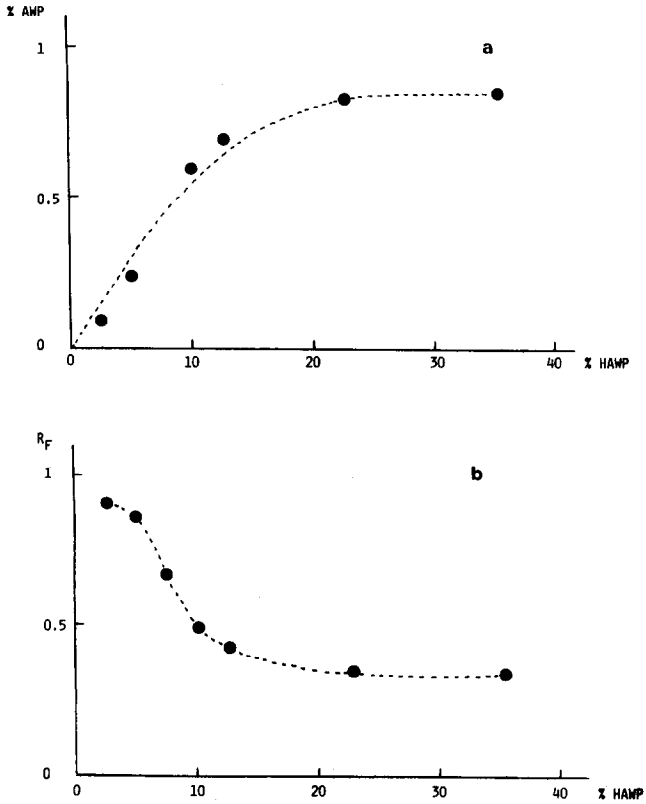

Fig. 1. (a) Percentage of AWP on silica *versus* percentage of HAWP in solution. (b) R_F values of tetraglycine on layers of AWP-coated silica *versus* percentage of HAWP in solution. Eluent, 0.05 *M* phosphate buffer.

tetraglycine on layers with different amounts of AWP were obtained. All experiments were carried out with 0.05 *M* phosphate buffer solution (pH 2.1). A plot of R_F values against the percentage of HAWP in the solution is shown in Fig. 1b. In general, this behaviour is in agreement with the data in Fig. 1a; in addition, it shows that the influence of AWP becomes significant only at levels above 0.1%, corresponding to HAWP levels above 2.5%.

Degree of coating and selectivity of the stationary phase

In order to study the retention capacity and the selectivity of the new stationary phase, HPLC columns with silica alone and silica coated with different amounts of AWP were packed. Four columns were tested with aliphatic and aromatic compounds and particularly with oligomers of glycine up to four residues and of tyrosine up to three residues. All the samples were eluted with 0.05 *M* phosphate buffer solution. The choice of these compounds was suggested by their different chromatographic behaviour observed on AWP thin layers[11]. Fig. 2 shows the chromatograms of (a) glycine and (b) tyrosine oligomers on columns of (1) silica, (2) silica + 0.3% AWP, (3) silica + 0.48% AWP and (4) silica + 0.65% AWP, corresponding to percentages of HAWP in solution of 0, 5, 7.5 and 15%, respectively (see Fig. 1a). On silica columns the members of the two series of oligomers are not retained and give rise to one peak only. An increase in the amount of AWP on the silica results in an

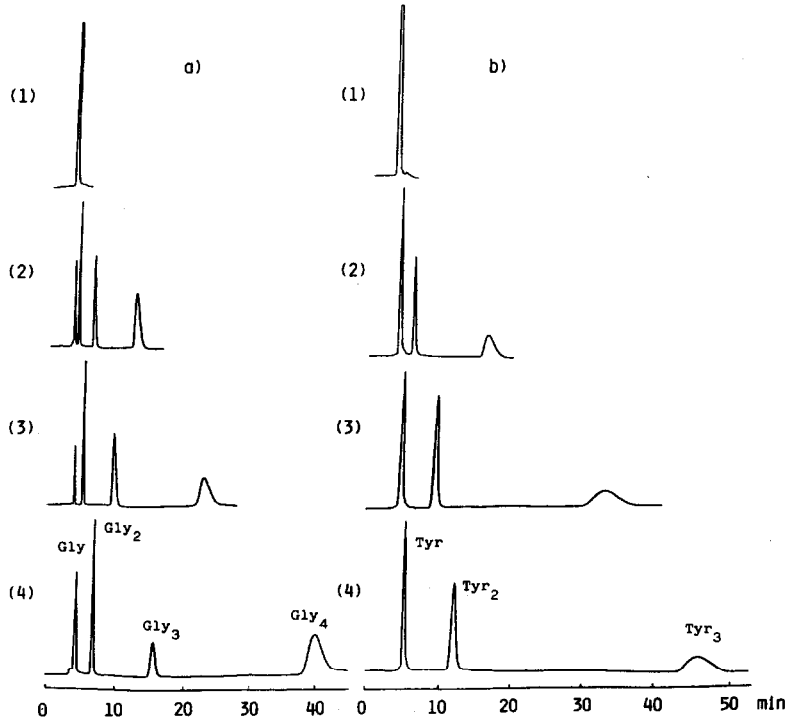

Fig. 2. Chromatograms of (a) glycine and (b) tyrosine oligomers on columns of (1) SiO_2, (2) SiO_2 + 0.3% AWP, (3) SiO_2 + 0.48% AWP and (4) SiO_2 + 0.65% AWP. Mobile phase, 0.05 *M* phosphate buffer (pH 2.1); column, 25 × 0.46 cm, I.D.; flow-rate, 1 ml/min; detection, UV (210 nm).

TABLE I

SELECTIVITIES OF SILICA GEL WITH DIFFERENT DEGREES OF COATING FOR PAIRS OF AMINO ACIDS AND PEPTIDES

Pair	α_2*	α_3*	α_4*
Gly_2–Gly	3.5	4.3	4.1
Gly_3–Gly_2	3.5	3.7	3.7
Gly_4–Gly_3	3.0	3.1	3.1
Tyr_2–Tyr	4.1	5.6	5.4
Tyr_3–Tyr_2	5.1	5.5	5.2
Tyr–Gly	2.4	2.6	2.1

* α_2, α_3 and α_4 refer to columns 2, 3 and 4 in Fig. 2.

increase in retention for all the compounds. This increase is greater for tyrosine than for glycine oligomers and for both series the retention increases considerably with increasing number of amino acid residues. Both glycine and tyrosine oligomers can also be well separated on columns packed with material with a low degree of coating (see column 2). This behaviour demonstrates the unusual retention capacity even of small amounts of this ion exchanger and its great selectivity, as shown in Table I. The increase in the degree of coating of silica does not involve a corresponding change in selectivity for either aliphatic or aromatic compounds. The greatest variations of α values are found for the pairs Gly_2–Gly and Tyr_2–Tyr.

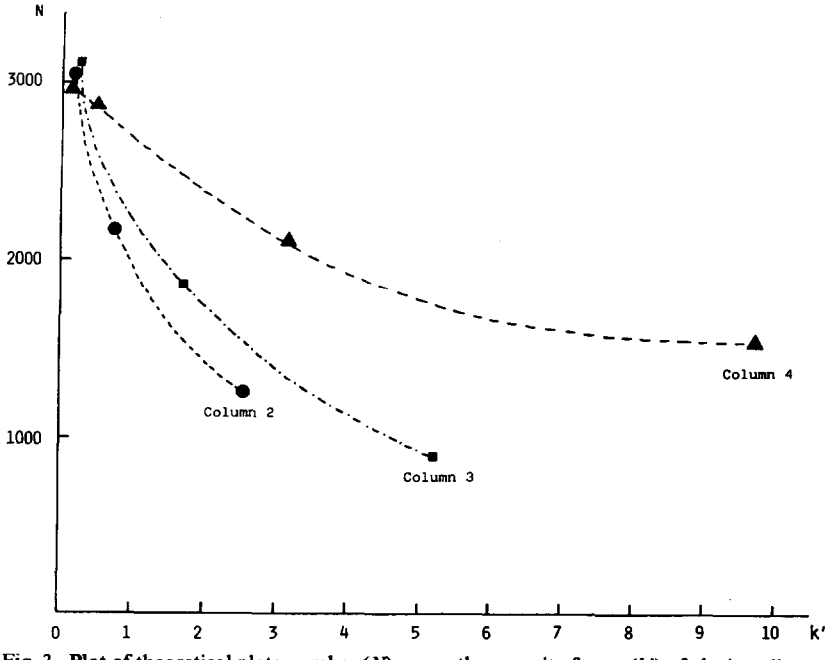

Fig. 3. Plot of theoretical plate number (*N*) *versus* the capacity factor (*k'*) of glycine oligomers on columns 2, 3 and 4. Mobile phase, 0.05 *M* phosphate buffer (pH 2.1); flow-rate, 1 ml/min.

Column efficiency

The theoretical plate numbers (N) were obtained for three different columns using glycine oligomers. In Fig. 3 the capacity factors (k') are plotted against the theoretical plate numbers for columns 2, 3 and 4. Data relative to glycine are reported only for column 4, as this compound is eluted near the void volume on the first two columns and the corresponding k' values are not reliable.

All the curves show a decrease in N with increasing k'. The decrease is greater when the degree of coating of the silica is low. The differences in N for the three columns are very slight for compounds having no appreciable retention. The maximum theoretical plate number, for example, is 3000 (or 12 000 plates/m) on all the columns.

The curves obtained by plotting HETP (height equivalent to a theoretical plate) values against the linear flow-rate (u ml/min) of the eluent are in agreement with the Van Deemter equation and reach their minima at a mobile phase flow-rate of *ca.* 0.3 ml/min. The high selectivity of this stationary phase gives good separations of oligomer mixtures even when using a flow-rate much higher than the optimal value, as shown in the chromatogram in Fig. 4.

Aqueous–organic solutions

The addition to the mobile phase of increasing amounts of acetonitrile results in no appreciable change in the chromatographic behaviour of glycine and tyrosine oligomers. For example, on eluting column 4 with phosphate buffer–acetonitrile (60:40), the retention times of glycine and tyrosine increase from 0.75 to 1.35 min and from 1.48 to 1.59 min, respectively, without any analytical advantage. In order to avoid salt precipitation and consequent obstruction of the column, acetonitrile concentrations above 40% are inadvisable.

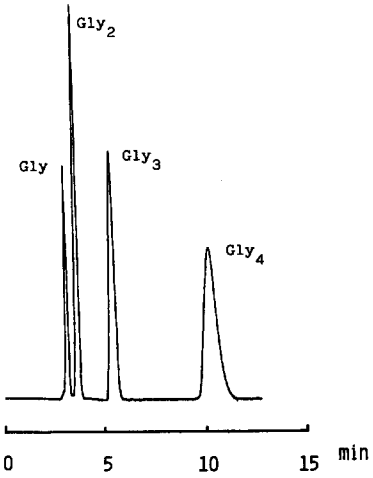

Fig. 4. Chromatogram of glycine oligomers on column 2. Mobile phase, 0.05 *M* phosphate buffer; flow-rate, 2.5 ml/min; detection, UV (210 nm).

Mechanism of retention

As the stationary phase and solutes contain groups able to undergo ion-exchange reactions, we wanted to investigate whether the ion-exchange process was the primary mechanism for the retention of amino acids and peptides. For this purpose we performed retention measurements on column 4 at different concentrations of ammonium ion at constant pH. The results are reported in Fig. 5, where log k' is plotted against $-\log[NH_4^+]$.

Peptides give straight lines with slopes between 0.85 and 1.03, in agreement with a retention process controlled by an ion-exchange mechanism. With amino acids, the mechanism of solute retention is more complex. The log k' *versus* $-\log[NH_4^+]$ trend for glycine is not well characterized, as this compound is eluted with the void volume using 0.1 *M* phosphate buffer. A linear trend can be identified for tyrosine but the slope is much lower (0.57) than that for peptides.

Such a behaviour indicates that no single retention mechanism is operative with amino acids, whose chromatographic characteristics are probably dependent on both an ion-exchange process and non-ionic interactions. The log k' *versus* $-\log[NH_4^+]$ trends for benzene sulphonic and acetic acids confirm such a hypothesis. These compounds, in fact, are lacking an NH_4^+ group and are not affected by changes in the ionic strength of the eluent.

Further information on the behaviour of the new stationary phase with aqueous eluents is derived from the retention data for several aliphatic and aromatic organic acids and for the corresponding amino acids. The results in Table II indicate that silica coated with AWP behaves as a reversed phase under the experimental

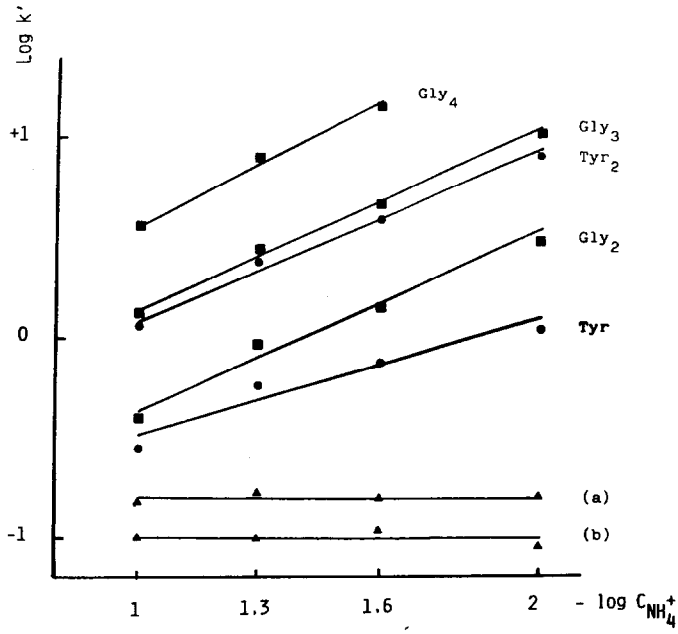

Fig. 5. Plot of log k' *versus* $-\log[NH_4^+]$ for amino acids peptides and organic acids on column 4. (a) Benzene sulphonic acid; (b) acetic acid.

TABLE II

RETENTION TIME (t_R) OF ORGANIC ACIDS AND OF THE CORRESPONDING AMINO ACIDS ON COLUMN 4 (SEE FIG. 2) ON ELUTING WITH 0.05 *M* PHOSPHATE BUFFER

Organic acid	t_R *(min)*	*Amino acid*	t_R *(min)*
HCOOH	3.74	NH_2CH_2COOH	4.18
CH_3COOH	3.80	$CH_3CH(NH_2)COOH$	4.09
CH_3CH_2COOH	4.16	$HOCHCH(NH_2)COOH$	3.97
$CH_3CH(OH)COOH$	3.62	$CH_3CH_2CH(NH_2)COOH$	4.31
$CH_3(CH_2)_2COOH$	4.74	$CH_3(CH_2)_2CH(NH_2)COOH$	4.87
$CH_3(CH_2)_3COOH$	5.15	$CH_3(CH_2)_3CH(NH_2)COOH$	5.49
C_6H_5COOH	5.42	$C_6H_5CH_2CH(NH_2)COOH$	6.73
$C_6H_5CH_2COOH$	4.83		
$C_6H_5SO_3H$	4.01		

conditions used. In fact, organic acids with more marked hydrophobic characteristics are the most retained. The same behaviour is shown by amino acids, even if their retention is also affected by an ion-exchange process that determines the reversal in retention between glycine and alanine, probably owing to the steric hindrance of the methyl group on the NH_4^+.

Reproducibility of the technique

As regards the lifetime and reproducibility of the columns, we verified that after rinsing with 3000 column volumes of acqueous solutions of different ionic strength (0.01–0.1 *M*) and pH (1–3), no appreciable change in the capacity factors of the test compounds was observed.

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