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STUDIES ON IODINATED COMPOUNDS

VI. SEPARATION CHARACTERISTICS OF IODOHISTIDINES ON REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of iodohistidines [monoiodohistidine (MIH) and diiodohistidine (DIH)] by reversed-phase high-performance liquid chromatography on octadecylsilylsilica columns was attempted in comparison with the separation of iodotyrosines. The retention of iodohistidines with the phosphate buffer used as the mobile phase was relatively small, and less pH dependent. However, the retention increased with the addition of hydrophobic ion-pairing agents to the mobile phase. When acetic acid was used in the mobile phase, DIH was eluted by reversed-phase elution, MIH by polar-phase elution. The separation of iodohistidines was largely dependent on the properties of the stationary phase, silanophilic interactions being influential. It may be concluded that the separation of iodohistidines will be best accomplished when an high ionic strength, low pH mobile phase containing an alkanesulphonate is used.

INTRODUCTION

Analyses of iodoamino acids are essential for the study of thyroid hormones[1–3] and iodination of proteins[4]. Iodinated derivatives of tyrosine (Tyr) and thyronine have been the main target of the investigation. There are two molecular species of iodotyrosines: that is, 3-iodotyrosine (MIT) and 3,5-diiodotyrosine (DIT). There are more molecular species of iodothyronines: triiodothyronine and thyroxine and many other isomers depending on the positions of iodine substitution in the thyronine. Recently, reversed-phase high-performance liquid chromatography (RP-HPLC) has been widely used for the separation of such iodoamino acids[5–7].

On the other hand, iodinated derivatives of histidine (His) are known. There are two molecular species of iodohistidines as in the case of iodotyrosines: that is, 4(5)-iodohistidine (MIH) and 2,4(5)-diiodohistidine (DIH) (Fig. 1). Separations of these iodohistidines were performed by paper chromatography[8], thin-layer chromatography[9] and open column chromatography[10,11], but HPLC separation has not been attempted. Therefore, the separation of these iodohistidines by HPLC was examined

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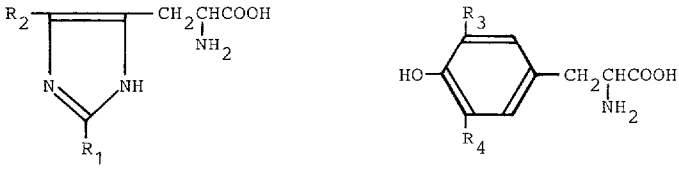

His: R_1 = H, R_2 = H
MIH: R_1 = H, R_2 = I
DIH: R_1 = I, R_2 = I

Tyr: R_3 = H, R_4 = H
MIT: R_3 = I, R_4 = H
DIT: R_3 = I, R_4 = I

Fig. 1. Structures of the compounds studied.

in the present study, since some physiologically activity of these compounds has been suggested[11,12]. Comparison of the separations of iodohistidines and iodotyrosines by HPLC may be of interest, since the imidazolyl group of His and the hydroxyphenyl group of Tyr have markedly different chemical properties. Thus in the present study, the separation of iodohistidines by RP-HPLC on an octadecylsilylsilica (ODS) column was attempted in comparison with the separation of iodotyrosines.

EXPERIMENTAL

Apparatus

The HPLC equipment consisted of a Waters Model 6000A pump, U6K universal injector and Model NS-310A variable-wavelength UV absorbance detector from Nippon Seimitsu Kagaku. The HPLC column was a Waters radial compression separation system, with cartridges of Radialpak Novapak C_{18} (NC_{18}) (100 mm × 8 mm I.D.), μBondapak C_{18} (MC_{18}) (100 mm × 8 mm I.D.) or C_{18} (RC_{18}) (100 mm × 8 mm I.D.).

Reagents

MIH (hydrochloride) and DIH (hydrochloride) were synthesized in our laboratory by the reported methods[13,14]. MIT and DIT were obtained from Sigma, His (hydrochloride, hydrate), potassium iodide (KI) and 0.5 *M* tetra-*n*-butylammonium hydroxide (TBAOH) solution from Wako and Tyr and 1-heptanesulphonic acid (HSA) (sodium salt) from Nakarai. Water used for the mobile phase component was deionized and distilled, and methanol and acetonitrile were of HPLC grade. Other reagents were of commercial guaranteed grade. Stock solutions of iodinated compounds were prepared by dissolving them in water or in 5 m*M* HCl: His, MIH and DIH (0.5 mg/ml in water); Tyr, MIT, DIT (0.5 mg/ml in 5 m*M* HCl) and KI (0.1 mg/ml in water).

Methods

HPLC was performed at room temperature (*ca.* 20°C). The flow-rate of the mobile phase was 2.5 ml/min when the NC_{18} column was used, and 3.0 ml/min when MC_{18} and RC_{18} were used. Detection of the solute was performed at 225 nm in general, and when an acetic acid-containing mobile phase was used, His was detected at 235 nm and other iodinated compounds at 254 nm. Ammonium phosphate buffer,

50 m*M*, used as the mobile phase was prepared by mixing 50 m*M* $NH_4H_2PO_4$, 50 m*M* $(NH_4)_2PO_4$ or 50 m*M* H_3PO_4, and the pH was adjusted using a pH meter. All solvents for the mobile phase were filtered through a 0.45-μm filter before use. Retention of compounds was determined by injecting 2–5 μl of the stock solutions. The capacity factor, k', was calculated by $k' = (V - V_0)/V_0$, where V is the retention volume of the solute, and V_0 is the hold up volume of the column. The hold up volume of the column was obtained from the retention volume of nitrate ion after injection of $NaNO_3$ using 60% acetonitrile as the mobile phase.

RESULTS AND DISCUSSION

Influence of the pH of the mobile phase

A solvent mixture of methanol (or acetonitrile)–water–phosphoric acid has been used as the mobile phase for RP-HPLC for the separation of iodotyrosines and iodothyronines[5,6]. The relationships between the retention of iodoamino acids with the above solvent mixture and their hydrophobicities, pH of the mobile phase and compositions of organic solvents have been reported[15–17]. Based on knowledge of the conditions for HPLC separation of these iodoamino acids, and the effect of the pH of mobile phase systems composed of methanol–50 m*M* ammonium phosphate buffer, the retention of iodohistidines was examined using three different ODS columns (Fig. 2).

The pH of the mobile phase was altered within the range allowed for the ODS column (pH 3.0–7.0). Under these conditions, the elution of iodohistidines was in the order of decreasing iodine substitution of the histidine, which might reflect that the hydrophobicity of histidine is increased, as in tyrosine, with increasing iodine substitution. The retention of iodohistidines and iodotyrosines having the same numbers of iodine groups was compared, and it was found that the retention of iodohistidine

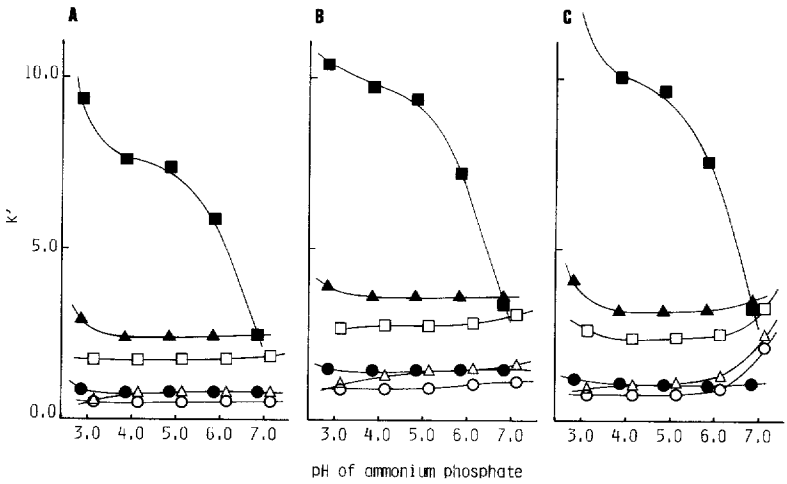

Fig. 2. Influence of pH on retention. Columns: (A) NC_{18}; (B) MC_{18}; (C) RC_{18}. Mobile phase: 25% methanol in 50 m*M* ammonium phosphate (pH 3.0–7.0). Compounds: ○, His; △, MIH; □, DIH; ●, Tyr; ▲, MIT; ■, DIT.

was less than that of the corresponding iodotyrosine. This may be due to the lower hydrophobicity of the histidine (imidazole moiety) than the tyrosine (hydroxyphenol moiety)[18]. No change in the retention behaviour of iodohistidines with the pH of the mobile phase was observed except in the case of the RC_{18} column. However, in general the retention of the solutes is dependent on the degree of solute ionization, so that the degree of ionization of iodohistidines at various pH should be taken into consideration. The degree of solute ionization, which is influenced by various factors, for example the organic solvent content in the mobile phase, may be assumed briefly from the pK_a value of the dissociable groups. There are three dissociable groups on iodohistidines, and the pK_a values of each were reported by Brunings[14] [although Brunings determined the pK_a of MIH for 2(5)-iodohistidine, the structure of MIH was corrected to 4(5)-iodohistidine by Bensusan and Naidu[19]] (Table I).

The pK_a value of the carboxyl group of His tends to be shifted toward lower values on iodination. However, the maximum pK_a of the carboxyl group of these compounds, which is the highest in His, is below 2.0, so that it may be assumed that the carboxyl group of the iodohistidines would be mostly dissociated in these pH regions. The pK_a value of the amino group of His will also be shifted to lower values on iodination. However, the lowest pK_a value of the amino group in these compounds, which is lowest in DIH, is above 8.0, thus the amino group of iodohistidines is assumed to be mostly dissociated in these pH regions. Therefore, the carboxyl and amino groups of His and iodohistidines are in the mostly ionized state, and no change in the retention behaviour due to a change of the extent of ionization of these groups is expected.

The influence of the pH of the mobile phase on the ionization of iodohistidines is marked for the third dissociable group, imidazolyl. The imidazolyl group is basic in nature, and its dissociation increases at pH lower than the pK_a value. The opposite is true for the hydroxyphenyl group of the iodotyrosines, which functions as an acid and its dissociation increases at pH higher than the pK_a value. However, the pK_a value of the imidazolyl of His is markedly lowered on iodination as in the case of Tyr. Therefore, the imidazolyl groups of His and MIH are mostly ionized at pH lower than 6.0 and 4.2, respectively, but the imidazolyl group of DIH would not be ionized within the pH range used in the present study. Based on such considerations, the

TABLE I

pK_a VALUES OF IODOAMINO ACIDS

Compound	pK_a[14,20,21]			
	Imidazolyl	*Hydroxyphenyl*	*–COOH*	*$–NH_2$*
His	6.00		1.82	9.17
MIH	4.18		1.72	8.62
DIH	2.72		–[a]	8.18
Tyr		10.13	2.20	9.11
MIT		8.20	–[a]	–[a]
DIT		6.48	2.12	7.82

[a] Not in literature.

ionization of the imidazolyl groups of His and MIH is expected to change the retention behaviour. However, no significant change in the retention as seen for DIT was observed on NC_{18} and MC_{18}, while the retention of DIT decreased at pH from 5.0 to 7.0 because of increasing dissociation of the hydroxyphenyl group. A change in the degree of ionization of the imidazolyl group might not contribute to the retention behaviour of iodohistidines since the hydrophobicity of the imidazolyl group is very small even when the imidazolyl group is not dissociated. Therefore, even if the dissocation of the imidazolyl group is suppressed at high pH in the mobile phase, a significant increase in the retention would not be expected. A high pH of the mobile phase poses a problem of the influence of the stationary phase as will be mentioned below.

Influence of the stationary phase

The separation of iodohistidines was not dependent on the nature of the stationary phase at lower pH of the mobile phase. However, as the pH of the mobile phase increased, the efficiency of the separation of iodohistidines became less, as in the case of iodotyrosines. This was seen mainly from the shape of the peaks: when the NC_{18} column was used, good peak shape was obtained for iodohistidines and iodotyrosines at pH 7, but when MC_{18} and RC_{18} columns were used, broadening and tailing of the peaks occurred. When the RC_{18} column was used the retention of iodohistidines was specifically increased as the pH of the mobile phase increased (Fig. 2C).

The properties of the column packings need to be known in order to discuss the difference in separation of iodohistidines ascribed to the stationary phase: particle sizes (NC_{18}, 4; MC_{18}, 10; RC_{18}, 10 μm); particle shapes (NC_{18}, spherical; MC_{18}, irregular; RC_{18}, spherical); modifications of the residual silanol groups (NC_{18}, endcapped; MC_{18}, endcapped; RC_{18}, non-endcapped); nominal pore sizes [range] (NC_{18}, 60 [60–100]; MC_{18}, 125 [50–300]; RC_{18}, 90 [60–175] Å) and carbon load (NC_{18}, 7; MC_{18}, 10; RC_{18}, 12%)[22]. The relatively small particle size and less carbon load of the NC_{18} column might be responsible for the good peak shape of iodohistidines and iodotyrosines at higher pH. Some ionic interactions between silanol groups on the stationary phase and iodohistidines might contribute to the increase in retention of iodohistidines on the RC_{18} column. The silanophilic interaction might be weak in acidic conditions because dissociation of the silanol groups may be suppressed, and the dissociation is increased as the pH is increased. Such an increase in the retention was not observed with iodotyrosines, so that the ionic interaction between iodoamino acids and silanol groups might be specific for the imidazolyl group on the iodohistidines.

Influence of organic solvent modifiers

The relationship between the ratio of organic solvent in the mobile phase and retention of iodohistidines on the NC_{18} column was examined using methanol–phosphate buffer (pH 3.0 or 7.0) as the mobile phase (Fig. 3).

As the ratio of methanol decreased at both pH values the retention of DIH as well as of iodotyrosines increased. However, little change was observed in the retention of His and MIH, which might imply that sufficient separation of His and MIH may not be accomplished with a lower ratio of the organic solvent modifier.

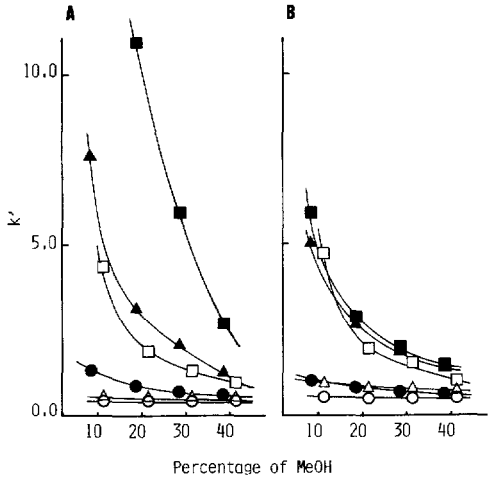

Fig. 3. Influence of the ratio of methanol on retention. Mobile phases: (A) methanol in 50 m*M* ammonium phosphate (pH 3.0), (B) methanol in 50 m*M* ammonium phosphate (pH 7.0). Column: NC_{18}. Compounds: as in Fig. 2.

When the ratio of the organic solvent modifiers was the same, less retention was obtained with acetonitrile (not shown) than with methanol.

Influence of hydrophobic ion-pairing agents

It is known that phosphate ions act as hydrophilic ion-pairing agents to decrease the retention of solutes when a mobile phase containing phosphates is used[5,23]. However, this property is not favourable for the separation of iodohistidines, the retention of which on the reversed stationary phase is weak. For the purpose of increasing the retention of iodohistidines, hydrophobic ion-pairing agents HSA and TBAOH were added to the mobile phase. The pH of the mobile phase was that generally used for HPLC (Table II).

When HSA was added to the mobile phase on the NC_{18} column an increase in the retention of iodohistidines was observed. As a result, a satisfactory separation of I^-, His, MIH and DIH was accomplished. A similar separation was observed with columns other than NC_{18} (Fig. 4).

Similarly, when TBAOH was added to the mobile phase on the NC_{18} column an increase in the retention of iodohistidines was observed. In this system, the retention of inorganic I^- together with iodoamino acids increased characteristically. The separation of I^- and iodoamino acids with a mobile phase containing alkylammonium will be reported elsewhere in detail. The separation of iodohistidines in the presence of TBAOH was largely dependent on the nature of the columns, as in the case of iodotyrosines (Fig. 5).

The shapes of the peak of iodohistidines were good when the NC_{18} column was used, but marked broadening and tailing were observed with other columns, and thus good separation was difficult. The effect of the stationary phase on the peak shapes was not dependent on the addition of TBAOH, but on the pH of the mobile phase, because the shapes were not affected by the addition of TBAOH at lower pH.

TABLE II

INFLUENCE OF ADDITION OF HYDROPHOBIC ION-PAIRING AGENTS

Mobile phase: A, 25% methanol in 50 m*M* ammonium phosphate (pH 3.0); A', 25% methanol in 50 m*M* ammonium phosphate (pH 3.0) containing 5 m*M* HSA; B, 25% methanol in 50 m*M* ammonium phosphate (pH 7.0); B', 25% methanol in 50 m*M* ammonium phosphate (pH 7.0) containing 5 m*M* TBAOH.

Compound	*k'*			
	A	*A'*	*B*	*B'*
I^-	0.55	0.34	0.59	3.30
His	0.49	1.54	0.54	0.74
MIH	0.59	1.99	0.80	1.24
DIH	1.74	4.09	1.84	3.09
Tyr	0.83	1.76	0.80	1.07
MIT	2.91	8.22	2.42	3.47
DIT	9.39	28.01	2.41	4.64

Retention behaviours in the presence of acetic acid

Acetic acid was added to the mobile phase in the place of phosphoric acid for the separation of iodotyrosines and iodothyronines[7]. Acetate ion behaves as a hydrophobic ion-pairing agent like alkanesulphonic acids[23]. Thus, an increase in the retention of iodohistidines was expected, and satisfactory separation should be accomplished. A mixture of methanol (or acetonitrile)–50 m*M* acetic acid, pH 3.0, was prepared as the mobile phase and the relationships between the ratio of organic

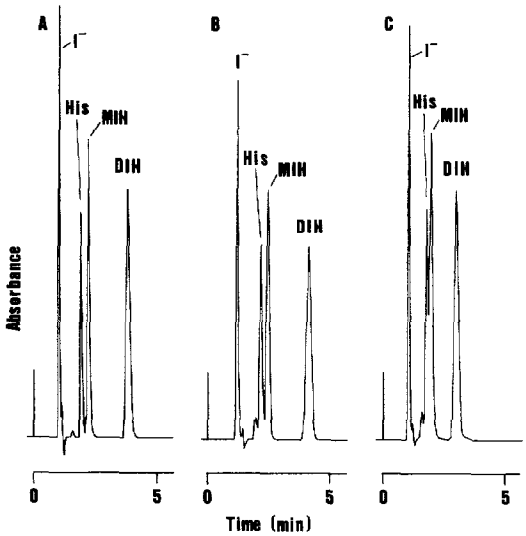

Fig. 4. HPLC separation of iodohistidines on different columns: (A) NC_{18}; (B) MC_{18}; (C) RC_{18}. Mobile phase: 25% methanol in 50 m*M* ammonium phosphate (pH 3.0) containing 5 m*M* HSA. Flow-rates: (A) 2.5; (B) 3.0; (C) 3.0 ml/min. Detector: wavelength, 225 nm; sensitivity, 0.1 a.u.f.s. Amounts injected: His, 1; MIH, 1; DIH, 1; I^-, 0.2 μg.

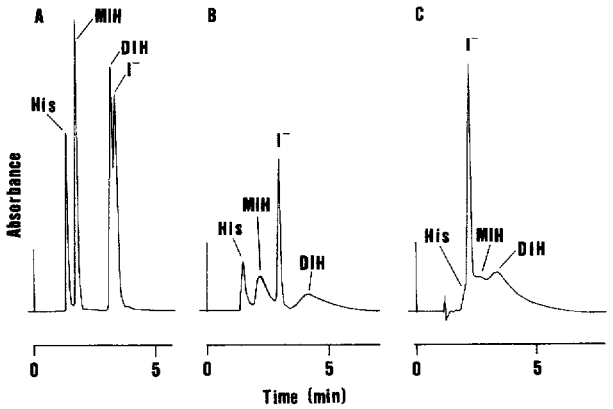

Fig. 5. HPLC separation of iodohistidines on different columns. Mobile phase: 25% methanol in 50 m*M* ammonium phosphate (pH 7.0) containing 5 m*M* TBAOH. Other conditions as in Fig. 4.

solvent modifier in the mobile phase and the retention of iodohistidines on the NC_{18} column were examined (Fig. 6).

The retention of DIH as well as iodotyrosines using the acetate mobile phase was larger than with the corresponding phosphate one, methanol (or acetonitrile)–50 m*M* phosphate buffer, pH 3.0, and an increase in the ratio of the organic solvent modifier resulted in a decrease in the retention, as generally observed in RP-HPLC. On the contrary, the retention behaviours of His and MIH with the acetate mobile phase was entirely different from that with the phosphate system. Thus, His and MIH showed an increase in retention when the ratio of organic solvent modifier was increased, and greater retention was obtained with acetonitrile than with methanol. When the mobile phase contained more than 40% methanol or more than 20% acetonitrile the elution sequence was entirely reversed, to DIH, MIH and His, which

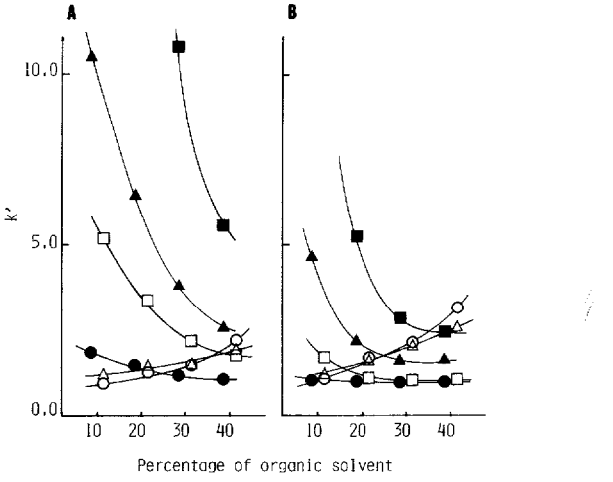

Fig. 6. Influence of the ratio of the organic solvent on retention. Mobile phases: (A) methanol in 50 m*M* acetic acid; (B) acetonitrile in 50 m*M* acetic acid. Column: NC_{18}. Compounds as in Fig. 2.

was unexpected based on the hydrophobicities of these compounds. The mode of retention of His and MIH was entirely different from that anticipated in the reversed-phase elution profile, and the elution pattern observed was that of a polar phase elution. Such retention behaviour was reported in the separation of iodotyrosines and iodothyronines with a mobile phase system containing a high ratio of organic solvent modifier[17]. It was also observed for His and MIH when a propionic acid-containing solution or methanol (or acetonitrile)–water without salt was used as the mobile phase. However, normal reversed-phase elution was observed when trifluoroacetic acid, having a dissociation constant higher than those of acetic acid and propionic acid, was used, or an inorganic salt like NaCl was added to the mobile phase, methanol (or acetonitrile)–50 m*M* acetic acid. Therefore, a polar phase elution mode might be observed in a mobile phase with low ionic strength.

The retention behaviour of iodohistidines was dependent on the nature of the stationary phase when the mobile phase containing acetic acid was used. The separation of iodohistidines was compared on three different columns using 30% acetonitrile in 50 m*M* acetic acid as the mobile phase (Fig. 7).

The retention of iodohistidines as well as iodotyrosines on the NC_{18} column was smaller than that on the RC_{18} column, and larger than that on the MC_{18} column. The marked increase in the retention of iodohistidines on RC_{18} might be due to strong ionic interaction between the silanol groups on the stationary phase and the iodohistidines. The different retention behaviours with phosphate and acetate are notable: that is, when 30% acetonitrile in 50 m*M* phosphate buffer, pH 3.0, was used as the mobile phase, the separation mode was little altered by the stationary phase,

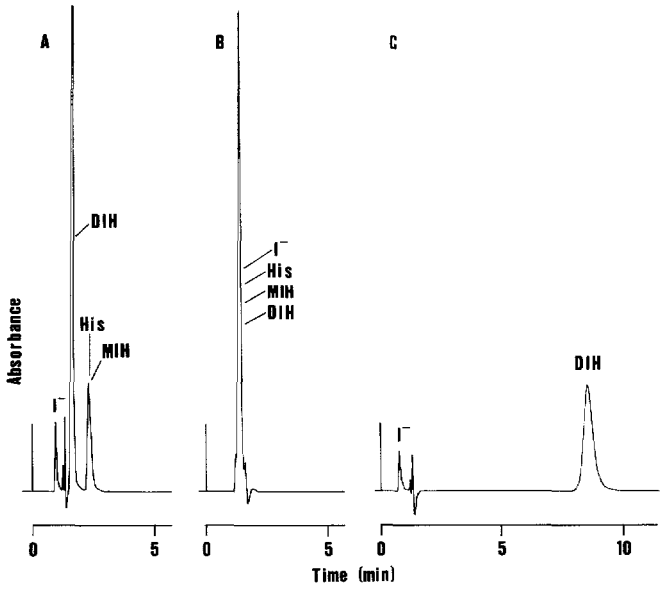

Fig. 7. HPLC separation of iodohistidines on different columns. Mobile phase: 30% acetonitrile in 50 m*M* acetic acid. Detector: wavelength, 254 nm; sensitivity, 0.02 a.u.f.s. The elution position of His was confirmed by separate injection and detection at 235 nm. Other conditions as in Fig. 4. His and MIH were not eluted within 10 min on the RC_{18} column.

while when acetate was used the separation was altered by the column used. Thus, the physicochemical properties of the stationary phase might be influential with a mobile phase solvent of low ionic strength at lower pH.

The relatively large retention of iodohistidines due to the polar phase elution mode or interaction with silanol groups is accompanied by broadening and tailing of the peaks, so that this system may not be adequate for practical purposes.

CONCLUSIONS

The separation characteristics of iodohistidines in RP-HPLC were investigated in comparison with iodotyrosines. A mobile phase composed of phosphate buffer did not give sufficient retention upon altering the pH and the ratio of the mobile phase solvents, so that satisfactory separation of iodohistidines was not achieved. Therefore, it is necessary to add adequate hydrophobic ion-pairing agents to increase the retention for a better separation. The characteristics of the stationary phase may be the influential factors when a mobile phase of about neutral pH or with low ionic strength is used. From the retention behaviours of iodohistidines in RP-HPLC, it may be concluded that an acidic mobile phase with high ionic strength, containing alkanesulphonic acid, should be the most suitable for the present purpose.

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