Journal of Chromatography, 322 (1985) 117–128
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,462

SEPARATION AND PURIFICATION OF DIASTEREOMERS OF ANGIOTENSIN I BY WEAK ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received December 4th, 1984)

SUMMARY

Several diastereomers of angiotensin I were resolved by weak anion-exchange high-performance liquid chromatography (HPLC). All of the diastereomers which were examined contained significant amounts of peptides whose amino acid composition differed from the designated diastereomer of angiotensin I. The des-Asp[1] forms of angiotensin I and the impurities were weakly retained on the anion-exchange column and were well separated from the rest of the peptides. Many of the impurities contained fractional amounts of amino acids suggesting that peptides which contained variable amounts of histidine and arginine are not resolved by this method. The results are compared with the results of separations of the same peptides by reversed-phase HPLC. This comparison strongly suggests that the two HPLC methods, utilizing different separation principles, are complementary; hence their combined use leads to a more confident assessment of the purity of a given peptide preparation.

INTRODUCTION

The resolution of biologically active peptides from closely related compounds and the characterization and quantification of these compounds is essential for quality control in peptide synthesis. It has been demonstrated that synthetic peptides can contain significant quantities of closely related substances[1]. These peptide "impurities" represent variations in amino acid sequence which may be caused by amino acid additions or deletions, racemizations leading to D-amino acids, or peptide bond formation with β- or γ-carboxyl groups. Reversed-phase high-performance liquid chromatography (RP-HPLC) has been used extensively to evaluate the purity of peptides[2–16]. Recent studies[17] have shown that diastereoisomers of angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) can be resolved by RP-HPLC from one another, and from impurities which were present in each of the peptide preparations. The peptide impurities, which were detected in the angiotensin I samples, represented either deletions or additions of amino acids. Several of the impurities exhibited un-

usual amino acid compositions, which suggested that they contained more than one impurity. This observation led us to examine other HPLC methods for evaluating the purity of these diastereomers of angiotensin I. Furthermore, examination of the purity of a substance by a second method based on a different principle of separation is additional assurance that impurities have not been strongly bound to a particular support medium, and hence not detected.

Dizdaroglu and co-workers[18–23] have demonstrated the use of weak anion-exchange HPLC for peptide separations. This method separated closely related peptides, such as analogues of angiotensins and neurotensins as well as their diastereomers[20,21,23]. In the present work, we demonstrate the application of this method to the separation of several diastereomers of angiotensin I and some of the impurities present in these samples. These results will then permit the comparison of reversed-phase[10] and anion-exchange[18–23] HPLC methods of peptide separation.

EXPERIMENTAL*

Separations were performed according to the method reported by Dizdaroglu *et al.*[19]. The apparatus and materials were as described previously[19]. The column was equilibrated with a solution containing acetonitrile (solvent A)–0.01 *M* triethylammonium acetate (TEAA) buffer, pH 6.0 (solvent B) (1:3, v/v), and the peptides were eluted with either a two-step linear gradient starting from 24% B with a rate of 0.08% B/min for 25 min, then 0.25% B/min for 30 min; or a linear gradient starting at 25% B with a rate of 0.5% B/min. Specific conditions are described in the figure captions. Peaks were collected as indicated on the chromatograms in Figs. 3–7, and their amino acid composition was analyzed according to the method of Benson and Hare[17] as adapted by Margolis and Konash[10]. The peptides containing D-amino acids were a gift from Dr. Susan Oparil of the University of Alabama Medical School, Birmingham, AL (U.S.A.). All peptides were stored at −20°C and were dissolved at the time of analysis in distilled water to a concentration of 1 mg/ml. The D-amino acid content of each sample had been determined previously[10]. The mobile phase used for the amino acid determinations (Femto Buffers) did not permit the analysis for proline.

RESULTS

The diastereomers of angiotensin I (AI) were resolved from one another and from AI (Fig. 1). Although baseline resolution was not achieved between AI and (D-Pro[7])-AI the use of a less steep gradient may separate these compounds more completely. It is apparent from this chromatogram that there are a number of other materials present in this mixture of six peptides. Consequently, we examined their purity in greater detail. The only pure peptide was the angiotensin I which has been certified as Standard Reference Material 998 (fig. 2).

The chromatograms of the individual diastereomers are illustrated in Figs.

* Identification of any commercial products or equipment does not imply recommendation or endorsement by the National Bureau of Standards nor does it imply that the material or equipment identified is necessarily the best for the purpose.

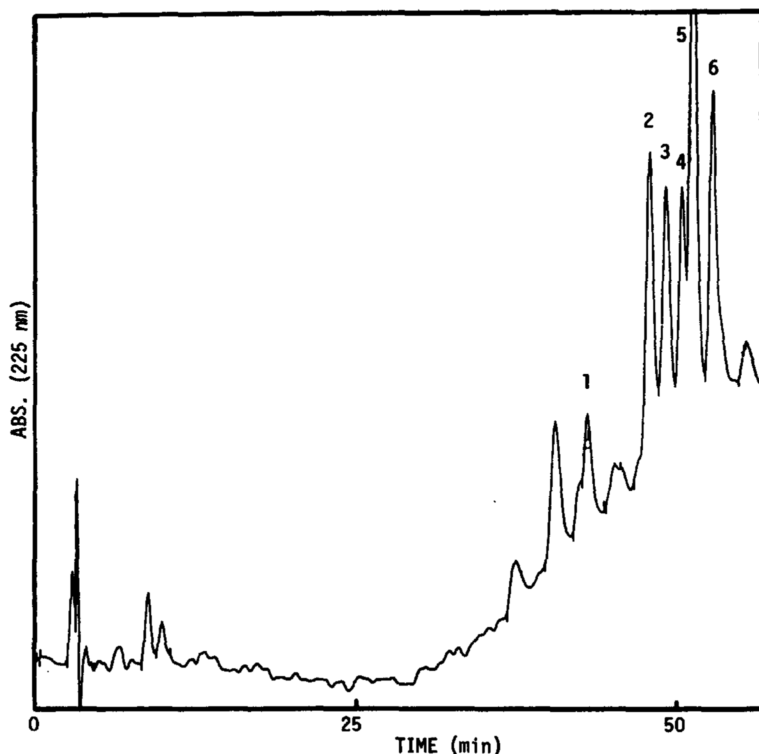

Fig. 1. Separation of some diastereomers of angiotensin I. Column, MicroPak AX-10 (10 μm), 30 × 0.4 cm. Temperature, 50°C. Eluent: A, acetonitrile; B, 0.01 *M* triethylammonium acetate buffer (pH 6.0). Gradient program: linear starting from 24% B with a rate of 0.08% B/min for 25 min, then 0.25% B/min. Flow-rate, 1 ml/min. a.u.f.s., 0.1 at 225 nm. Peaks: 1 = (D-*allo*-Ile^5)-AI; 2 = (D-Leu^{10})-AI; 3 = (D-Phe^8)-AI; 4 = (D-Pro^7)-AI; 5 = AI; 6 = (D-Asp^1-Ile^8)-AI.

3–7, showing that none of the peptide samples are homogenous. The errors in amino acid composition found in the impurities in these synthetic diastereomers of AI fall into four general classes; deletions, additions, fractional reductions and fractional additions of specific amino acids. Peaks 1–4 and 6 of (D-Phe^8)-AI (Fig. 3) are characterized by fractional additions of combinations of Asp, Tyr or Arg, and fractional deletions of His (Table I). Peak 8 contains reduced amounts of Ile, Val and Tyr. (D-Asp^1-Ile^8)-AI contained two major impurities (Fig. 4); peak 1 which contains a deletion error of one mole of Ile and one mole of Asp, and the addition of one mole of Leu; peak 2 which contains a deletion error of one mole of Ile and one mole of Tyr, a fractional reduction in His and a fractional increase in Arg (Table II). Similar results are seen in the analyses of the impurities of (D-Pro^7)-AI, (*allo*-Ile^5)-AI, and (D-Leu^{10})-AI (Figs. 5, 6 and 7, and Tables III, IV and V, respectively). Peak 1 from (D-Pro^7)-AI lacks Asp, peaks 2–4 and 6 contain fractional deletions of Tyr and Phe, and a fractional increase in His (Table III). Peak 1 from (D-Leu^{10})-AI contained no Tyr and a fractional deletion of His (Table V). Peak 1 from (*allo*-Ile^5)-AI lacks Tyr, while peaks 4 and 5 contain fractional increases in Phe, His and Arg, peak 2 contains an additional mole of Arg, and peak 4 contains an additional mole each of Arg and

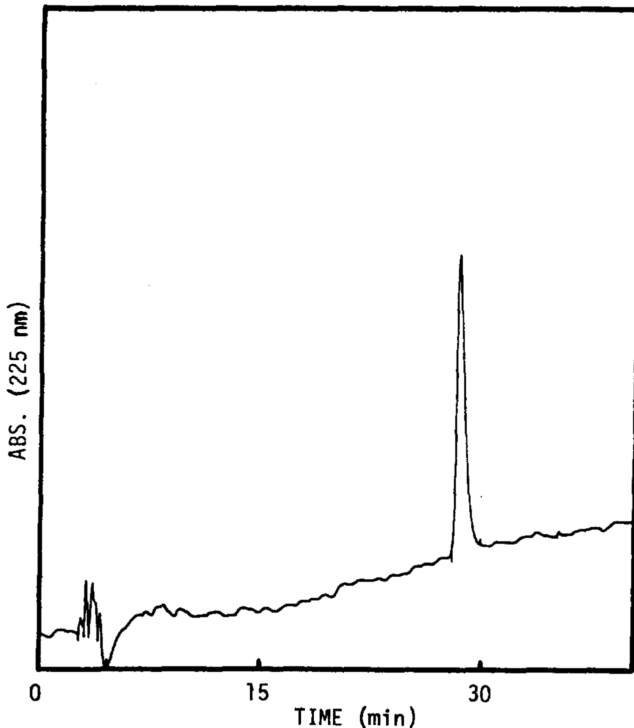

Fig. 2. Chromatographic analysis of the purity of AI (SRM 998). Column details as in Fig. 1 except temperature, 30°C and gradient program starting from 25% B with a rate of 0.5% B/min.

Leu (Table IV). In every case, the major component in a given chromatogram is the peptide with the assigned composition. This peak represents 46–77% of the total peptide content.

The presence of the indicated peptide as the predominant form and the wide range of non-terminal additions and deletions is evidence that it is unlikely that this method of amino acid analysis generates errors in the determination of the amino acid composition. This conclusion is further substantiated by the absence of fractional deletions and additions in both peptides represented by peaks 1 of (D-Pro^7)-AI and (*allo*-Ile^5)-AI in Tables III and IV. In both cases, the peptides are present in small amounts (4.5 and 13.7%, respectively), and both peptides are characterized only by the complete deletion of a single amino acid. Finally, it is important to note that: (a) the peptides identified in each table were hydrolyzed at the same time as a standard containing equimolar amounts of each amino acid; and (b) that the variation from the stated composition is not a systematic error resulting from the hydrolysis procedure or the amino acid analysis of the peptides.

Each peptide contained a number of impurities which were present in very small amounts. This accounts for the incomplete recoveries of total peptide material, as assessed by peak areas of the chromatograms. This is most evident in the chromatograms for the D-Phe^8 and the D-Pro^7 diastereomers (Figs. 3 and 5, respectively). The number of impurities was so complex that it was possible to resolve completely

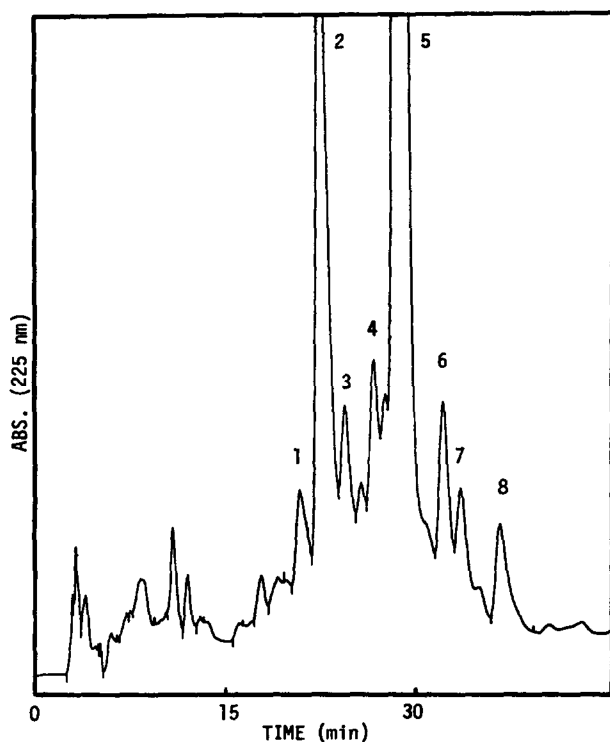

Fig. 3. Separation of (D-Phe[8])-AI from its impurities. Column details as in Fig. 1 except temperature, 30°C and gradient program starting from 25% B with a rate of 0.5% B/min. Peak identification is given in Table I.

TABLE I

AMINO ACID COMPOSITION OF PEAKS FROM THE CHROMATOGRAM OF (D-Phe[8]) ANGIOTENSIN I

The values in this table represent the relative number of moles of each amino acid.

Amino acid	*Peak number*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Asp	0.96	0.97	1.08	0.98	1.11	1.39	1.81	1.09
Val	0.98	0.89	0.96	0.95	0.88	0.93	0.87	0.22
Ile	1.07	0.99	1.02	1.02	0.92	0.98	1.08	0.26
Leu	1.01	1.05	1.06	1.08	1.09	1.09	1.08	0.95
Tyr	0.69	0.36	0.74	0.82	1.03	0.93	0.87	0.20
Phe	0.97	1.06	0.99	0.98	1.06	1.07	0.97	1.00
His	1.60	2.06	1.92	1.97	1.95	2.58	2.23	0.97
Arg	1.59	1.67	1.52	1.33	1.04	1.94	1.95	1.00
Met	0.60							
allo-Ile			0.10					
Peak area*	1.4	13.1	2.7	3.1	65.1	3.1	2.3	1.8
Retention time	20.8	22.5	24.3	26.6	28.6	32.0	33.4	36.4

* Value equals percentage of the total peak area of chromatogram.

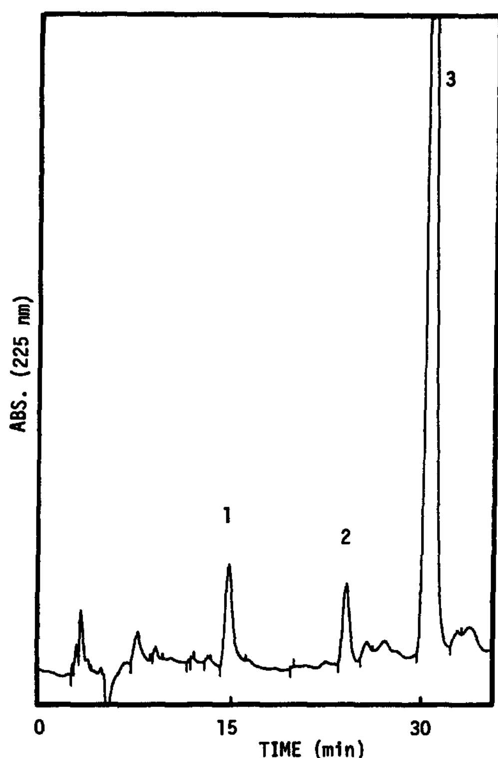

Fig. 4. Separation of (D-Asp[1]-Ile[8])-AI from its impurities. Column details as in Fig. 2. Peak identification is given in Table II.

TABLE II

AMINO ACID COMPOSITION OF PEAKS FROM THE CHROMATOGRAM OF (D-Asp[1]-Ile[8]) ANGIOTENSIN I

The values in this table represent the relative number of moles of each amino acid.

Amino acid	*Peak number*		
	1	*2*	*3*
Asp	0	0.91	1.01
Val	0.98	0.94	0.89
Ile	0.94	1.03	1.81
Leu	2.18	1.03	1.09
Tyr	0.99	0	0.96
His	1.75	1.36	1.95
Arg	1.75	1.30	1.13
Peak area*	4.4	4.8	77.4
Retention time	14.8	24.2	30.7

* Value equals percentage of the total peak area of chromatogram.

TABLE III

AMINO ACID COMPOSITION OF PEAKS FROM THE CHROMATOGRAM OF (D-Pro[7]) ANGIOTENSIN I

The values in this table represent the relative number of moles of each amino acid.

Amino acid	*Peak number*					
	1	*2*	*3*	*4*	*5*	*6*
Asp	0	0.99	1.14	0.94	1.08	1.42
Val	1.02	0.98	0.99	0.91	0.95	0.91
Ile	1.08	1.05	0.92	1.01	0.87	0.92
Leu	1.02	1.04	0.92	1.04	1.10	1.15
Tyr	0.83	0.16	0.24	0.61	0.98	0.86
Phe	0.97	1.01	0.39	0.64	1.01	0.60
His	1.96	2.04	1.84	2.02	1.96	2.32
Arg	1.08	1.04	1.12	1.10	1.01	1.33
Peak area*	4.6	15.9	2.8	4.5	46.2	4.9
Retention time	8.9	23.2	26.4	27.6	29.6	33.8

* Value equals percent of the total area of the chromatogram.

and identify all of them. This inability to resolve all of the minor constituents is believed to be the reason for the observation of fractional deletions and additions.

This method readily resolves the des-Asp[1] peptides from the rest of the impurities (retention times of 14.8 and 8.9 min in Figs. 4 and 5, and in Tables II and III, respectively). The remaining peptides are grouped in a narrow region with retention times between 20 and 36 min. The inability to resolve completely closely related peptides that contain a terminal aspartic acid residue indicates a limitation of this method. This problem is most dramatically illustrated by inspection of the chro-

TABLE IV

AMINO ACID COMPOSITION OF PEAKS FROM THE CHROMATOGRAM OF (*allo*-Ile[5]) ANGIOTENSIN I

The values in this table represent the relative number of moles of each amino acid.

Amino acid	*Peak number*				
	1	*2*	*3*	*4*	*5*
Asp	1.07	1.05	1.03	1.97	1.38
Val	1.00	1.04	0.89	0.95	0.99
allo-Ile	1.00	0.97	1.03	0.94	1.00
Leu	1.08	1.06	1.02	1.80	1.05
Tyr	0	0.94	1.04	0.92	0.98
Phe	1.03	1.01	1.01	1.10	1.31
His	1.82	2.04	2.04	2.08	2.84
Arg	0.92	1.87	0.89	1.73	1.34
Peak area*	13.7	7.7	57.6	5.8	8.8
Retention time	20.3	21.6	25.7	27.3	30.7

* Value equals percent of the total area of the chromatogram.

TABLE V

AMINO ACID COMPOSITION OF PEAKS FROM THE CHROMATOGRAM OF (D-Leu[10]) ANGIOTENSIN I

The values in this table represent the relative numbers of moles of each amino acid.

Amino acid	*Peak number*	
	1	*2*
Asp	1.05	0.93
Val	0.94	0.91
Ile	0.95	1.05
Leu	1.00	1.08
Tyr	0	0.92
Phe	0.95	1.01
His	1.74	2.18
Arg	0.98	1.01
Peak area*	9.3	74.2
Retention time	20.2	27.4

* Value equals percent of the total area of the chromatogram.

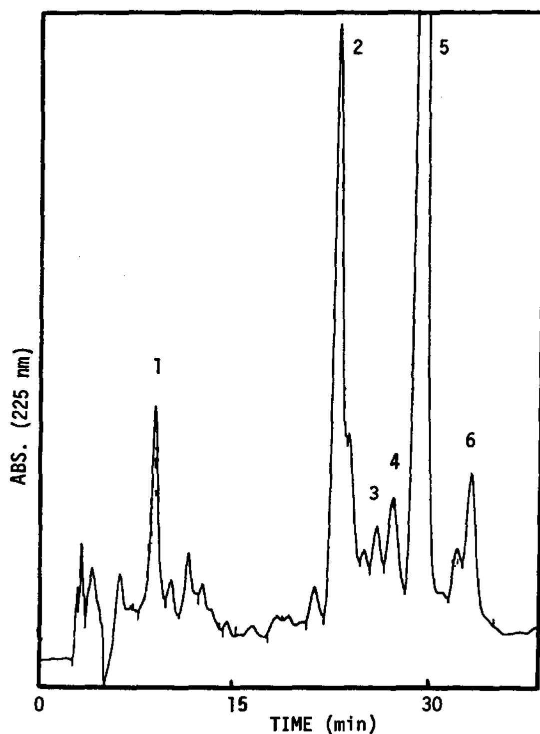

Fig. 5. Separation of (D-Pro[7])-AI from its impurities. Column details as in Fig. 2. Peak identification is given in Table III.

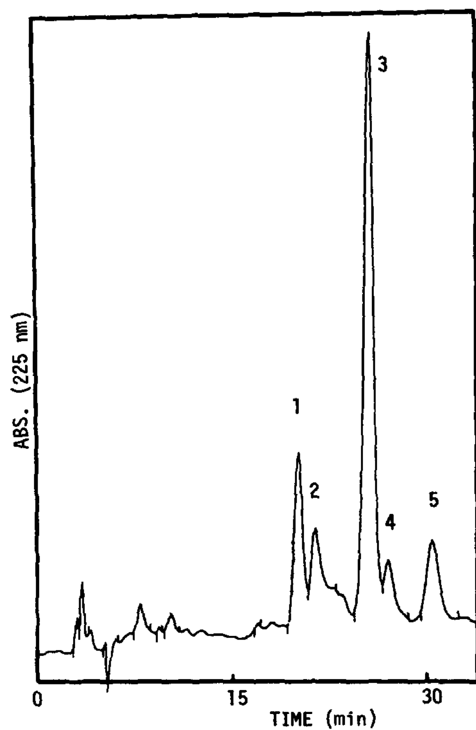

Fig. 6. Separation of (D-*allo*-Ile⁵)-AI from its impurities. Column details as in Fig. 2. Peak identification is given in Table IV.

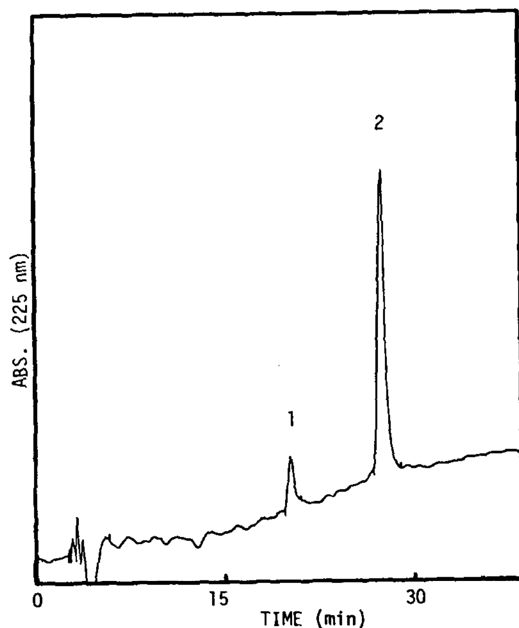

Fig. 7. Separation of (D-Leu¹⁰)-AI from its impurity. Column details as in Fig. 2. Peak identification is given in Table V.

TABLE VI

COMPARISON OF THE RETENTION TIMES OF THE DIASTEREOMERS OF ANGIOTENSIN I ON REVERSED-PHASE AND ION-EXCHANGE HPLC COLUMNS

Angiotensin I diastereomer	*Retention time (min)*	
	*Reversed-phase**	*Ion-exchange*
(D-Asp[1])-AI	8.5	30.7
(D-Leu[10])-AI	10.1	27.4
AI	17.2	30.0
(*allo*-Ile[5])-AI	21.2	25.7
(D-Phe[8])-AI	28.0	28.6
(D-Pro[7])-AI	30.0	29.6

* Ref. 10.

matogram of (D-Phe[8])-AI (Fig. 3) and of the summary of the amino acid analyses of the constituent peptide peaks (Table I). The chromatograms of the four other sample peptides are less complex; however, even peak 2 from (D-Asp[1]-Ile[8])-AI (Fig. 4) (which appears as a single symmetrical peak) contains two peptides which differ by one amino acid in their histidine and arginine content (Table II). These results suggest that the weak anion-exchange resin which was used for this analysis does not strongly retain peptides that do not contain an acidic amino acid. Further, this resin does not effectively resolve peptides that differ by one amino acid in Arg and/or His content, a characteristic of nearly every unresolved group of peptides.

DISCUSSION

The objective of this study is to demonstrate that weak anion-exchange HPLC[19] successfully resolves the same group of diastereomers of AI that was resolved by RP-HPLC[10]. The results demonstrate that weak anion-exchange HPLC resolves a distinct constellation of impurities, the composition of which is very difficult to correlate with those impurities resolved by RP-HPLC[10]. These results strongly support the concept that a single HPLC method is insufficient to assess the purity of a given peptide preparation as a recent study suggests[22], even though a number of reports document remarkable resolving powers for both types of HPLC methods[11,23].

A comparison of the retention times of the diastereomers of AI for each of the HPLC methods is given in Table VI. There is no apparent correlation between the structure and the retention time observed for each compound with each method. (D-Asp[1]-Ile[8])-AI has the shortest retention time with the reversed-phase method and the longest retention time with the anion-exchange method, whereas (*allo*-Ile[5])-AI has the shortest retention time with the anion-exchange method and a mid range retention time with RP-HPLC. The degree of resolution of the various diastereomers by each HPLC method appears to be equivalent with some diastereomers not being completely resolved. It is apparent that the combined use of these methods yields increased information on the stereochemical purity of the peptides.

Both methods indicate that these peptides (except for AI) are not homoge-

neous. From the results summarized herein, it is apparent that the peptide fractions which were separated by RP-HPLC were not homogeneous. It is probable that almost every collected peptide fraction contained more than one peptide (including the allegedly "pure" fractions). A greater number of peptide fractions were obtained by the anion-exchange HPLC method indicating that this method more effectively evaluates the composition of the impurities that may occur in incompletely purified samples of angiotensin I. Amino acid analysis of these fractions indicates that with possibly few exceptions each of the fractions contains more than one species of peptide.

Many of the peptide fractions accounted for less than ten percent of the total area of the peptide peaks. Since the fractions were detected at 225 nm, peptides including those lacking the aromatic amino acids were detected. Even at this wavelength the extinction coefficients of the individual amino acids differ significantly and, therefore, it is invalid to quantitatively compare the amount of peptide in each peak on the basis of peak area. However, the presence of an impurity at the 5% level in a given peptide fraction would go undetected in the present study because of the level of precision of the amino acid analysis (Tables I–V). It is clear that the anion-exchange HPLC method resolves a larger number of impurities than the reversed-phase method.

There are compounds that elute during the first 15 min [*e.g.*, (D-Phe^{8})-AI, Fig. 2] which were not collected or analyzed. All of the Asp deficient peptides were eluted from the anion-exchange column during the first 10 min. This is consistent with previous reports and most likely accounts for the discrepancy between the two HPLC methods in the content of Asp-deficient peptides.

It is unlikely that these impurities are the result of column artifacts or peptide degradation, since the Standard Reference Material (SRM) AI chromatographed as a single peak and had a correct amino acid composition. The extent of degradation of some amino acids is known to be affected by the hydrolytic conditions used[14]. Of the amino acids composing angiotensin, this concern applies particularly to tyrosine. However, in our analysis, we simultaneously hydrolyzed all of the peptide fractions from one or more chromatographic runs to assure the similarity of hydrolytic conditions. Further, a standard equimolar mixture of amino acids was included in each hydrolytic run. The amino acid content of each of the unknown peptide fractions was normalized to the standard mixture to correct for the degradation of amino acids such as tyrosine. Thus, the validity of the comparisons of the amino acid composition of peak fractions from a single or group of chromatographic runs was established.

These results indicate that anion-exchange HPLC resolves the tested diastereomers of angiotensin I. This method also resolves a significant number of peptide impurities from each diastereomer. A comparison of these results to those obtained by RP-HPLC[10] indicates that the order of elution of the diastereomers is different and that different groups of peptide impurities are resolved. Together the results of these studies, using different separation principles, indicate that these methods are complementary and both should be used in the assessment of peptide homogeniety.

ACKNOWLEDGEMENTS

We wish to thank Dr. Susan Oparil of the University of Alabama School of Medicine for her generous supply of synthetic peptides and Robert Weker for his technical assistance.

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