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U.S. DEPARTMENT OF COMMERCE

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Introduction

During the tenure of this contract, a series of methods were evaluated and some were developed for assessing peptide purity, particularly the purity of Angiotensin I. Our objectives were: 1) the detection, characterization and quantitation of impurities (peptide and non-peptide) in Angiotensin I samples; 2) the selection of methods for peptide quantitation; and, 3) the development of specifications and criteria by which a peptide may be assessed for certification as a Standard Reference Material (SRM).

Thin-layer chromatography (TLC) methods and high performance liquid chromatography (HPLC) methods were evaluated for use in separating the impurities from Angiotensin I. Substances separated from Angiotensin I were analyzed for their amino acid content and composition by the methods described in the attached reprints. Unfractionated peptide was analyzed for D stereoisomeric amino acids by three methods. Total peptide was measured by three methods. Non-peptide substances were studied to determine their possible origin, their relative and total proportion, their possible contribution to A_{254} nm absorbing components in the samples, and their physical and chemical properties when these materials were amenable to analysis.

Some of the results reported herein were obtained with the cooperation of Drs. Jean Sealey (Cornell Medical Center, N.Y., N.Y.), Mahesh Khosla (Cleveland Clinic, Cleveland, OH) and P. E. Hare (Carneige Institute, Washington, D.C.).

Methods for Analysis of Peptide Purity

We have developed a high performance liquid chromatography (HPLC) method [2] that is capable of resolving very closely related peptides. By modifying the solvent system that was used in the method of Rivier [1]. Our method is able to resolve such closely related peptides as the [D-Pro]-, [D-Phe,]-, [aIle]-, [D-Leu]-, and [D-asp,Ile^8]- analogs of Angiotensin I and provides an important new means of determining the purity of Angiotensin I and Angiotensin II. The presence of the D amino acid was confirmed by GC analysis of the peptide hydrolysate on a chiral column [3]. In addition to resolving closely related peptides, peptides characterized by omissions and duplications of one or more amino acids, can be resolved and characterized. We were not able to test the resolution of the method for truncated "Angiotensin I peptides" or those with duplicate amino acids. because the appropriate analog peptides are not available. However, such analogs of Angiotensin II are available, and they were tested.

A. Resolution of Peptides with Amino Acid Composition Similar to Angiotensin I

The capability of the HPLC for resolving peptide products differing because of errors in peptide synthesis has not been exhaustively studied, but Table I illustrates the resolving capacity of the acetonitrile-triethylamine phosphate buffer system for peptides closely related to Angiotensin I. Diasterioisomers of Angiotensin I containing either D-Pro, D-Phe, or aIle can be separated from each other and from Angiotensin I (figure 1 and Table 1). Also [des Asp] [aIle]- Angiotensin I can be resolved from [aIle]-Angiotensin I.

[D-Phe]-Angiotensin I was resolved from a peptide having 50 percent less aspartic acid. This aspartic acid deficient peptide may be composed of either identical sequences on the α and β carboxyl groups of the Asp or a sequence with duplication of each residue except the N-terminal amino acid (figure 1, Table 1, peak 5 vs. 4). A similar error is seen in the comparison of peaks 10 and 11. The resolution of [des Asp],[Ile 8]- from [Ile 8]-Angiotensin I suggests that this HPLC technique can also separate the des[Asp]-nonapeptide analog of Angiotensin I. The resolution of a group of impurities from [D-Pro]-Angiotensin I having reduced proportions of Phe and extra His and Arg demonstrates

that products resulting from extensive errors in peptide sequence may be easily separated from Angiotensin I. [D-Leu]-Antiotensin I (Figure 1 peaks 2 and 3) partially coeluted with products having an erroneous composition; however, it was adequately separated from Angiotensin I. Deviations in the amino acid composition of these impurities demonstrate the need for analyzing the amino acid composition of each peak. These observations suggests that impurities having a retention time identical to that of Angiotensin I may exist; however, our compositional analysis of the Angiotensin I peak suggest that none are present.

B. Resolution of Truncated Peptides Related to Angiotensin II

Des[Asp], Des[Asp, Arg], and des[Asp, Arg, Val] Angiotensin II exhibit retention times which indicate that they can be separated from each other and from Angiotensin II (figure 2, Table II). Similarly, des[Phe]-Val^5-Angiotensin II was separated from [Val^5]-Angiotensin II. These samples also contained at least one additional peptide which exhibited either a duplication of an amino acid (His in peak 11) or truncation of the peptide (particularly the Tyr in peaks 3 and 6 and possibly in peak 11 as well as His in peak 9). By HPLC it was also possible to separate [Val^5]-Angiotensin isomers that differed in one amide linkage, i.e., of the Asp by the amide bond of either the α or β carboxyl group to the α amino group of the Arg (Figure 3). Furthermore, the β Asp

isomer of [val^5] Angiotensin II contained a small amount of the α isomer.

C. Commerical Angiotensin I and II: Analysis by HPLC and Identification of the Impurities

The purity of commercial Angiotensins I and II was examined by our HPLC method. The results of these studies are reported in the appended reprints. Briefly, Angiotensin I and II contain one peptide impurity each. Each impurity appears to form during storage of the peptides. Angiotensin I was found to contain also a trace component, detectable only by radioimmunoassay (RIA) (Table III Fraction 12-14). The trace RIA-positive material cochromatographed with 3 ultraviolet (UV)-positive (254 nm) components which were peptide-free by amino acid analysis. These UV positive components (12, 13, 15 min. ret. time) were not present in crude Angiotensin I; therefore, we believe, they were introduced during the purification step and are not related to the RIA positive material (see appended preprint). Four additional non-peptide components with absorbance at 254 nm were detected; however, they were found only after the aqueous solution of the peptide was allowed to come into contact with the rubber stopper of the vial. The presence of HPLC peaks due to these materials could be avoided by not exposing the peptide solution to the stopper. The chemical composition of the other non-peptide components was not pursued when it was found that they evaporated with the aqueous solvent at 100 °C under a stream of nitrogen.

The peptide impurity in commercial Angiotensin I had the same amino acid composition as Angiotensin I (Table IV). An analogous result was found for the peptide impurity in Angiotensin II (Table V).

Angiotensin I and the peptide impurity were separated on the octadecylsilane column and collected. Each sample was dried under N_2 at 100 °C, dissolved in a neutral bicarbonate buffer and tested in a pressor assay on rats by Dr. M. Khosla. In the biological assay, the peptide impurity from the Angiotensin I preparation exhibited approximately 30 percent of the expected specific activity and the Angiotensin I exhibited approximately 20 percent of the expected biological specific activity thus indicating that the materials eluted from both peaks were biologically active peptides. The loss of biological activity probably occured during the concentration of the samples prior to bioassay. This biological active impurity was found in Angiotensin I that had been stored in its original dry form for more than one month at 4 °C It was not detected in either of two different lots of Angiotensin I from Beckman obtained 9 months apart when they were analyzed immediately upon receipt. (The samples were examined on the same column). For fresh and stored Angiotensin I the recovery of the Angiotensin I in the HPLC experiments was 95 percent of the total amount of peptide applied to the column based on the amount of peptide

stated on the label, when the recovery was determined either by amino acid analysis or by RIA. Furthermore, the Angiotensin I measured by RIA in the collected chromatographed peaks was equivalent to that measured in the unfractionated material, indicating that the fractionated impurities which are not included in the weight stated on the label do not modify the RIA method of analysis.

D. The Analysis of Commerical Angiotensin I by TLC and Electrophoresis

The lack of peptide impurities in commercial Angiotensin I is consistent with the general findings obtained by TLC (Figure 4). With the possible exception of a peptide impurity, evident as a small extended tail which was barely detectable on cellulose using n-butanol, pyridine, water (65:35:35) as the solvent and the Pauly reagent for detection, there was no other evidence of peptide impurities.

Only a single spot was observed on cellulose using n-butanol, acetic acid, water (4:1:5); n-butanol, pyridine, water (65:35:65) and n-butanol, acetic acid, water, pyridine (15:3:12:10).

A single peptide impurity which migrated slightly slower than Angiotensin I was detected by electrophoresis at pH 8.6. A single spot was observed at pH 2.5.

E. Angiotensin I Analysis for D-Amino Acids in the Peptide

The presence of "Angiotensin I peptides" containing D amino acids should be of serious concern because these

types of diastereomers differ in biological activity from Angiotensin I. Several such diasteriomers of Angiotensin I can be separated from Angiotensin I by HPLC (section A). Kohsla has demonstrated that [D-His]-angiotensin I is separable from the [L-His]-diasteriomer by TLC on cellulose in n-butanol, pyridine, water (65:35:35) [5]. Frank et al [3] demonstrated that chromatography of derivatives of hydrolysates of peptides on a chiral column can provide a rapid quantitative method for the separation and measurement of the D & L amino acids. The examination of Peninsular and Beckman Angiotensins by TLC revealed no detectable [D-His]-Angiotensin I (Figure 5) (interpretation of M. Khosla). Also, examination of the 2-OH propyl TFA derivatives of the amino acids from hydrolysates of new and aged Beckman Angiotensin I did not reveal significant amounts of D amino acids (i.e., greater than 1%) above the amount generated by the racemization that occurs during hydrolysis. This method was not useful for the analysis of D and L His or Arg. We expect to use a method being developed by E. Gil-Av for these amino acids. Thus three independent methods failed to reveal the presence of diasteriomers.

F. Angiotensin I Analysis for Non-Peptide Impurities

Analyses for non-peptide impurities were performed by three independent methods: 1) detection of HPLC peaks absorbing UV light at 254 and 280 nm; 2) mass spectrometric

analysis, using a programmed volatilization for materials that evaporate in the mass spectrometer between 30 and 300 °C; 3) amino acid analysis by the method of standard additions. The UV absorbing non-peptide impurities which are described in section C and the appended reprint arise from two sources: a group of three HPLC peaks eluting before Angiotensin I that appear to be introduced during the purification steps, and a group of four HPLC peaks eluting after Angiotensin I that are from the rubber stoppers.

The mass spectrometric analysis indicated the presence of dimethyl siloxane polymers, triethylamine, acetic acid, and a compound which decomposes above 300 °C with the release of benzyl alcohol. The first three of these compounds absorb little or no UV light and thus are not detected by simply monitoring the UV absorbance at 254 nm or 280 nm.

The total amount of impurity in the Angiotensin I was determined by a standard addition experiment. Lyophilized Angiotensin I was weighed and dissolved in distilled water. Phe was weighed and dissolved in 0.1 mol/L KOH in a volumetric flask. The concentration of this solution was confirmed by measuring its absorbance at 258 nm and calculating its concentration by use of the published molar extinction coefficient 209.6. Phe was added to aliquots of an Angiotensin I solution in amounts equal to 0, 1, 2, 3 and 4 times the approximate content. The 5 solutions were dried

in a stream of nitrogen, hydrolyzed for 20 min in 6 mol/L HCl at 155 °C, dried again, taken up in a suitable amount of solvent and analyzed on the amino acid analyzer. The Phe content of each sample was normalized against each of four amino acids in the Angiotensin I hydrolysate and then plotted against the calculated amount of Phe added to each sample. The data were analyzed by linear regression and the amount of Phe present in the Angiotensin I was determined from the intercept of the line with the x axis (concentration scale). From this number the Angiotensin I content was calculated and compared to the weight of Angiotensin I that had been added to each sample. The results of this calculation indicate that 5 percent of the weight of the Angiotensin I was non-peptide (Table VI). This value was found to be independent of the amino acid used to normalize the Phe concentrations. This 5 percent non-peptide impurity value includes those non-peptide impurities detected by HPLC and MS.

G. Angiotensin I – A survey of UV Impurities in Six Commerical Lots

To accomplish the objective of surveying the purity of commercial Angiotensin I, samples from various lots obtained from several commercial sources were dissolved in water to a final concentration of 1 mg/mL (based upon the suppliers stated peptide content). Thus these results reflect both the peptide impurities and non-peptide

impurities that absorb at 254, 280, or 220 nm and inaccuracies and variation in peptide content between vials and between lots. The impurities detected at 254 nm also absorbed at 280 and 220 nm. The non-peptide impurities exhibited greater absorbance at 280 nm while the peptides exhibited greater absorbance at 220 nm.

We evaluated the purity of 6 lots of Angiotensin I from three different primary sources. One to three vials were examined from each lot. Each value is the average of duplicate measurements. We attempted to isolate and identify the impurities but were unsuccessful in our attempts to concentrate them after they were collected. Therefore, the peak areas reflect only a concentration relative to the total absorbance, since the chemical structure and molar absorbtivity of the material in these peaks is unknown.

Two independent HPLC systems of analysis were used. One system employed an aminosilane column (Waters Associates) with 0.001 mol/L acetic acid:methanol, 3:7 (V/V) as the mobile phase and the other system utilized an octadecylsilane column (DuPont Zorbax ODS) with 0.1 mol/L triethylamine phosphate (pH 3.5):acetonitrile, 81:19 (V/V) as the mobile phase. Table VII summarizes the relative peak areas of the Angiotensin peak and the two non-peptide peaks that were resolved by use of the aminosilane column. The three peaks were analyzed separately

for their amino acid composition to determine the amount and nature of the peptide. They were analyzed also by RIA for Angiotensin I by Dr. Jean Sealey. Both the RIA and amino acid analyses indicated that 100 percent (within 2%) of the original peptide was recovered after chromatography in the peak labeled Angiotensin I. No peptide was detected in the solvent front.

A comparison of the data observed for the samples which were examined indicate that the relative amount of the non-peptide impurity varies from vial to vial in a given lot, (e.g., Lots 1A and 1B) and from lot to lot (e.g., lot 1A vs lot 2A). The cleanest material appears to be the lot obtained from Source 2 (the Protein Research Foundation). In addition to the variations in the content of non-peptide peaks, the amount of peptide varies from lot to lot - from 256 ± 11 (Lot 3A) to 328 ± 12 (Lot 3C) in relative area units, i.e., a difference of 72 units or 28 percent of the lower value.

The material fractionated by the second method on an octadecylsilane column was also analyzed for peptide composition, RIA-detectable material and biological activity. As mentioned previously, the non-peptide peaks contained approximately 0.3 percent of the total RIA-detectable material. The peaks at 12, 13 and 15 min in Table VIII contained no detectable peptide by amino acid analysis. The magnitudes of these peaks

varied in a manner that paralleled the inter-lot and inter-vial variation of the non-peptide peaks listed in Table VII. All of the Angiotensin I samples that were examined in this survey were at least 3 months old. These samples also fractionated into 2 peptide-containing peaks (retention times 20 and 25 min) each of which had the amino acid composition of Angiotensin I and exhibited pressor activity in the rat assay. The peak at 25 min was equivalent to 9-13 percent of the area of the peak at 20 min. The relative magnitude of these peaks showed little intervial and interlot variation. The peak at 25 min was not detected in samples that were examined within 1-3 weeks after receipt. On the other hand, the actual area of the two peaks did exhibit inter-vial and inter-lot variation similar to the variation observed in the area of the peptide peak when the Angiotensin I was analyzed on the aminosilane column (Table VII vs. Table VIII).

H. Quantitation of the Peptide

All peptide samples were disolved in distilled water at a concentration of 1 mg/mL based upon the weight stated on the vial. Measurement of the areas of the peaks from HPLC of different lots of Angiotensin I indicated a large inter-lot variability in peptide content (see section G). A smaller variability was found in the

inter-vial peptide content. Results for Peninsular Angiotensin I (Lot 3A) based upon amino acid analysis of the collected HPLC peak indicated that this preparation contained between 95 and 97 µg per 100 mL when the total contents of the vial were dissolved in water to a final concentration of 1 mg/mL (the mass of peptide was based on the value given on the label). Thus samples with peak areas greater than or less than those for source 3 sample A contain more or less than the peptide content stated on their label.

The standard addition analysis provides an independent measure of the total peptide content (see section F), but has the disadvantage of being extremely time-consuming. The phthalaldehyde method [6] for measuring total peptide was evaluated (see addendum) and was found to be useful for the quantitative measurement of peptide content in the range of 12-120 µg/mL, and is thus adequate for reference samples containing 0.5 to 1.0 mg of peptide. This method is less time-consuming than the standard addition analysis. For peptide homogeneity studies, we believe that the standard addition analysis should be used to calibrate the peak area and the fluorometric assay methods if they are used as the analytical techniques for measuring the peptide and non-peptide content of the samples.

Discussion

An HPLC method has been developed for the analysis of Angiotensin I. This method is capable of resolving Angiotensin I from other peptides, such as D-amino acid diasteriomers and peptides resulting from errors in the Angiotensin I synthesis. des[Asp]-, des[Asp, Arg]-, and des[Asp, Arg, Val]-Angiotensin II and their impurities (except for peak 6) can be resolved from Angiotensin II by use of the HPLC method. [β-Asp, Val^5]-Angiotensin II can be distinguished from [α-Asp, Val^5]-Angiotensin II and from des[Asp]-Angiotensin II; des[Asp, a-Ile^5]-Angiotensin I can be separated from [a-Ile]-Angiotensin I. Thus, this system is capable of resolving the impurities which are of primary concern in the analysis of Angiotensin I. The presence or absence of D-diasteriomers can be determined by analysis for most of the D-amino acids using gas chromatography. The levels of D-Asp and D-Phe which were detected are typical of the levels generated during the hydrolysis procedure due to racemization.

Newly synthesized Angiotensin I chromatographs as a single peptide, showing one peak having the amino acid composition characteristic of Angiotensin I. HPLC of aged Angiotensin I (stored as received for 2-12 months at 4 °C) reveals 2 peptide peaks having identical amino acid compositions. The newly evident peak comprises 9-12 percent of the total peptide. Since this peptide has the requisite

amount of aspartic acid, the peptide cannot be des[Asp]-Angiotensin I. The following experimental facts suggest that it is [β-Asp]-Angiotensin I.

1. A significant peptide impurity is found on electrophoresis of Angiotensin I at pH 8.6. It migrates somewhat less rapidly than the major component. The impurity is not observed at pH 2.5. This observation is consistent with the resolution of [β-Asp]-Angiotensin I under slightly basic conditions [4].

2. A peptide impurity is found by TLC on cellulose with butanol, pyridine and water (65:35:35 V/V) which is consistent with the resolution of [β-Asp]-Angiotensin I under slightly basic conditions.

3. The greater retention time of this peptide impurity on an ODS column using TEAP:acetonitrile is consistent with the increased retention time found for [β-Asp, Val^5]-Angiotensin II as compared to the α isomer.

4. The presence of acetic acid in the lyophilized material would provide, in the presence of moisture, an appropriate concentrated acidic environment necessary for the catalysis of the α-β rearrangement of the Asp amide bond with the α amino group of the Arg.

5. The specific activity of the impurity as measured by bioassay was found to be 50 percent greater than that of the major peptide - a characteristic of β Asp

Angiotensin I [4]. Thus the evidence strongly supports the formation of β Asp Angiotensin I during aging.

The non-peptide impurities do not appear to significantly affect the Angiotensin I measurement by RIA. Their effects on the biological assay have not been assessed. The non-peptide impurities detected by HPLC appear to be present in only small proportions in two of the lots evaluated. Impurities derived from the stopper can be obviated by avoiding contact between the Angiotensin solution and the stopper. The dimethyl siloxane polymers that we found probably arise from the silanizing process used on the glass to minimize absorption of the peptide and therefore may not be avoidable. The total non-peptide impurities can be assessed by the standard addition method. The non-peptide impurities comprise approximately 5 percent of the dry weight of the peptide sample for the samples we analyzed.

The inter-vial homogeneity of peptide content can be measured by three methods: the standard addition method to obtain the absolute peptide content, the HPLC area measurement method, and the o-phthalaldehyde method. The last two methods can yield information only on the relative homogeneity, unless they are normalized measurements by the standard addition method which provides a more absolute basis for the comparative measurements.

Conclusion

The primary problem in the preparation of an Angiotensin I standard appears to be the spontaneous α to β rearrangement of the amide linkage of the carboxyl group of the aspartic acid residue. This rearrangement results in an enhanced biological activity of the Angiotensin I preparation in proportion to the content of the rearranged product. Since it is 50 percent more active than Angiotensin I, if present as 30 percent of the preparation, then samples would show 15 percent higher activity in the bioassay. However, RIA does not distinguish between the two forms (personal communication from Dr. J. Sealey); hence, activity by RIA would be unchanged regardless of the ratio of these isomers. The impurities present in the Angiotensin I do not appear to modify the sensitivity of the RIA assay for Angiotensin I. Thus if the peptide content is accurately stated then the peptides we have analyzed appear to be suitable for use as RIA standards, but not as standards for biological assay unless their content of [β-Asp]-Angiotensin I is periodically assessed. With this stipulation, Angiotensin I now available can be certified as a standard.

Specifications for Certification of an Angiotensin I SRM

These criteria are based on the studies summarized herein.

1. When examined by HPLC on a Zorbax ODS column using TEAP buffer 0.1 mol/L, pH 3.4: acetonitrile (81:19 v/v and 75:25 v/v), the area of all non-peptide peaks should be less than 1 percent of the area of the Angiotensin I peak. Samples should be examined separately at each of the solvent ratios.

2. Upon receipt, the peptide should be free of peptide impurities a) when examined by HPLC analysis as described in paragraph 1 of these specifications; b) when examined by TLC on cellulose using each of the following solvent systems:

Butanol, acetic acid, water (BAW) 4:1:5 v/v

Butanol, pyridine, water (BPW) 65:35:65 v/v

Butanol, pyridine, water (BBW) 65:35:35 v/v

Butanol, acetic acid, water, pyridine (BAWP) 15:3:12:10 v/v

and c) when examined by electrophoresis at pH 2.5 and pH 8.6.

3. The amino acid composition should be within ±3 percent of the theoretical composition for each amino acid.

4. The peptide should contain less than 1 percent of any [D] amino acid when examined by GC analysis on a chiral column [3] or by the new method of E. Gil Av (personal communication).

5. Total non-peptide material should be less than 5 percent when estimated by the standard addition method using Phe as the standard.

6. Each vial should contain between 500 and 525 µg of peptide.

Table 1

Amino Acid Analysis of D Amino Acid Peptides

Amino Acid Ratios for Chromatographic Peaks

	Peak Number									
Amino Acid	1[a]	2&3	4	5	6	7	8	9	10	11
Asp	.87	1.01	.47	.86	1.00	.92	0	1.00	.55	.99
Val	.99	.88	1.10	.96	1.10	1.02	1.04	.96	.92	1.02
aIle	0	0	0	0	0	0	.89	.83	0	0
Ile	.88	.93	.85	.89	.84	.92	0	0	.92	1.75
Leu	1.11	1.01	1.00	1.10	1.00	1.05	1.04	1.09	1.01	1.06
Tyr	.92	.52	.98	1.13	.84	.89	.85	.92	.92	.89
Phe	1.13	1.51	1.05	1.13	.66	1.02	1.10	1.15	0	0
His	2.15	3.13	2.07	2.09	2.50	2.13	1.95	1.87	2.20	2.22
Arg	1.00	1.15	1.00	1.13	4.00	1.05	.98	1.13	.92	1.08
Pro	1.03	1.02	.99	1.06	.95	.93	.90	.91	.95	1.01
Ret. Time (min.)	17.2	10,11	24.5	28	2	3	18.2	21.2	4.0	8.5

[a]Angiotensin I.

Table II

Amino Acid Analysis of Peaks from the Chromatograms of Truncated Forms of Angiotensin II

	Peak Number												
	1[a]	2	3	4	5[b]	6[b]	5[c]	6[c]	7	8	9	10	11
Asp	.99	.93	1.04	1.08	0	0	0	0	0	0	0	0	0
Val	1.00	1.07	1.82	2.13	.92	1.03	.91	.91	1.06	1.11	.93	0	0
Ile	.87	.91	0	0	.94	1.05	.91	.95	0	1.02	.92	.91	.87
Tyr	.83	.89	0.54	.88	.97	.73	.95	0.53	.83	.91	1.04	1.04	.75
Phe	1.11	1.13	0	0	1.20	1.24	1.13	1.07	1.08	1.04	1.25	1.04	1.16
His	.99	1.01	1.11	.97	1.02	1.13	1.09	1.07	1.08	.91	.83	1.01	2.23
Arg	.98	1.07	0.94	1.05	0.88	0.83	1.00	1.00	0	0	0	0	0
Pro	1.00	1.05	+	+	+	+	+	+	+	+	+	+	+
Ret. time (min.)	12.2	15.7	2.5	3.6	9.8	12.6	9.8	12.8	5.3	15	19	9	11.2

[a] Angiotensin II

[b] Sample one

[c] Sample two

[d] + indicates that proline was detected but not quantifiable.

Table III

Radioimmunoassay[a] of Angiotensin (100 µg) Fractionated on a C_{18} Column Using TEAP:Acetonitrile Solvent

Fraction Time Interval (Min)[b]	Ng Angiotensin I
0-2	23
2-4	17
4-6	14
6-8	20
8-10	22
10-12	16
12-14	140
14-16	89
16-18	79
18-20	53800
20-22	41500
22-24	364
24-26	245
26-28	188
28-30	131
30-32	98

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[a]Assay performed by Dr. J. Sealey

[b]Solvent flow rate 1mL/min.

Table IV

Amino Acid Ratios for Angiotensin I Peaks

Amino Acid	Retention Time 20 Min[a]	25 Min	
Asp	.87	.90	
Val	.99	1.06	
Ile	.88	.88	
Leu	1.11	1.13	
Tyr	.92	.89	
Phe	1.11	1.13	
His	2.08	2.15	
Arg	1.05	1.00	
Pro	1.03	1.01	
µg Peptide Recovered	45.9	3.3	95% Recovery

Solvent 81% TEAP: 19% Acetonitrile

[a] Angiotensin I

Table V

Amino Acid Ratios for Angiotensin II Peaks

	Retention Time		
Amino Acid	12.2 Min[a]	15.7 Min	
Asp	.99	.93	
Val	1.00	1.07	
Ile	.87	.91	
Tyr	.83	.89	
Phe	1.11	1.13	
His	.98	1.01	
Arg	.98	1.07	
Pro	1.00	1.05	
µg Peptide Recovered	32.7 µg	1.00 µg	86% Recovery

Solvent 82.5% TEAP: 17.5% Acetonitrile

[a]Angiotensin II

Table VI

Purity of Angiotensin I by Standard Addition

A I Added µg	Phe Added nmol	Peak Ht. Phe / Peak Ht. Val
26.67	0	0.207
26.67	17.52	0.410
26.67	35.05	0.569
26.67	52.52	0.783
26.67	70.09	0.959

By linear regression analysis R = 0.999

X Intercept 19.53 n mol = 25.33 µg protein

$$\% \text{ Purity} = \frac{25.33}{26.67} \times 100 = 94.98\%$$

Table VII

Magnitude of Impurity Peaks in Angiotensin I (AI)

Source	Lot	Relative Peak Areas of Impurities (AI = 1000)		Actual Peak Area AI
1	A	175	0	299
		280	18	286
		269	30	296
	B	37	3	312
2	A	15	34	262
		0	0	265
3	A	41	4	267
		129	43	256
		132	19	143
	B	53	15	323
		54	19	316
	C	24	19	318

Analysis conditions: NH_2 column 0.001 mol/L acetic acid: methanol 3:7 V/V. All peptide samples were dissolved in water just before analysis at a concentration of 1 mg/mL based on the peptide weight stated on the label.

Table VIII

Magnitude of Impurity Peaks in Angiotensin I (AI)

		Relative Peak Area (AI = 1000) Retention Time				Actual Peak Area (Ret. time = 20 min.)
Source	Lot	12 Min	13 Min	15 Min	25 Min	AI
1	A	20	159	61	133	540
		17	247	81	116	580
		10	108	65	105	611
	B	2	52	19	121	630
2	A	1	9	1	120	548
		1	1	1	128	550
3	A	2	69	19	118	566
		4	130	37	103	563
		9	100	27	116	558
	B	5	74	23	92	739
		5	69	21	116	773
	C	6	74	16	102	724
		7	64	16	103	685

Analysis Conditions: ODS Column 0.1M TEAP pH 3.5:acetonitrile 81:19 V/V.

All peptide samples were dissolved in water at the time of analysis at a concentration of 1 mg/mL based upon the weight stated on the label.

Addendum

Angiotensin I Fluorescence Study

D. Reeder and L. Sniegoski

Several conclusions may be reached as a result of this study: (1) Angiotensin I may be analyzed for homogeneity in its packaged form of 0.5 mg/vial, provided there are no extraneous amino acids. Adequate sensitivity is achieved by the fluorescence method in the 12-120 µg/mL range (4-40 µg/mL in final detection system). A suggested procedure is to reconstitute the contents of the 0.5 mg vial with 750 µL water, ad analyze 4 x 120 µL samples from each vial; (2) the sensitivity of the orthophthalaldehyde reagent for the peptide is about 10-fold less than that for the amino acids. This suggests the reagent is only reacting with the terminal amino acid; (3) Angiotensin II reacts slightly better in the fluorescence assay system than Angiotensin I; (4) standard curves for the individual amino acids may be generated to give correlation coefficients greater than 0.99. Proline does not react in this system. On a molar basis, tryosine is about 1/2 as reactive, and valine about 2/3 as reactive as nor-leucine. All other amino acids are approximately equal in reactivity, although isoleucine gives the highest readings.

Corrected μmoles/liter in Final Solution

	MW	mg/100 mL for 125 μmol/L	Actual Amount Weighed	m moles/ μmol	A	B	C	D	E	F	Slope m	Inter-cept b	Corr. Coeff. v
Arg	210.07	48.75	49.68	.2358	12.746	6.373	3.186	1.275	0.037	0.319	11.423	0.585	0.997
Asp	133.1	30.79	31.45	.2363	12.773	6.386	3.193	1.277	0.639	0.319	11.696	2.234	0.997
His	155.2	35.90	36.40	.2345	12.676	6.338	3.169	1.268	0.634	0.317	11.441	4.468	0.993
Iso	131.2	30.35	30.13	.2296	12.411	6.205	3.103	1.241	0.621	0.310	12.962	1.400	0.997
Leu	131.2	30.35	31.81	.2425	13.108	6.554	3.277	1.311	0.655	0.328	11.623	2.364	0.994
Nor	131.2	30.35	31.03	.2365	12.784	6.392	3.196	1.278	0.639	0.320	11.691	3.121	0.981
ϕAla	165.2	38.20	36.22	.2192	11.849	5.924	2.962	1.185	0.592	0.296	12.767	1.028	0.999
Pro	115.1	26.60	26.60	.2311	12.492	6.246	3.123	1.249	0.625	0.312	-0.116	1.890	0.127
Tyr	181.2	41.90	42.17	.2327	12.578	6.289	3.145	1.258	0.629	0.314	6.250	1.567	0.997
Val	117.2	27.10	27.33	.2332	12.605	6.303	3.151	1.261	0.630	0.315	9.698	5.635	0.970

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Reprints

1. S. A. Margolis and P. J. Longenbach, "Analysis of Purity of Commercial Peptides by High Resolution Liquid Chromatography" in The Role of Peptides in Neuronal Function, Eds. J. Barker and T. Smith, Marcel Dekker (in press).

2. S. A. Margolis and P. J. Longenbach, J. High Resolution Chromatogr. and Chromatogr. Commun., 2:255 (1979).

Figure Captions

Figure 1. Chromatograms of D-Diasteriomers of Angiotensin I. The peptides were dissolved in water at a concentration of 1 mg/mL. 50 µg was applied to an ODS column that was equibrated with 0.1 M triethanolamine phosphate, pH 3.5 (TEAP):acetonitrile 81:19 v/v at a flow rate of 1 mL/min. The fractions absorbing at 254 nm were collected and analyzed for amino acid content (Table 1). The D peptides were a gift of Dr. Susan Oparel.

Figure 2. Chromatograms of truncated form of Angiotensin II. The peptides were dissolved in water at a concentration of 1 mg/mL. 50 µg was applied to an ODS column that was equilibrated with TEAP buffer: acetonitrile (81:19 v/v) at a flow rate of 1.25 mL/min. The fractions absorbing at 254 nm were collected and analyzed for amino acid content (Table II).

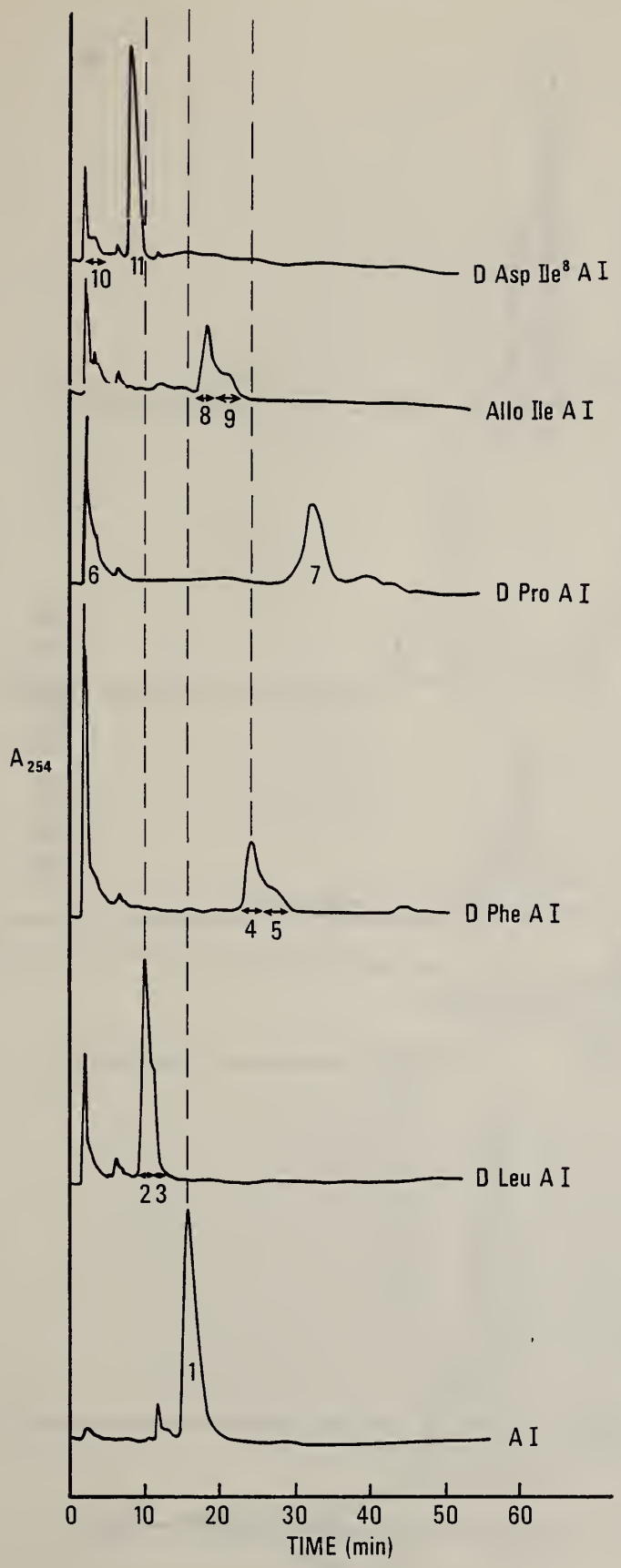
Figure 3. Chromatogram of α and β Asp Val^5 Angiotensin I The peptides were dissolved in water at a concentration of 1 mg/mL. 50 µg was applied to an ODS column that was equilibrated with TEAP buffer:acetonitrile (85:15 v/v) at a flow rate of 1 mL/min. The fractions absorbing at 280 nm were collected and analyzed for amino acid content. The β-asp Val^5 Angiotensin II was a gift of Dr. Michael Peach.

Figure 4. Thin layer chromatograms of Angiotensin I samples.

The chromatograms were prepared by Dr. M. Khosla [5] using cellulose plates and the solvent systems indicated on the chromatograms.

Figure 5. Electrophoretic chromatograms of Angiotensin I

samples. The chromatograms were prepared by Dr. M. Khosla using the standard methods in his laboratory at the pH's indicated on the chromatograms [5].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Allo Ile A I
D Pro A I
D Phe A I
D Leu A I
A I
10
11
8 9
6
7
4 5
2 3
1
A254
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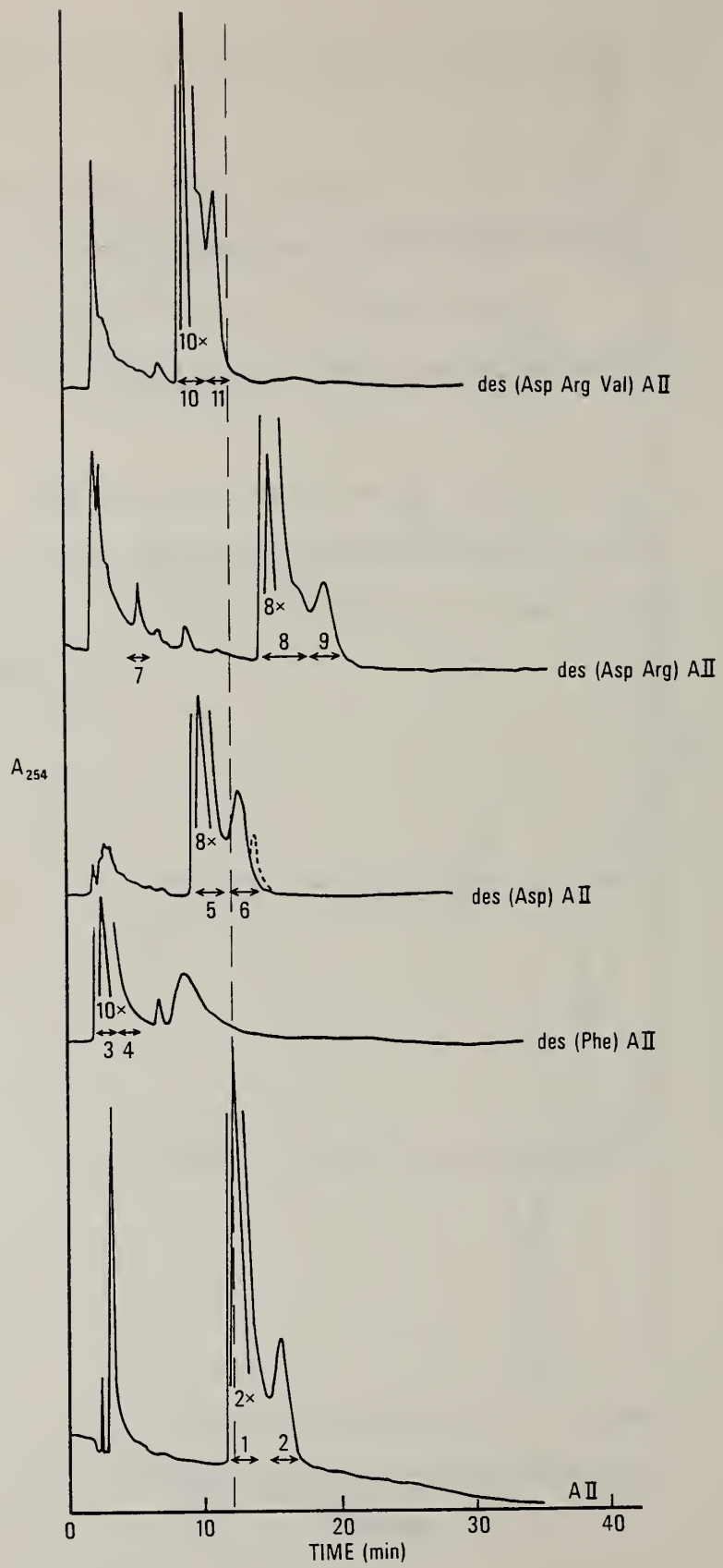
10
20
30
40
50
60
TIME (min)


10×
10 11
des (Asp Arg Val) AII
8×
8 9
7
des (Asp Arg) AIII
A254
8×
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2×
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AII
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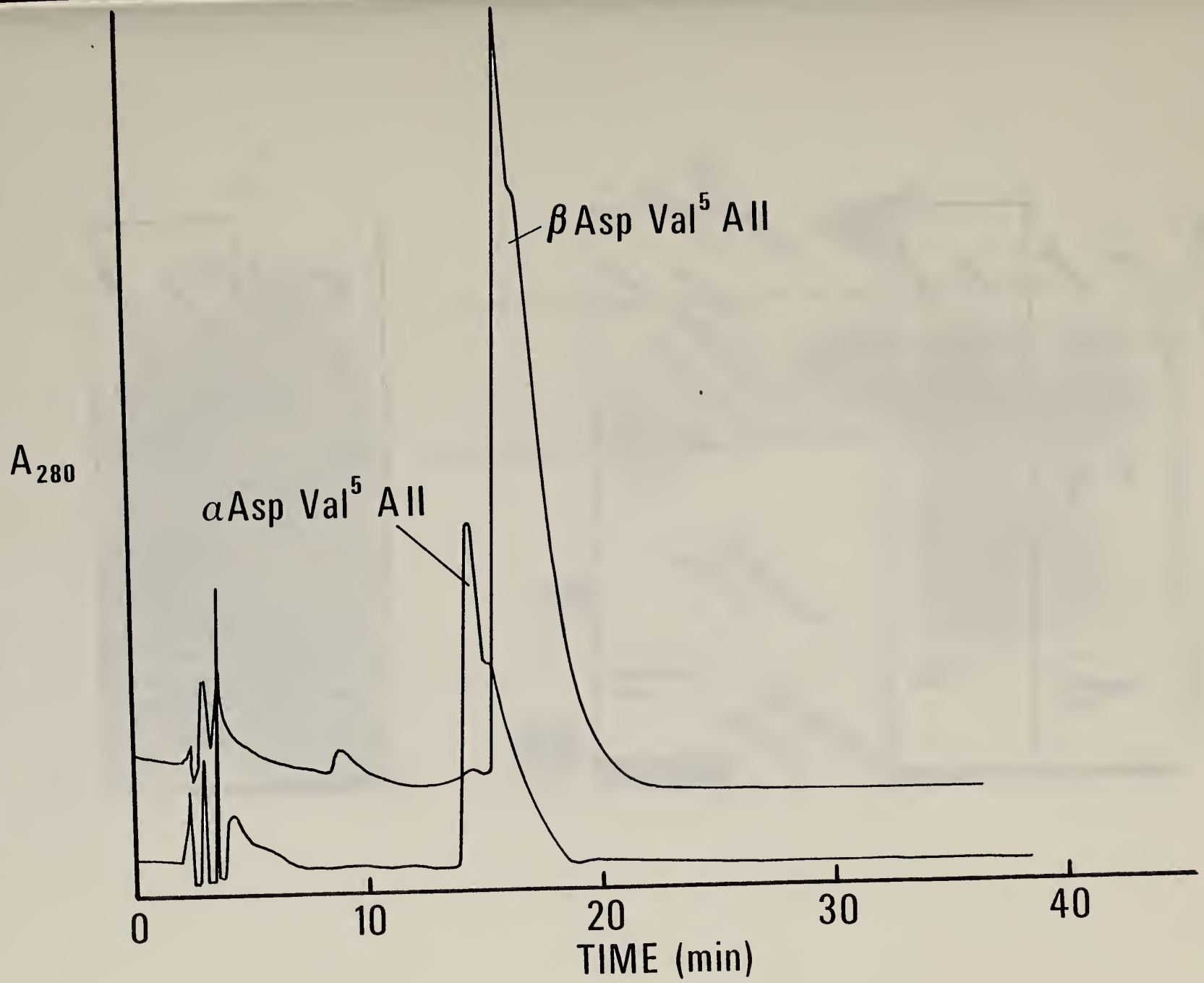
10
20
30
40
TIME (min)


βAsp Val5 AII
αAsp Val5 AII
A280
0
10
20
30
40
TIME (min)


BAWP
BPW
Buchnan
Pyrrole
Buchnan
Pyrrole


BPW
Buchnan
Pyrrole


BAW
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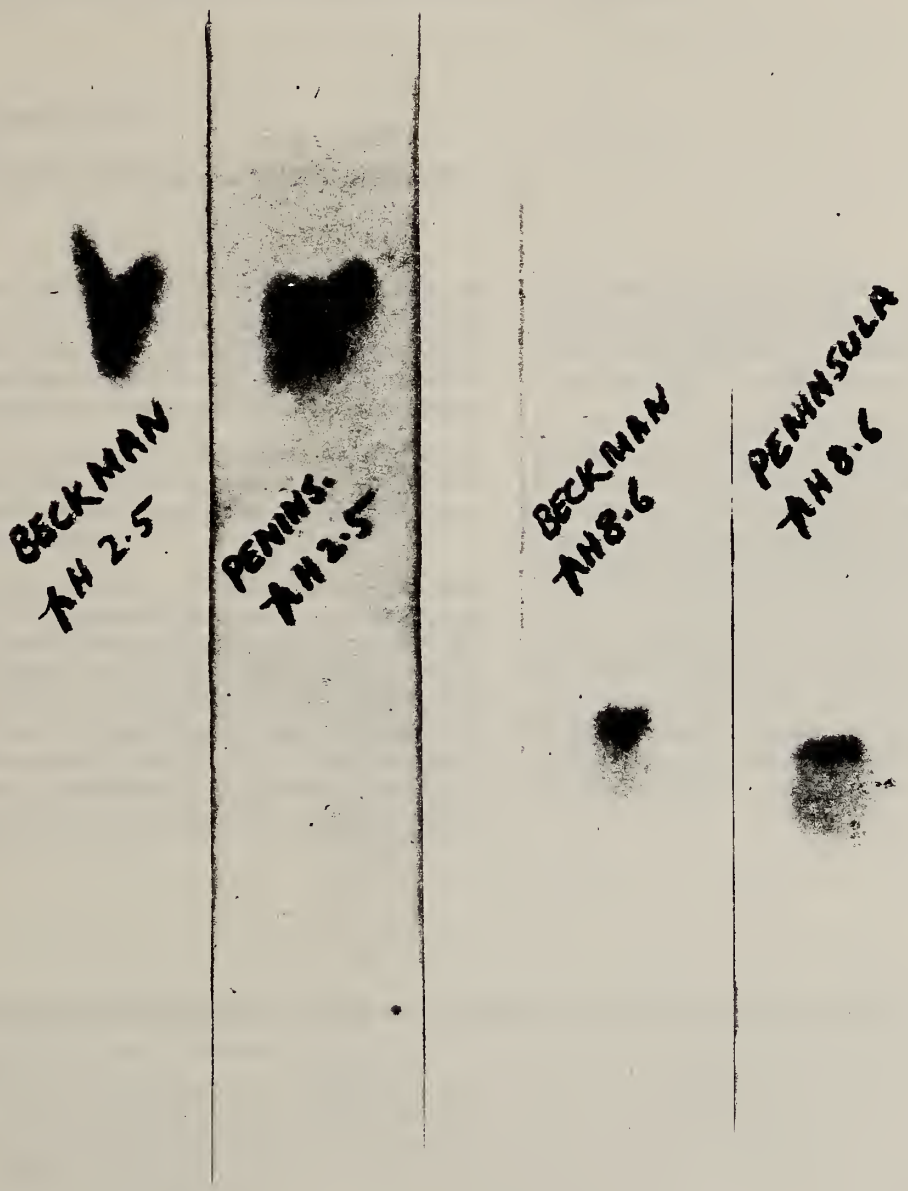
BPW
Pyrrole.
Pyrrole.

DEC 19, '78

ANGIOTENSIN I

BECKMAN & PENINSULA SAMPLES



DR. M. C. KHOSLA

Separation of Structually Similar, Biologically Active Peptides from Their Impurities

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Key Words:

Liquid Chromatography, HPLC
Peptide analysis
Angiotensin I and II, peptide hormones, resolution of impurities

Interest in the use of high resolution chromatography for the separation and analysis of peptides has grown considerably during the past few years [1-5]. The purity of biologically acitve peptides is of particular importance for meaningful studies of their pharmacological mechanism of action and use in humans.

A simple isocratic method that provides ample resolution of very closely related peptides as well as resolution of many non-peptide impurities has been developed by modifying the work originally reported by *Rivier* [5].

The peptides which were studied are listed in **Table 1**. They were either purchased from commercial sources or were supplied as gifts from Dr. J. Barker (National Institute of Neurological and Communicative Diseases and Stroke) and Dr. M. Khosla (Cleveland Clinic). Analyses were performed with a commercial chromatographic system on an octadecylsilane column. The solvents were composed by volume of either 17.5, 19, or 25% acetonitrile (glass distilled) and 0.1 mol/l triethylamine phosphate buffer, pH 3.5 (TEAP). Eluted peptide-containing peaks were identified by amino acid analysis.

This method is capable of separating the decapeptide angiotensin I from the octapeptide angiotensin II, resolving [ile^5]-angiotensin II from [val^5]-angiotension II (which differ only by a methylene group), and met enkephalin from leu enkephalin.

By 254 nm monitoring of preparations of individual peptides, several chromatographic peaks were resolved (e.g., see **Figure 1** for Angiotensin I). Different preparations of the same peptide were found to contain different groups of impurities (Table 1) and different proportions of the same impurity. The same peptide preparations revealed different impurities depending upon the solvent system employed (Figure 1).

Preliminary analysis of our data indicates that some peaks were non-peptide impurities from impure peptide; others were introduced during the purification; and some were contaminants from the serum stopper. Several peptide preparations were found to contain several percent of peptide impurities, for example, des [ile^5]-angiotensin II in angiotensin II.

Table 1

Values of K′ for Several Biologically Active Peptides and the Number of Other Peaks Detected at A_{254}.

Solvent composition [a]	17.5%		19%		25%	
	K′	other peaks	K′	other peaks	K′	other peaks
Peptide						
Somatostatin	b		b		8.5	5
Substance P	b		b		3.0	5
Angiotensin I	b		16.0	9	1.7	6
Eledoisin	8.0	5	6.4	5	<1.0	
[Leu]-Enkephalin (lot A)	11.4	5	6.2	5	<1.0	
[Leu]-Enkephalin (lot B)	11.5	6	6.2	5	<1.0	
[Ile^5]-Angiotensin II	7.0	6	4.1	3	1.0	3
[Met]-Enkephalin (lot C)	5.4	7	3.2	8	0.9	5
[Met]-Enkephalin (lot D)	5.4	9	3.0	5	<1.0	
LRH	4.5	1	2.6	1	<1.0	3
[Val^5]-Angiotensin II	3.0	1	2.1	5	<1.0	

(a) percent acetonitrile (by vol.) in 0.1 mol/l triethylamine phosphate (pH 3.5)
(b) not measurable

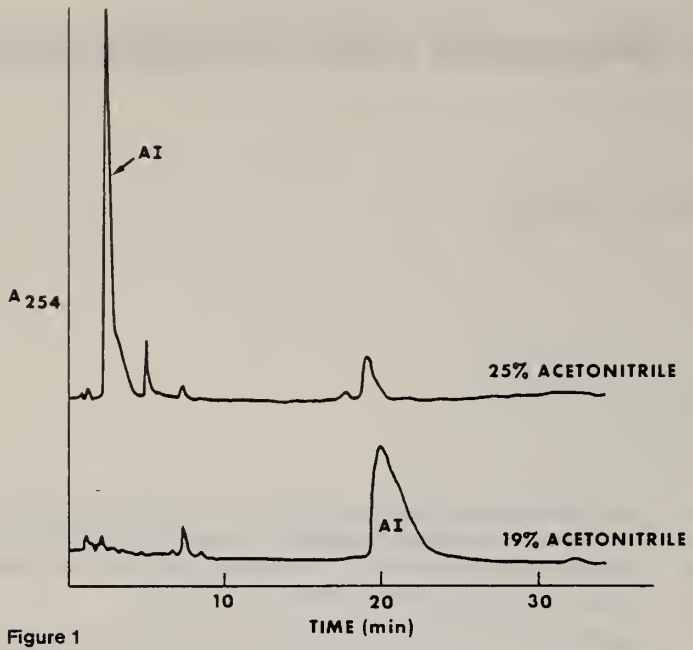

Figure 1

Resolution of impurities in Angiotensin I by different solvents. A 25 µg sample of angiotensin I dissolved in the eluting solvent was applied to the column and eluted isocratically with either acetonitrile: TEAP buffer 19 : 81 (A) or 25 : 75 (B). All eluted peaks were collected and analyzed for amino acid composition. Only the angiotensin I peak (AI) contained amino acids and these were in the correct stoichiometric ratio for AI. 94 percent of the AI was recovered. Detection was at 254 nm at 0.02 AUFS.

Investigators using commercially prepared peptides for pharmacological and physiological studies should be aware of these impurities and their possible effect on their experiments.

Acknowledgement

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MS received: 19 March 1979

Post-Column Derivatization Using HPLC Cassettes 10093

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Key Words:

Liquid Chromatography, HPLC
Detection by post-column derivatization
MICONN-T-pieces as flow-reaction chamber
Separation cassette principle

Photometric detectors – especially those with variable wavelength – are the most frequently used in HPLC. They have proven their value in a large number of applications. In some cases, however, their performance is inadequate, for example when the absorbance of the component of interest is too small or when traces are to be detected.

In such cases it is possible under certain circumstances to use other detection principles, e.g. refractometry or electrochemistry. Another possibility, which utilizes the advantages of photometric detection, is to perform post-column derivatization in order to produce compounds which are more easily detectable. Fundamental studies on this technique have been carried out by, e.g. *Frei et al* [1-3], *Snyder* [4] and *Zech* and *Völter* [5].

The column outlet T-piece of the HPLC cassette system (Figs. 1 and 2) provides a simple method of adding a reagent solution. The cassette system and the use of cassettes in HPLC is described in detail elsewhere [6,7].

The typical features of the system are the novel, problem-free method of connecting the cassettes ("needle seal") and the fact that the column is not fitted with the usual single inlet and single outlet, but with a system of four combined inlet/outlets (see **Figure 1**). These features lead to easy operation, increased performance and a high degree of flexibility.

Figure 2 shows a possible cassette arrangement for post-column derivatization. The sample is chromatographed in the usual way; the reagent solution is then added via the column outlet T-piece, e.g. by means of a second pump. Eluate and reagent are mixed in the T-piece and pass through a reaction capillary into the detector.

A chromatogram run in such a way is shown in **Figure 3**; it shows gentamicin after ion-pair chromatography, derivatization with o-phthalaldehyde and fluorescence detection (conditions according to [8-10] with minor modification, see Fig. 3). Gentamicin is an aminoglycoside antibiotic which is composed of three major and several minor components.

ANALYSIS OF PURITY OF COMMERCIAL PEPTIDES BY HIGH RESOLUTION LIQUID CHROMATOGRAPHY

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I. INTRODUCTION

The accurate description of the mechanism of action of a chemical on a physiological system - in this case neurological - requires the use of substances of defined chemical purity. It is essential that the test substance be free from other components capable of perturbing the test system. The purpose of these studies is to evaluate the state of purity of peptide hormones in an effort to increase investigator awareness of the types and sources of peptide and non-peptide components.

The major sources of peptide impurities that occur in peptide synthesis include: 1) alkylation of Trp, Tyr, or Met residues during the removal of protective groups; 2) formation of truncated peptides by incomplete coupling or by incomplete removal of the protective groups on the amines; 3) duplication of an amino acid, especially during classical synthesis procedures; 4) racemization of amino acids (esp. His); 5) modification of Met, Asn, or Gln during purification; and 6) rearrangement of peptide bonds with Ser, Thr, or Asp [1]. The major sources of non-peptide impurities are: 1) impure reagents used during peptide synthesis and purification; 2) glassware, caps and stoppers used for packaging the

peptide; and 3) reagents used for silanization of the storage vials.

II. APPRAISAL OF CURRENT METHODOLOGY FOR PEPTIDE ANALYSIS

A good method for peptide analysis should require a short analysis time, use small amounts of sample, employ multiple quantitative methods of detection, obtain complete sample recovery, and make available, with minimum dilution, separated components for further analysis of biological activity, quantitation by radioimmunoassay (RIA), or assessment of chemical composition. The analytical methods used today for examining peptide homogeneity - thin layer chromatography and paper electrophoresis - provide a high degree of resolution. However, amino acid sequence errors that occur during peptide manufacture may not always be detected by these methods. Neither may errors in the amino acid sequence of synthetic peptides be detected by amino acid analysis. Furthermore, the amino acid values reported in the literature often vary as much as 7 percent from the theoretical value. If a given peptide contains 2 moles of an amino acid, a seven percent error in analysis can translate into as much as fourteen percent of the peptide representing either a deletion or an addition error. Finally, very few of the analytical methods that are routinely used in peptide analysis detect non-peptide impurities. With the exception of the measurement of specific rotation and elemental analysis, essentially all the methods utilize detection techniques which are specific for amino acids or the peptide bond. Specific rotation measurements will detect only optically active impurities, and elemental analyses are often not reported. Only high resolution liquid chromatography uses the more general quantitative detection methods, such as ultraviolet absorption at nominal wavelengths of 215, 254, and 280 nm. Use of variable wavelength detectors can be used to give more versatility. Other more specific quantitative techniques, such as fluorescence measurement and post-column derivatization

reactions can be used to supplement the information obtained by use of ultraviolet detectors.

High resolution liquid chromatography (LC) can fulfill most of the requirements for a good analytical method for peptide analysis. Less than thirty minutes is usually required for the analysis of a 10-100 ug sample of a peptide. The components can be monitored and quantitated at specific wavelengths (usually 215, 254 or 280 nm), or by post-column derivatization techniques [2]. The entire sample can be recovered [3], hydrolyzed, and analyzed for amino acid composition by the method of Benson and Hare [4]; assessed for biological activity; or quantitated by RIA [3].

The utility of high resolution LC for analysis of peptide hormones was demonstrated by Rivier [5] for luteinizing hormone releasing hormone (LRH), somatostatin and thyrotropin releasing hormone (TRH), and by Molnar and Horvath [6] for angiotensins and melanocyte stimulating hormone (MSH). Other investigators have confirmed these results and have also examined other synthetic peptides [7-12]. Krumen and Frei [11] separated analogs of oxytocin and vasopressin [2]. Feldman [13,14] also chromatographed some of these hormones and demonstrated that more than one chromatographic peak can be found in commercially prepared peptides. The resolution of diastereomers of peptides was accomplished using an octadecylsilane column, with acetonitrile:ammonium acetate buffer as the mobile phase [15]. The [D-Trp^{8}]- and [D-Cys^{14}]- diastereomers of somatostatin were completely separated from somatostatin and the [D-Met^{14}]-bombesin from bombesin. Most recently Takai et al. [1] separated [Val^{5}]-, [Ile^{5}]- and [D-Tyr^{4}]-angiotensins II and demonstrated that the tryptic peptides of [D-His]-, [D-Tyr]- and [β Asp]-angiotensins II could be resolved from the tryptic hydrolysates of angiotensin II. On the basis of these studies, they demonstrated that their preparation of synthetic angiotensin II was free of diastereomers and the β-aspartyl derivative.

Almost every sample of commercial peptide that has been examined contained multiple impurity peaks which absorb light at 254 nm.

However, the origin, identity and structure of these components have not been elucidated. Only Rivier determined the amino acid composition of some of the components, that were separated by the chromatography process, and measured the recovery of peptide after chromatography [3].

Our objective was the assessment of the purity of commercial peptides under conditions, where essentially all of the peptide was recovered, by use of a method that fulfills the requirements that have been outlined for a good analytical procedure. This was achieved by modifying the solvent system of the high resolution LC method of Rivier [3]. Six of the nine biologically active peptides that we have examined were obtained from more than one source. Every peptide sample contained impurities. Some of these were errors in peptide synthesis, and some were non-peptide components which were introduced during purification or eluted from the stopper.

III. METHODS AND MATERIALS

Peptides were obtained from Beckman Inst. Inc., Boehringer Mannheim Biochemicals, Peninsula Laboratories Inc., Calbiochem-Behring Corp., and Pierce Chemical Co. The peptides were chromatographed on an octadecylsilane column using commercial instrumentation and they were detected with a fixed wavelength detector set at 254 nm. All solvents were degassed and filtered. The solvent system was composed of 0.1 mol/L aqueous triethylamine phosphate (TEAP), pH 3.5, and acetonitrile. Chromatography was done isocratically at room temperature, with acetonitrile representing 17.5, 19, or 25 percent by volume of the total solvent. Small changes in pH resulted in significant changes in resolution and retention times of some peptides. Fractions of the column effluent that contained material absorbing at 254 nm were collected, concentrated under nitrogen, hydrolyzed, and analyzed for amino acid composition by the method of Benson and Hare [4]. Recoveries were based on the

quantity of amino acid measured by this technique. Angiotensin I fractions were analyzed by RIA by Dr. Jean Sealey (Cornell University).

IV. APPLICATION OF HIGH RESOLUTION LIQUID CHROMATOGRAPHY TO THE PROBLEM OF PEPTIDE HORMONE PURITY

A. Properties of the Chromatographic Method

The chromatographic technique requires small samples of 25-100 µg and requires less than 30 minutes for the analysis of either angiotensin I, angiotensin II, or [Val^5]-angiotensin II (Figures 1 and 2). The detector is sensitive to peptides which contain Tyr, Phe, or Trp, as well as non-peptide compounds which absorb light at 254 nm. Greater than 95 percent of the angiotensin I or II applied to the column was recovered when the main peaks were collected and analyzed for amino acid composition. The identities of all the chromatographic peaks that contained peptide were established in this manner. The response of the detector at 254 nm to angiotensin I was linear for samples of 25 to 100 µg (Figure 3), indicating that the method is quantitative over this range. Furthermore, radioimmunoassay of the material in the angiotensin I peak at 20 minutes (Fig. 2) indicated that more than 95 percent of the RIA detectable peptide was located in this peak. Approximately 0.3 percent of the total RIA activity was located in the peaks at 12, 13, and 15 minutes.

The resolution of the chromatographic system is illustrated in Figures 1 and 2 where angiotensins I and II and [Val^5]-angiotensin II are completely resolved from each other. The baseline resolution of [Ile^5]- from [Val^5]-angiotensin II, which differ by a single methylene group, provides an example of the degree of resolution achieved by this method. Of equal importance is the baseline resolution of des[Ile^5]-angiotensin II from angiotensin II where the former (retention time 3 minutes) is present at one

twentieth the concentration of the angiotensin II (retention time 6.5 minutes) (Fig. 1). No amino acids or peptides were detected in the other peaks.

B. Types and Sources of the Impurities found with Angiotensin I

Angiotensin I was extensively studied by chromatography using several solvents in an effort to delineate the sources of impurities. Fresh solutions of angiotensin I were found to contain a single peptide component with the expected amino acid composition (Fig. 2) when chromatographed in TEAP-acetonitrile (81:19 v/v). A second peptide component (retention time 25 minutes) exhibiting pressor activity in rats and having the same amino acid composition as angiotensin I was detected after storage of the angiotensin I as the lyophilized product at 4 °C (Fig. 2). Although the basis for the formation of this peptide component is not understood, it appears that as the lyophilized angiotensin I ages it is partially converted to this new form. The magnitude of the chromatographic peak for this second component relative to that of the angiotensin I peak is the same whether the lyophilized sample is dissolved either in water or in the chromatographic solvent prior to the chromatographic analysis. Furthermore, the formation of this component seems to be independent of the source of the angiotensin I (Table I). The other peaks in the angiotensin I chromatogram represent predominantly non-peptide impurities. Peptide was not detected in these peaks by amino acid analysis. However, a small amount of material measurable by RIA (approx. 0.3%) was detected in the three peaks which have retention times between 12 and 15 minutes (Fig. 2). The minimum amount of peptide required for this RIA measurement (100 pg) is not detectable by our amino acid analysis method and that amount of peptide would not account for the magnitude of the absorbance of these components. These peaks between 12 and 15 minutes are absent or very low in the crude angiotensin I peptide obtained after HF cleavage of the peptide

from its solid phase support (Fig. 4). Their intensities differ markedly between lots from the same company and vary somewhat between samples of the same lot (Table I). The angiotensin I from source 3 contained only traces of these impurities. Thus, it appears that non-peptide impurities are introduced during the purification steps which may co-elute with trace amounts of material measurable by RIA.

Chromatography of angiotensin I in TEAP:acetonitrile (75:25 v/v) resolved a second set of four impurities (retention times, 5, 7.5, 18, and 19.5 minutes) which elute after angiotensin I (Fig. 5). These impurities were found to be contaminants from the serum stopper and were present in trace amounts in the lyophilized material. When the angiotension I was dissolved in water that was prepared specially free of organic impurities [16] and the solution was not permited to come in contact with the serum stopper, only trace amounts of these impurites were detected. Subsequent exposure of the angiotensin I solution to the stopper increased the height of these peaks two- to four-fold (Fig. 5, Trace B). When the stopper was transferred to a new vial and exposed to the chromatography solvent, additional amounts of these impurities were eluted from the stopper, (Fig. 5, Trace C). Other peptides such as somatostatin showed similar contamination with impurities originating from the serum stopper (Fig. 6).

C. Purity of Other Commercial Peptides

A series of peptides which are important to the neurophysiologist were also evaluated by these methods. Somatostatin and substance P were chromatographed in TEAP:acetonitrile (75:25 v/v). Somatostatin contained between 8 and 10 impurities. One impurity (retention time 16 minutes) was the deletion error peptide des-[Lys Phe Thr]-somatostain (Fig. 6). The other peaks contained no peptide and four of these peaks were due to the same impurities of stopper origin as those found in angiotensin I.

Substance P contained no other peptides. However, the peak containing the peptide appears to be associated with a partially resolved component (Fig. 7) and must be studied further. More than eight non-peptide peaks were also evident.

LRH is one of the purer peptides evaluated with respect to the number of additional peaks. However, 5-6 small non-peptide peaks were detected in addition to one major impurity peak (retention time 26 minutes) that accounted for seven percent of the peptide material, as estimated by amino acid analysis. This peptide impurity contained ~~one~~ 5 extra Gly, one extra Glu and one extra Ser in addition to the expected amino acid composition of LRH (Fig. 8). A second lot of LRH also contained a peptide impurity - des-[Gly]-LRH- which represented nine percent of the total peptide as estimated by amino acid analysis.

The eledoisin sample contained only a single peptide peak which had the proper amino acid composition. However, it contained eight or more components that were devoid of amino acid content (Fig. 9).

[Leu]- and [Met]-enkephalin samples appeared to be the purest of the peptides which we examined. Each sample contained a single peptide peak and five to six very small peaks which did not contain peptide or amino acids (Fig. 10). The peptides obtained from source 1 appeared purer than those obtained from sources 2 and 3.

V. DISCUSSION AND CONCLUSIONS

The use of the TEAP-acetonitrile solvent system with an octadecylsilane column confirms and extends the observations of Rivier [3]. Peptides collected after chromatography in this buffer can be analyzed and quantitated directly by RIA, and preliminary results with angiotensin I and II indicate that with pretreatment (solvent removal) these samples can also be analyzed by bioassay procedures for biological activity. The peptide fractions can be dried under nitrogen, hydrolyzed directly in 6 mol/L HCl and analyzed for amino acid composition. More than 95 percent of the peptide is recovered.

The magnitude of the ultraviolet absorbance is proportional to the concentration in the range of 25 to 100 μg of peptide and the analysis time is less than 60 minutes.

The resolution of this method is demonstrated by the separation of [Val^5]- and [Ile^5]-angiotensin II and by the resolution of such impurities as des-[Ile^5]-angiotensin I, des-[Gly]-LRH, LRH with four extra amino acids, des-[Lys, Phe, Thr]-somatostatin, des-[Ser]-somatostatin and the several non-peptide impurities that are present in each of these hormone preparations which, according to the suppliers, were pure and homogenous. The lack of peptide purity or the presence of a number of chromatographically distinct components confirms the results of other investigators who examined commercial material [3,9-14]. However, these investigators did not examine the chemical composition of these peaks.

The component in angiotensin I which has a retention time of 25 minutes and appears to form upon storage of the lyophilized material, does not seem to be a chromatographic artifact since the other peptides that we analyzed did not exhibit similar behavior (Fig. 6-10). This second component of angiotensin I may be β-[Asp]-angiotensin I. It has the same amino acid analysis as angiotensin I and its position in the chromatogram relative to angiotensin I is similar to that of the β-[Asp]- derivative of [Asp-Arg-Val-Tyr] relative to its α derivative [1].

Several of the non-peptide impurities in angiotensin I appeared to have been introduced during the purification of the peptide. This was indicated by their absence in crude angiotensin I. Furthermore, each lot of peptide contained a different proportion of each impurity peak. The variation in the amount of the non-peptide components is illustrated in Table I for the impurity peaks at 12, 13, and 15 minutes. Both inter-lot and inter-vial variations were observed in the peak areas and one lot contained negligible amounts of these peaks. When the components in the angiotensin I impurity peaks eluting at 12, 13, and 15 minutes were concentrated under nitrogen and chromatographed, they were not

detected. Thus, these peaks probably represent volatile impurities that were introduced during the peptide purification process and were incompletely removed to various degrees by lyophilization.

Another set of non-peptide impurities found in several different peptide preparations was eluted from the stopper. The small amounts of these stopper impurities, which were present in the lyophilized mass of peptide, may have been introduced during the filling or lyophilization process, or may have been present in the final peptide solution and adsorbed onto the stopper during the lyophilization process. Their presence is not restricted to angiotensin I. They have been observed also in somatostatin and other peptide preparations, particularly after the peptide solution was permitted to come in contact with the serum stopper.

Because of the volatility of the peaks at 12, 13, and 15 minutes that were observed in angiotensin I samples, we have been unable to establish their identity. However, these components exhibited very small but significant amounts of material measurable by the radioimmunoassay for angiotensin I; suggesting the presence of trace amounts of peptide. However, no peptide was detected in these peaks by amino acid analysis. Therefore, the absorbance of these peaks was due to something other than peptide, leading to the conclusion that these three peaks represent trace peptide impurities which co-chromatograph with non-peptide components.

We have attempted to identify some of these impurities by use of mass spectrometry. Preliminary evidence for angiotensin I indicates that the lyophilized mass of peptide contains, in addition to the peptide, dimethylsiloxane polymers, acetic acid, triethylamine, and material which decomposes above 300 °C releasing benzyl alcohol.

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TABLE I

Magnitude of Impurity Peaks in Angiotensin I

Source	Lot	Relative Peak Area[a] (AI[b] = 1000) 12 min.	13 min.	15 min.	25 min.	Actual Peak Area AI[b]
1	A	20	159	61	133	540
		17	247	81	116	580
		20	208	65	105	611
	B	2	52	19	121	630
2	A	2	69	19	118	566
		4	130	37	103	563
		9	100	27	116	558
	B	5	74	23	92	739
		5	69	21	116	773
	C	6	74	16	102	724
		7	64	16	103	685
3	A	<1	9	<1	120	548
		<1	<1	<1	128	550

[a]Each value represents the average of similar values of two separate determinations on the same sample. Every sample was dissolved in water free of trace organic compounds. The concentration of the peptide was 0.5 mg/mL based on the peptide content assigned by the supplier. A 50 µL aliquot of each solution was chromatographed for each analytical determination.

[b]AI = angiotensin I (ret. time = 20 min.)

FIGURE CAPTIONS

FIG. 1. Resolution of [Ile^5]- and [Val^5]-angiotensin II. Twenty-five µg of peptide, dissolved in the chromatographic solvent, was applied to an octadecylsilane column that was equilibrated with acetonitrile:TEAP (19:81 v/v) at a flow rate of 1 mL/min. Detection was at 0.02 absorbance units full scale (AUFS). The peptide peaks were identified by amino acid analysis.

FIG. 2. The Effect of Storage at 4 °C on Commercial Lyophilized Angiotensin I. Twenty-five µg of peptide, dissolved in the chromatographic solvent, was analyzed on an octadecylsilane column that was equilibrated with acetonitrile:TEAP (19:81 v/v) at a flow rate of 1 mL/min. Detection was at 0.02 AUFS. Left trace: angiotensin I at the time of receipt. Right trace: angiotensin I after eight weeks of storage at 4 °C.

FIG. 3. Load - Response Curve for Angiotensin I. Incremental quantities of angiotensin I were chromatographed as described in Figure 1.

FIG. 4. Chromatogram of Crude Angiotensin I. Twenty-five µg of lyophilized peptide, obtained by HF cleavage from the solid phase, was dissolved in the chromatographic solvent and applied to an octadecylsilane column that was equilibrated with acetonitrile:TEAP (19:81 v/v) at a flow rate of 1 mL/min. Detection was at 0.02 AUFS.

FIG. 5. Identification of the Angiotensin I Impurity Peaks Which are Eluted From the Serum Stopper. Fifty microliters of each sample was chromatographed on an octadecylsilane column equilibrated with acetonitrile:TEAP (25:75 v/v) at a flow rate of 1.7 mL/min. Detection was at 0.02 AUFS. Trace A: Angiotensin I dissolved in organic compound free water at a concentration of 0.5 mg/mL without contact with the serum stopper. Trace B: Angiotensin I solution part of which was used in Trace A, after contact with the serum stopper. Trace C: Chromatographic solvent after contact with the stopper that was used in the preparation of the sample for Trace B.

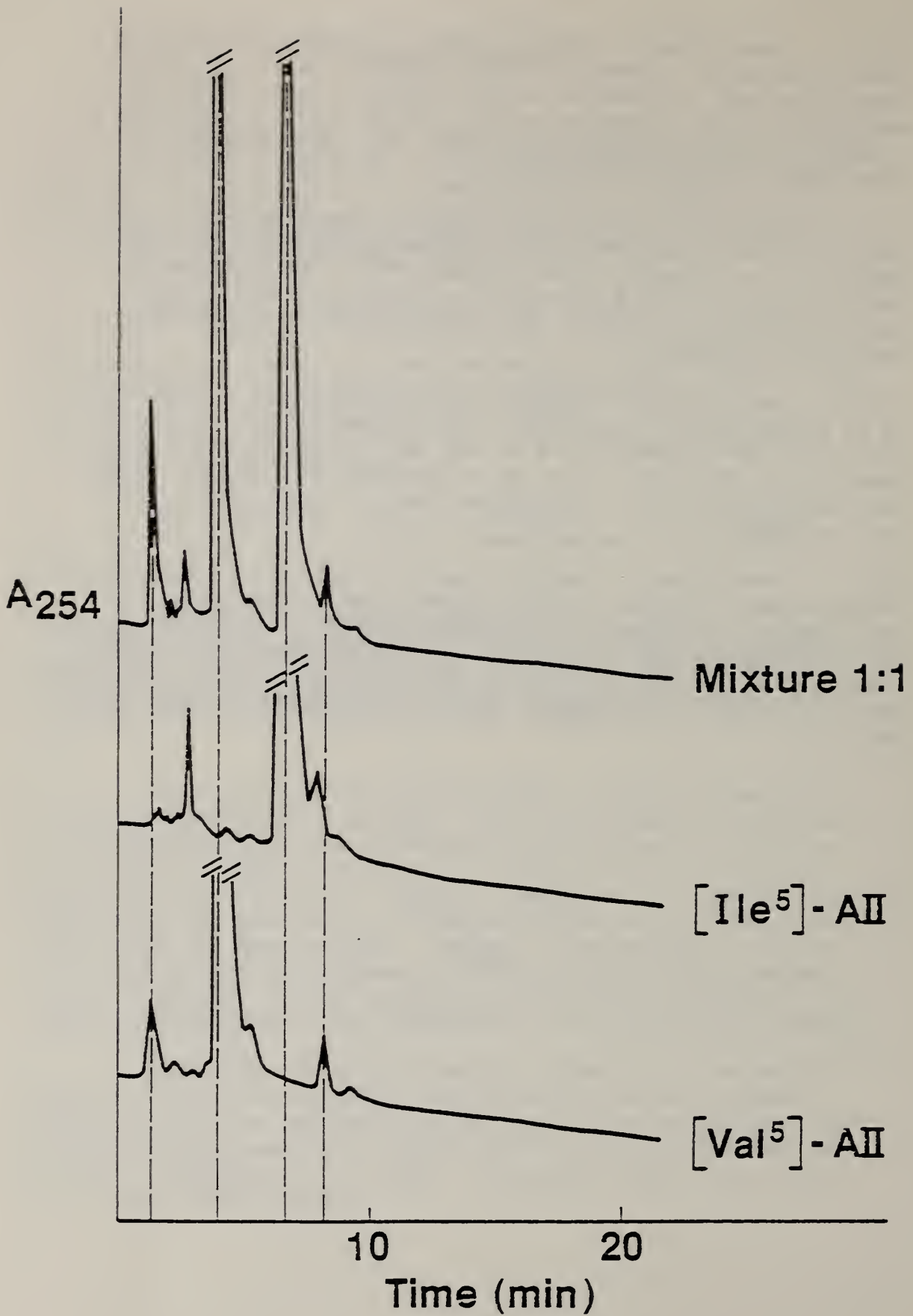
FIG. 6. Resolution of Impurity Peaks in Commercial Somatostatin. Twenty-five µg of somatostatin (SS), dissolved in the chromatographic solvent, was applied to an octadecylsilane column that was equilibrated with acetonitrile:TEAP (25:75 v/v) at a flow rate of 1 mL/min. Detection at 0.05 AUFS. The peptide peaks were identified by amino acid analysis.

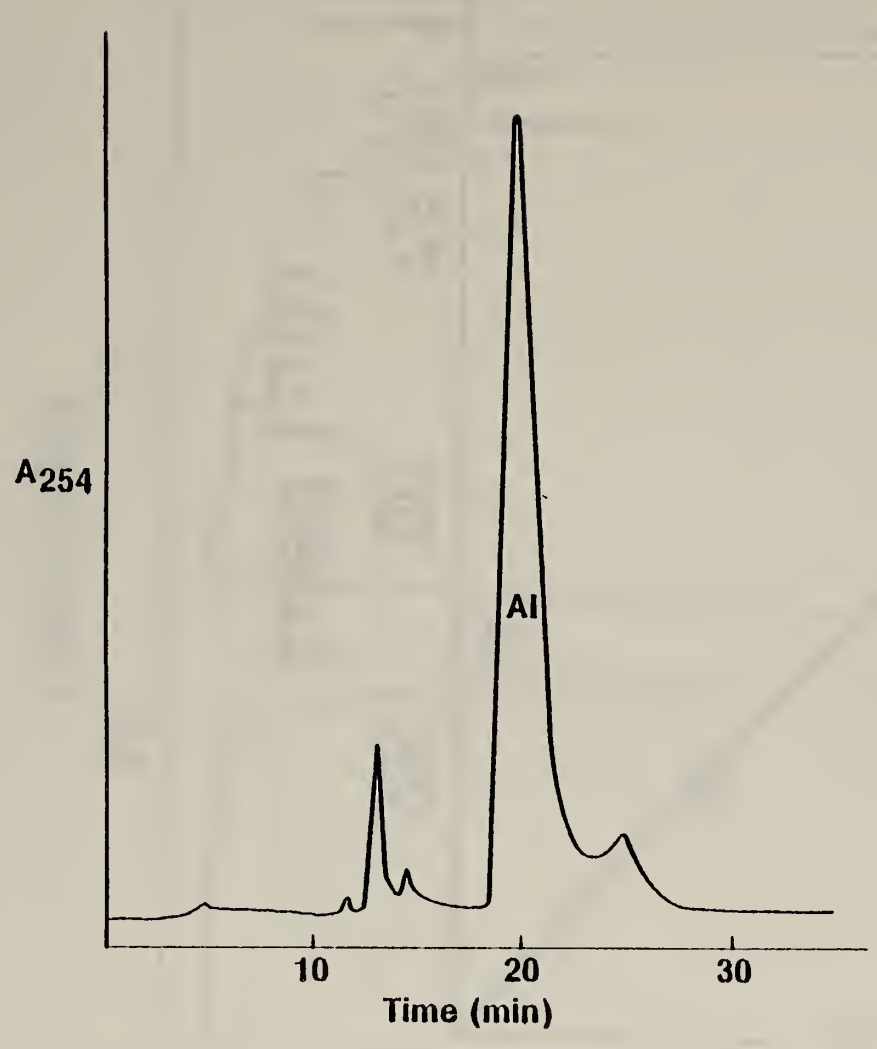
FIG. 7. Resolution of Impurity Peaks in Commercial Substance P. Twenty-five ug of substance P (SP), dissolved in the chromatographic solvent, was applied to an octadecylsilane column that was equilibrated with acetonitrile:TEAP (25:75 v/v) at a flow rate of 1 mL/min. Detection was at 0.01 AUFS. The peptide peaks were identified by amino acid analysis.

FIG. 8. Resolution of Impurity Peaks in Commercial LRH. Twenty-five ug of LRH, dissolved in the chromatographic solvent, was applied to an octadecylsilane column that was equilibrated with acetonitrile:TEAP (17.5:82.5 v/v) at a flow rate of 1 mL/min. Detection was at 0.05 AUFS. The peptide peaks were identified by amino acid analysis.

FIG. 9. Resolution of Impurity Peaks in Eledoisin. Twenty-five ug of Eledoisin (E), dissolved in the chromatographic solvent, was applied to an octadecylsilane column that was equilibrated with acetonitrile:TEAP (19:81 v/v) at a flow rate of 1 mL/min. Detection was at 0.02 AUFS. The peptide peak was identified by amino acid analysis.

FIG. 10. Resolution of Impurity Peaks in Different Commercial Preparations of [Met]- and [Leu]-Enkaphalins. Twenty-five ug of each peptide, dissolved in the chromatographic solvent, was applied to an octadecylsilane column that was equilibrated with acetonitrile:TEAP (17.5:82.5 v/v) at a flow rate of 1 mL/min. Detection was at 0.02 AUFS. The peptide peak was identified by amino acid analysis.


A254
Mixture 1:1
[Ile5]-AII
[Val5]-AII
10
20
Time (min)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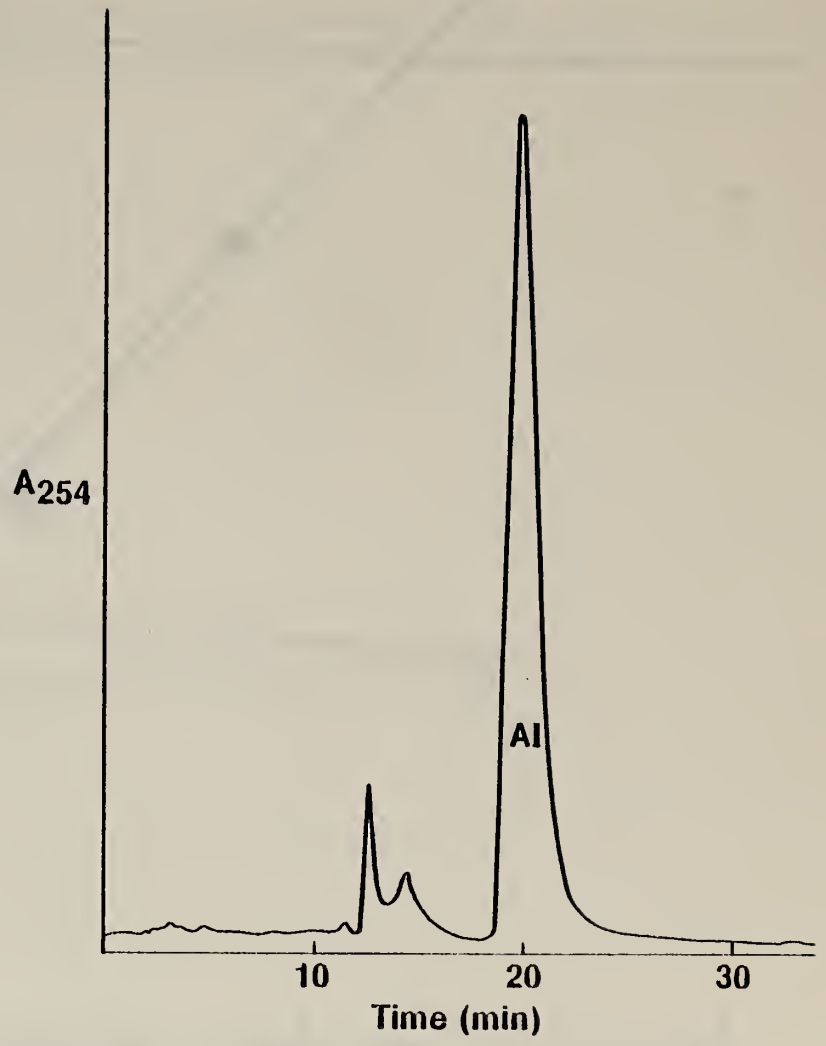
20
30
Time (min)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10
20
30
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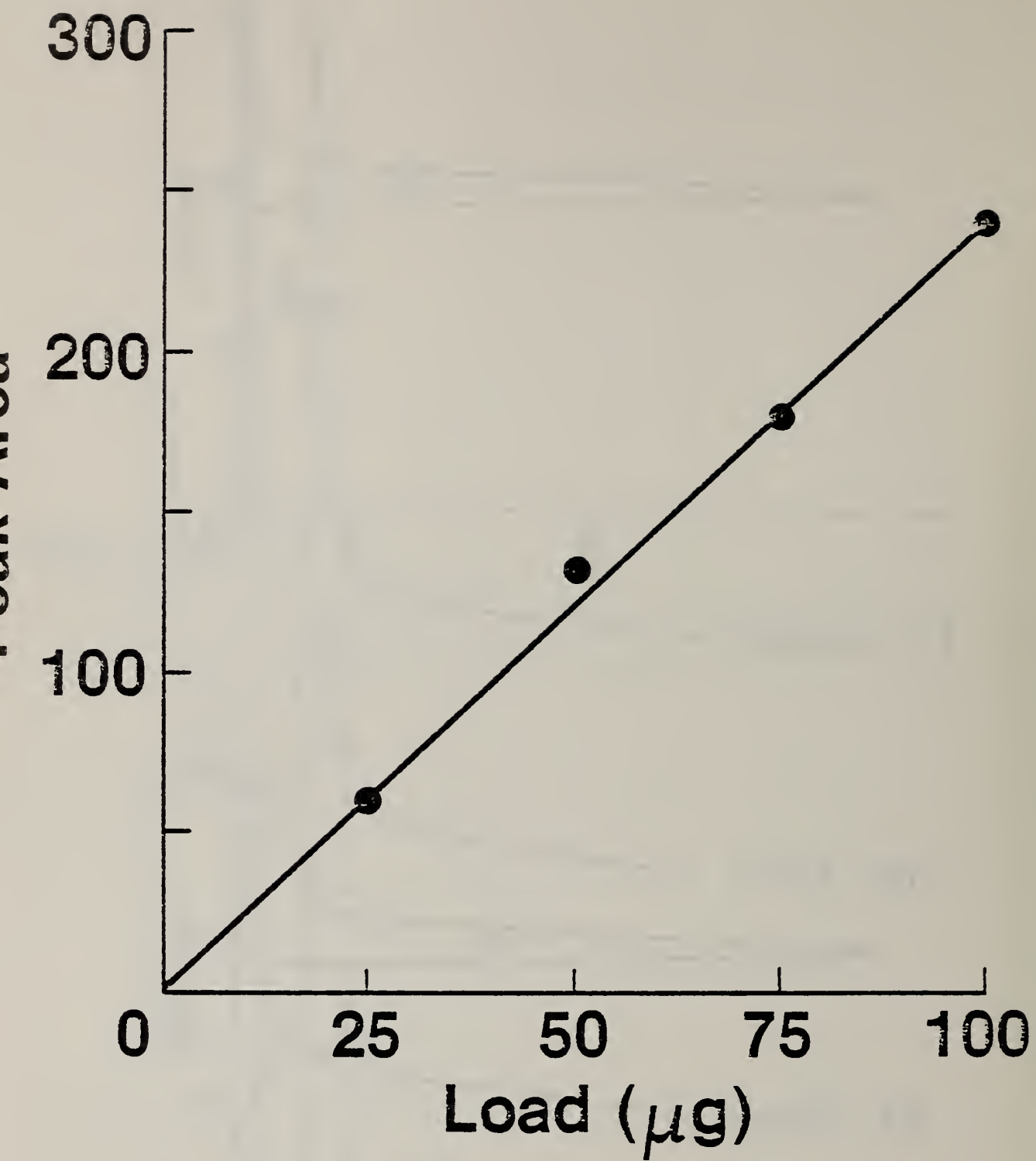
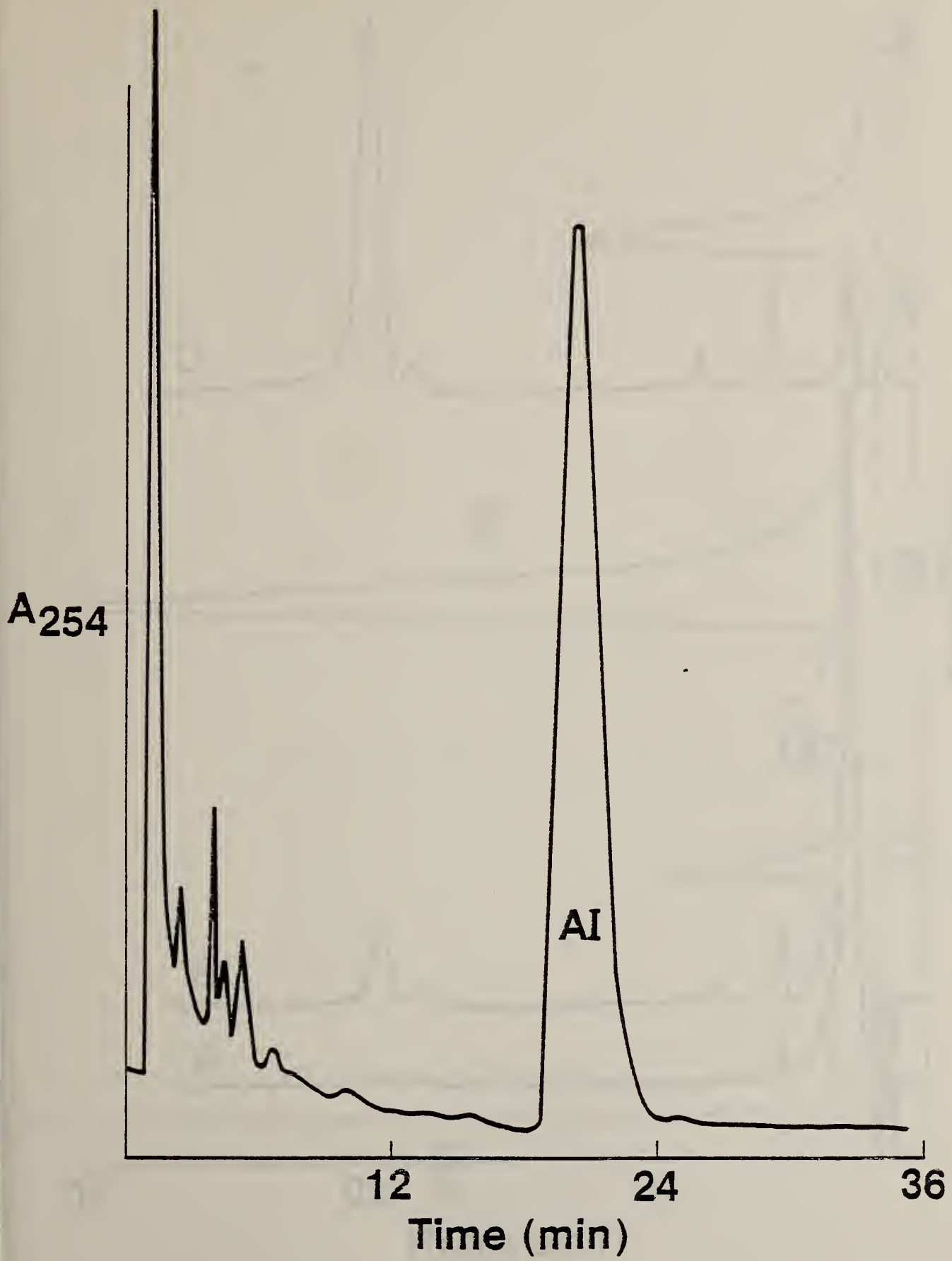
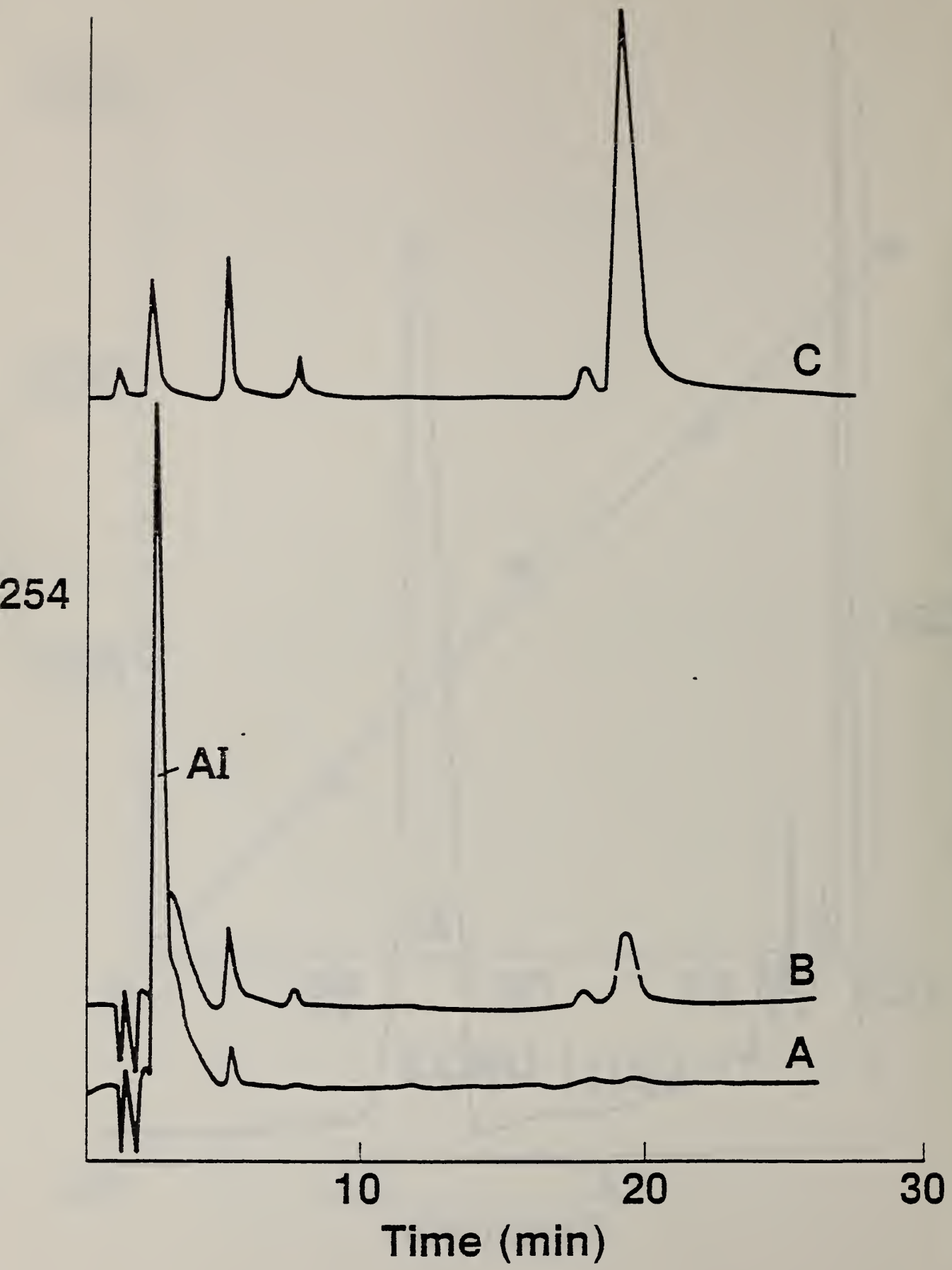

300
200
100
0
25
50
75
100
Load (μg)


A254
AI
12
24
36
Time (min)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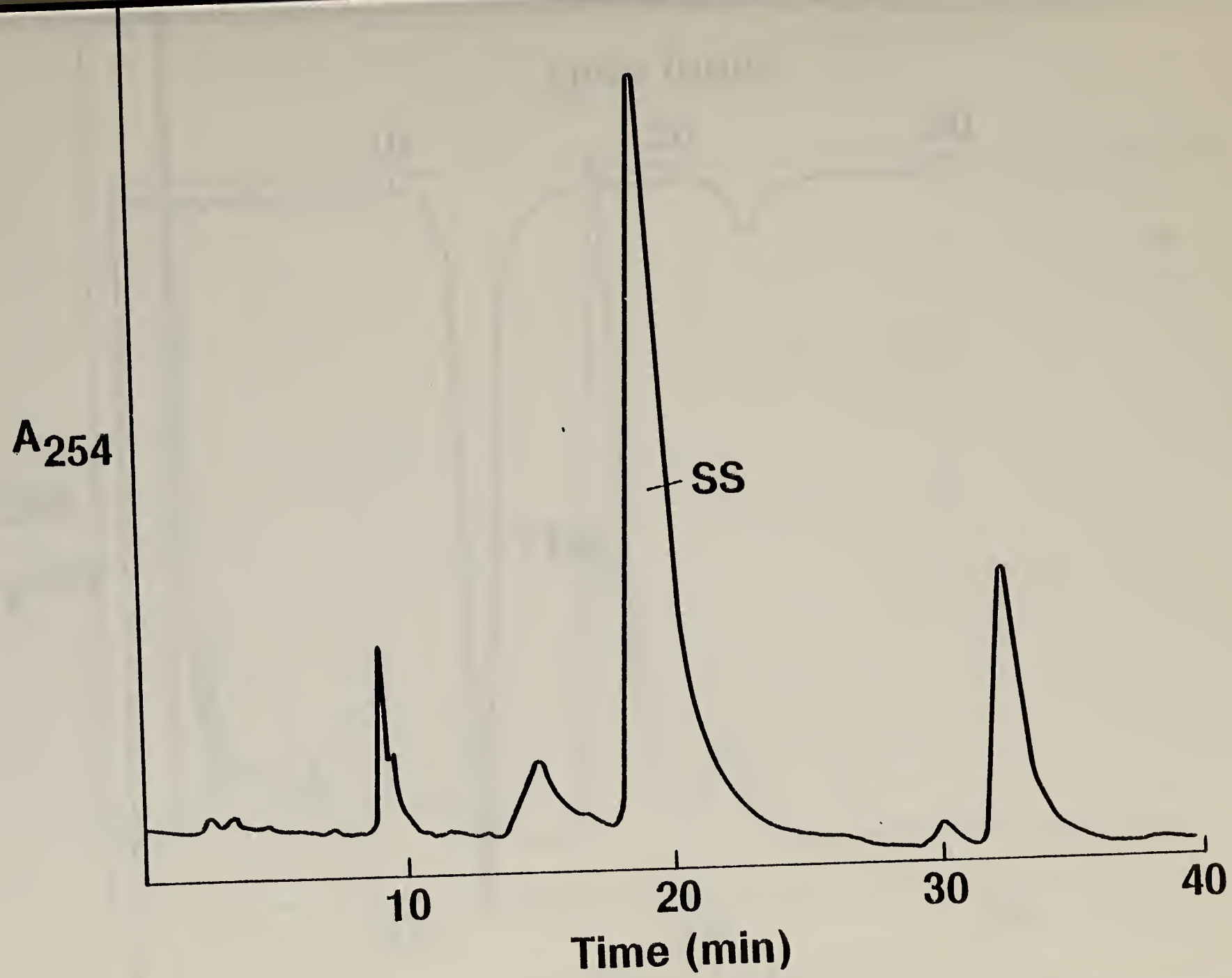
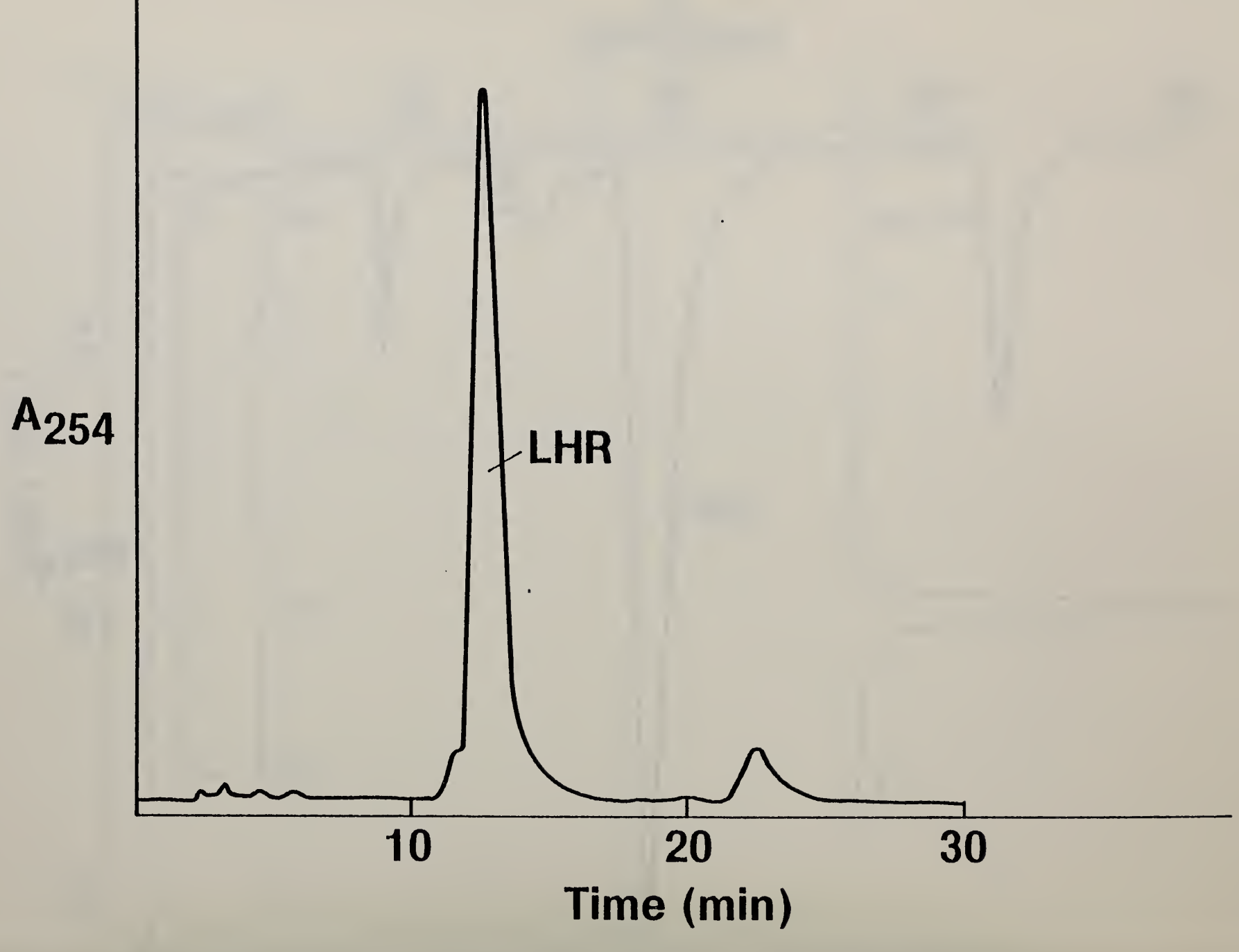
254
AI
B
A
10
20
30
Time (min)


A_{254}
SS
10
20
30
40
Time (min)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20
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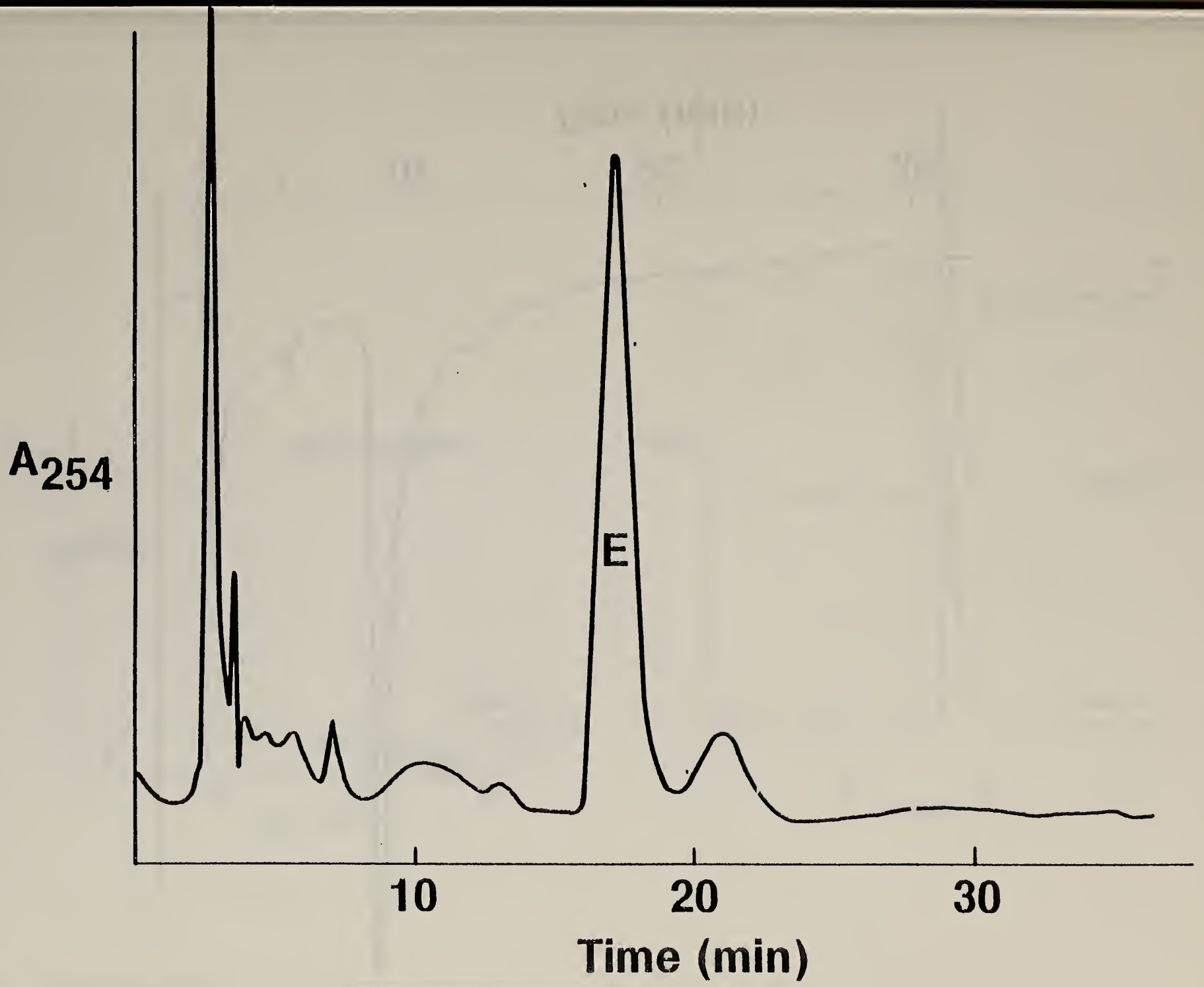
Time (min)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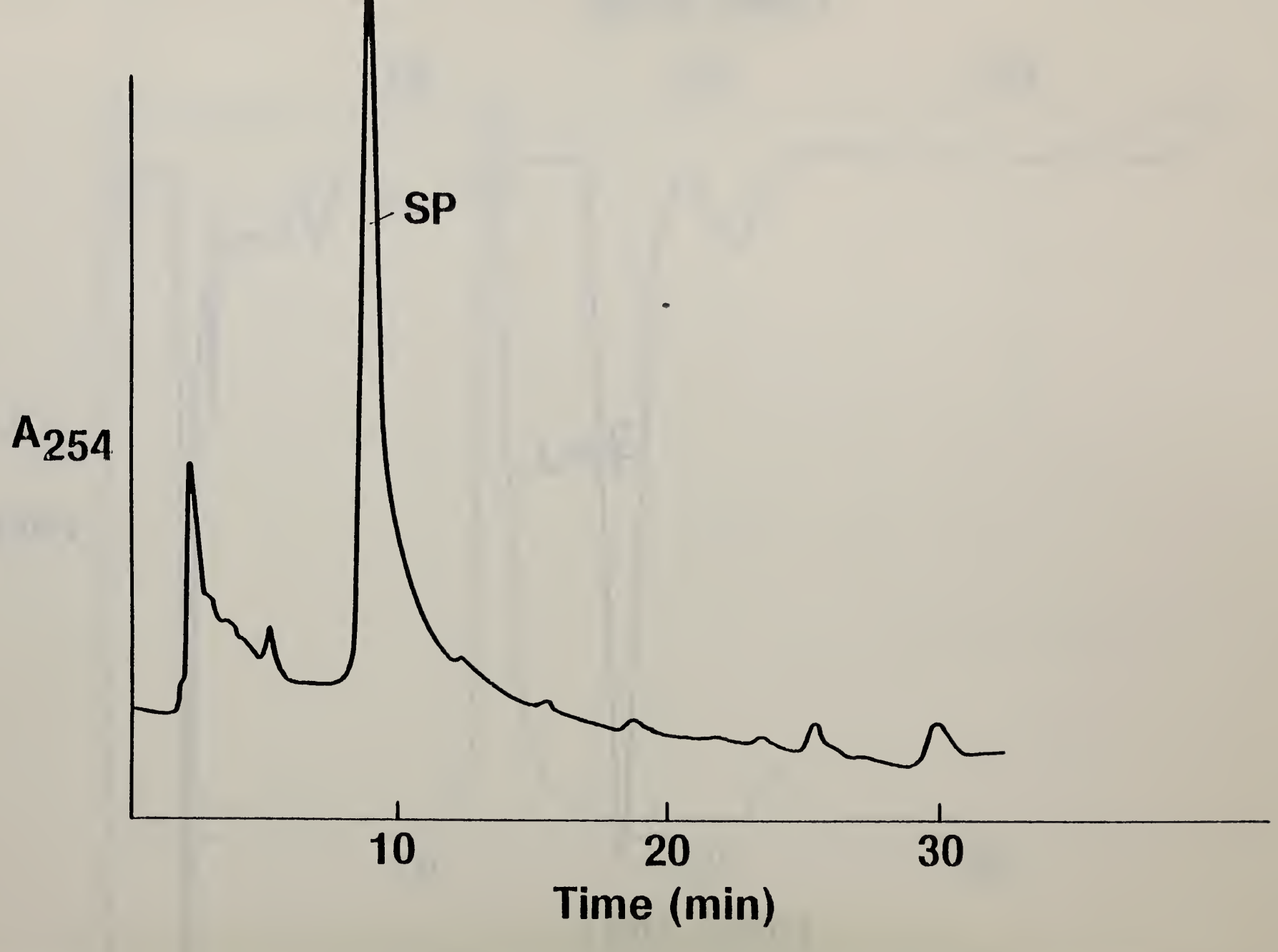
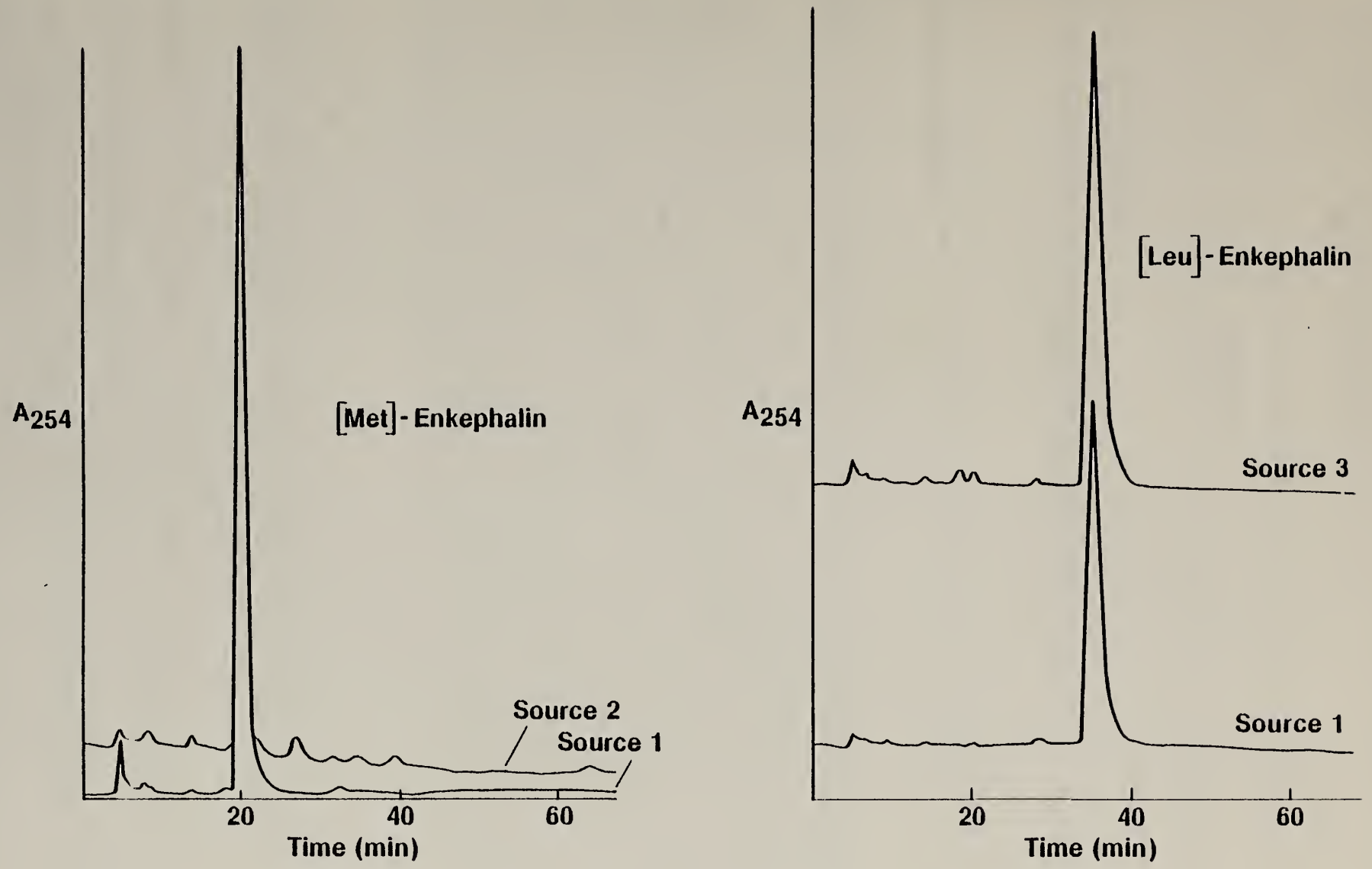
E
10
20
30
Time (min)


SP
A_{254}
10
20
30
Time (min)


A254
[Met]-Enkephalin
Source 2
Source 1
20
40
60
Time (min)
A254
[Leu]-Enkephalin
Source 3
Source 1
20
40
60
Time (min)

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16. ABSTRACT *(A 200-word or less factual summary of most significant information. If document includes a significant bibliography or literature survey, mention it here.)*

The primary problem in the preparation of an Angiotensin I standard appears to be the spontaneous α to β rearrangement of the amide linkage of the carboxyl group of the aspartic acid residue. This rearrangement results in an enhanced biological activity of the Angiotensin I preparation in proportion to the content of the rearranged product. Since it is 50 percent more active than Angiotensin I, if present as 30 percent of the preparation, then samples would show 15 percent higher activity in the bioassay. However, RIA does not distinguish between the two forms (personal communication from Dr. J. Sealey); hence, activity by RIA would be unchanged regardless of the ratio of these isomers. The impurities present in the Angiotensin I do not appear to modify the sensitivity of the RIA assay for Angiotensin I. Thus if the peptide content is accurately stated then the peptides we have analyzed appear to be suitable for use as RIA standards, but not as standards for biological assay unless their content of [β-Asp] - Angiotensin I is periodically assessed. With this stipulation, Angiotensin I now available can be certified as a standard.

17. KEY WORDS *(six to twelve entries; alphabetical order; capitalize only the first letter of the first key word unless a proper name; separated by semicolons)* Angiotensins; criteria for SRM; HPLC; peptide purity.

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