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Note

Separation of biologically important angiotensin peptides by high-performance liquid chromatography on a weak cationic exchange bonded phase

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Members of the angiotensin peptide group have a variety of biological actions including pressor responses involving vascular receptors producing systemic vasoconstriction [1], pressor responses mediated by angiotensin receptors in the central nervous system (CNS) [2], influences on water and salt intake [3] as well as the control of adrenal steroid secretion [4]. Biological production of active angiotensin involves cleavage of a circulating precursor glycoprotein, angiotensinogen, by the enzyme, renin. The decapeptide product, angiotensin I, is cleaved at the C-terminal to produce the octapeptide, angiotensin II, the most significant biologically active member of the group [5]. Further metabolism of both angiotensin I and angiotensin II may yield peptides with additional biological function [5]. Production of angiotensin in the periphery is an extracellular process occurring in the circulating blood. Blood plasma or serum has been analyzed for angiotensin immunoreactivity by radioimmunoassay (RIA); typical normal ranges of human arterial plasma angiotensin II immunoreactivity are 10—50 fmol/ml [6].

Angiotensin may also be produced in the CNS [7]. Production of the peptide here may be an intracellular process [8], with active peptide released in a neuromodulatory role [2]. Angiotensin has been detected extracellularly in the CNS by both RIA and high-performance liquid chromatography (HPLC) [9, 10] and cell surface receptors have been characterized in the CNS [11]. Estimates of normal and abnormal levels of angiotensin II in cerebrospinal fluid have relied on RIA estimates and have produced disparate results [9].

Clinical measurements of the activity of the renin—angiotensin system have relied predominantly on estimates of plasma renin activity (PRA) by RIA quantification of angiotensin I production in plasma samples incubated with

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excess renin substrate. Although angiotensin II levels would be the most relevant for clinical diagnosis of angiotensin effects on cardiovascular function, and the correlation between PRA and angiotensin II levels is weak [6], RIA of angiotensin II has not been widely used because of the influence of cross-reactive precursors or metabolites, particularly in venous blood samples. Further investigation into the production, function and degradation of angiotensin peptides requires techniques which are capable not only of quantifying angiotensins in the femtomolar range, but also of discriminating between the structurally closely related precursors, active components and metabolites. For this reason a system has been established to accomplish rapid and efficient separation of immunoreactive angiotensins by HPLC. Furthermore, since present HPLC detection methods do not permit accurate quantification of peptides at biological levels in femtomolar concentrations, the HPLC system was developed so that peptides in samples fractionated by HPLC could subsequently be quantified by RIA.

MATERIALS AND METHODS

Peptides used were synthetic and had a specified purity in excess of 95%. Angiotensin I, angiotensin III, angiotensin C-terminal hexapeptide and C-terminal peptapeptide were prepared by Cambridge Research Biochemicals (Atlantic Beach, NY, U.S.A.). Des. Asp^1 angiotensin I, angiotensin II and angiotensin C-terminal tetrapeptide were obtained from Bachem (Torrance, CA, U.S.A.). Amino acid composition of the peptides are shown in Table I. Solvents used in the gradient elution system were: solvent A, 10 mmol/l aqueous ammonium formate—acetonitrile (90:10, v/v) pH 4.2 with formic acid; and solvent B, 50 mmol/l aqueous ammonium formate—acetonitrile (80:20, v/v), pH 4.2 with formic acid. Ammonium formate and formic acid were reagent grade from Sigma (St. Louis, MO, U.S.A.), acetonitrile was HPLC grade from Fisher Scientific (Plano, TX, U.S.A.) and water was either HPLC grade from J.T. Baker (Phillipsburg, NJ, U.S.A.) or double-glass-distilled water.

Chromatography was performed using a Waters HPLC system comprising microprocessor-based gradient controller, twin pumps (Model 6000A), sample injector (Model U6K) and a fixed-wavelength ultraviolet absorbance detector

TABLE I

AMINO ACID SEQUENCE OF ANGIOTENSIN PEPTIDES

N-Terminal									C-Terminal	Angiotensin peptide
1	2	3	4	5	6	7	8	9	10	
H-	Asp-	Arg-	Val-	Tyr-	Ile-	His-	Pro-	Phe-	His-Leu-OH	Angiotensin I
H	-	Arg-	Val-	Tyr-	Ile-	His-	Pro-	Phe-	His-Leu-OH	des. Asp^1 Angiotensin I
H-	Asp-	Arg-	Val-	Tyr-	Ile-	His-	Pro-	Phe-	OH	Angiotensin II
H	-	Arg-	Val-	Tyr-	Ile-	His-	Pro-	Phe-	OH	Angiotensin III
	H	-	Val-	Tyr-	Ile-	His-	Pro-	Phe-	OH	C-Terminal hexapeptide
		H	-	Tyr-	Ile-	His-	Pro-	Phe-	OH	C-Terminal pentapeptide
			H	-	Ile-	His-	Pro-	Phe-	OH	C-Terminal tetrapeptide

at 254 nm (Model 440). The column was a 25 cm × 4.6 mm weak cation exchanger comprised of carboxylic acid functional sites fixed on 5-μm spherical silica particles (CBA, Analytichem International, Harbor City, CA, U.S.A.). Solvent flow-rate was 2 ml/min. The column was equilibrated with solvent A prior to sample injection. Samples were 25 μl of aqueous solutions of the peptides. Following sample injection, solvent A was run isocratically for 5 min. A linear gradient from 100% solvent A to 100% solvent B was performed between 5 and 6 min after injection. The remainder of the elution was performed under isocratic conditions using 100% solvent B. Ultraviolet absorbance at 254 nm was continuously monitored and output recorded on a Texas Instruments strip-chart recorder.

RESULTS

The separation of a multicomponent mixture of angiotensin peptides on a carboxylic acid weak cation-exchange column is illustrated in Fig. 1. All peaks were satisfactorily separated from adjacent peaks and demonstrate good symmetry. At pH 4.2, amino acid residues in angiotensins are predominantly protonated enhancing functional interaction with the carboxylic acid groups of the ion exchanger. Chromatographic separation involved modification of this interaction over a gradient of salt strength. However, satisfactory separation of peptides could not be accomplished without the presence of a gradient of the polar organic solvent, acetonitrile. This latter observation suggests the possibility of both ion-exchange and hydrophobic interactions (perhaps with exposed silica subsurface sites not blocked by carboxylic acid groups) between the analyte and the stationary phase.

The contribution of functional groups associated with amino acid residues in each of the peptides to the interaction between the peptide and the stationary

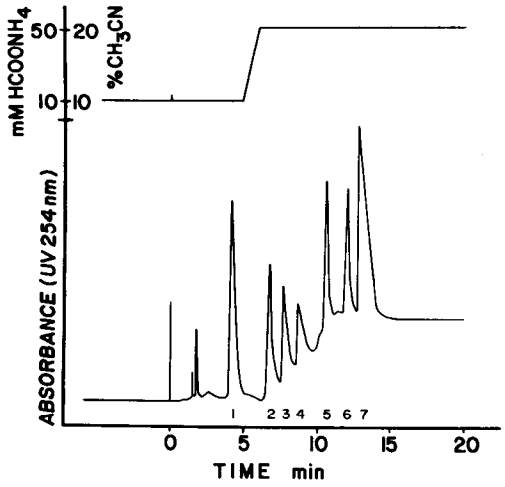

Fig. 1. Separation of seven angiotensin group peptides (25 pmol each) using gradients of salt strength and acetonitrile concentration over a weak cation exchanger (carboxylic acid). Peaks: 1 = angiotensin C-terminal tetrapeptide; 2 = angiotensin C-terminal pentapeptide; 3 = angiotensin II; 4 = angiotensin C-terminal hexapeptide; 5 = angiotensin III; 6 = angiotensin I; and 7 = des. Asp' angiotensin I.

phase is revealed to a certain extent by the elution sequence of the peptides (see Table I for peptide composition). Presence of the basic residue of arginine at the exposed N-terminal such as in des. Asp' angiotensin I and angiotensin III was associated with increased retention compared to the retention of angiotensin I and II, respectively, where the N-terminal residue is the less positively charged aspartate. However, the complexities of concurrent hydrophobic and ion-exchange interactions make further analysis uncertain.

The compatibility of the present separation system with RIA of angiotensin II has been evaluated by comparison of the assay standard curve of angiotensin II obtained in the presence and absence of the non-volatile HPLC solvent residue. The only non-volatile component of the solvent system was ammonium formate. The effect of 1 ml of dried solvent from a variety of solvent mixtures on the RIA estimation of angiotensin II was assessed. Fig. 2 shows the displacement of ^{125}I-labelled angiotensin II in the normal standard curve range, and the displacement of ^{125}I-labelled angiotensin assayed in tubes containing 1 ml of dried solvent B. The superimposition of the curves indicates clearly that accurate estimates of angiotensin II can be made by RIA of peptide in samples fractionated in the present chromatographic system.

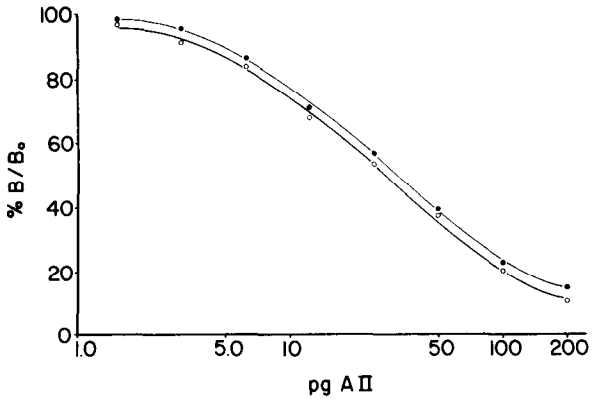

Fig. 2. RIA standard curve of angiotensin II (AII) in absence (•) and presence (○) of dried residue of 1 ml of HPLC solvent B.

Recovery tests have been performed to evaluate the recovery of angiotensin II standards fractionated in the HPLC system. Mean (± S.E.M.) recovery of 50-fmol samples of angiotensin II fractionated by HPLC and subsequently quantified by RIA was 89.6 ± 17.5% (n = 8).

DISCUSSION

Several methods have been previously described for the chromatographic separation of some angiotensin peptides [12—16]. Methods used have included paper chromatography [12], thin-layer chromatography on silica gels [13], and HPLC [14—16]. While some of these methods have been potentially useful in permitting both separation and subsequent quantification of some angiotensins, none has permitted the rapid and routine analysis of the principal immunoreactive precursors and metabolites of angiotensin II. The present

method systematically separates each of seven peptides all of which possess a greater or lesser cross-reactivity in RIA measurement of angiotensin II.

Other authors [15, 16] report the use of both reversed-phase C_{18} and weak anion exchangers in HPLC analysis of angiotensins. While these methods document satisfactory separation of some of the peptides used in the present study, the peptides do not cover the entirety of the closely related, broad group of peptides used here. Early attempts in this laboratory using ion-pairing techniques with halogenated organic acids in C_{18} systems yielded only partial separations of the peptides examined here. The results of the present study indicate that weak cation exchangers may yield a more suitable substrate for the separation of these and possibly other peptides and their metabolites. Suitability for application to quantitative assessments has been further demonstrated by the finding that HPLC solvent components do not interfere with RIA measurements. At present, studies are underway to further evaluate the application of this system to quantification of angiotensins in biological fluids under a variety of physiological conditions.

In conclusion, the HPLC separation of major immunoreactive angiotensin peptides has been satisfactorily accomplished. The separation method provides a means of rapidly separating peptides of biological origin and subsequently quantifying peptides by RIA. Many potential applications of the method can be envisaged ranging from an assessment of the metabolism of angiotensins in the blood to studies examining the role of angiotensin in the central nervous system.

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