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High-performance liquid chromatographic separation of renin–angiotensin system peptides and most of their metabolic fragments ☆

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Abstract

We describe here a gradient HPLC procedure for the separation, and quantification by UV absorption of renin tri- and tetradecapeptide substrates, angiotensins I, II, III, IV and V, angiotensin-derived peptides, and peptidase inhibitors including amastatin, bestatin, pepstatin, lisinopril, a renin peptide inhibitor, *Z*-Pro-prolinal, *N*-[1-(*R*,*S*)-carboxy-2-phenylethyl]-L-Ala-L-Ala-L-Phe-*p*-aminobenzoate, and phosphoramidon. Most peptides and peptidase inhibitors were baseline-resolved within 32 min. The overall intra- and inter-assay precisions ranged from 0.8 to 5.9 ($n=6$) and 2 to 13% ($n=6$), respectively. There was a linear relationship (correlation coefficients≥0.9660) between peak height and peptide amount injected. In conclusion, the present method when combined with a peptidase-inhibitor paradigm can lead to the identification of renin–angiotensin system metabolizing enzymes, and when combined with radioimmunoassay can enhance the specificity of angiotensin measurement.

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1. Introduction

The renin–angiotensin system (RAS) has been implicated in many physiological functions [1–3]. Angiotensin (Ang) II (Asp^1–Arg^2–Val^3–Tyr^4–Ile^5–His^6–Pro^7–Phe^8) has long been considered the main biologically active RAS peptide. However, angiotensin III [4] and angiotensin-related peptides appear to participate in some RAS functions [5,6]. For example, Ang IV [Ang II (3–8)] seems to play a role in the acquisition of spatial search strategies and memories [6], and Ang III [4] and [Des Phe^8]-Ang II [Ang II (1–7)] [7] have been implicated in vasopressin release. Some angiotensin fragments, such as Ang II (5–8), can elicit neuronal firing at concentrations fivefold greater than those of AII [8].

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Peptidase inhibitors have been used to identify the involvement of some RAS peptides in biological function [9], and their sites of cleavage [10,11]. However, most of the RAS peptides are substrates for more than one peptidase [12]. Thus, analysis of a RAS peptide processing depends on the characterization of the peptide and/or amino acid products formed with or without peptidase inhibitors. There are several methods that could be used to accomplish this task. Some RAS peptides can be selectively measured by radioimmunoassay after a previous high-performance liquid chromatography (HPLC) separation. Radioimmunoassay can not be used to detect most RAS peptides and their proteolytic fragments because available antisera usually cross-react with several of them [13]. More recently, the combined use of liquid chromatography and mass spectrometry has greatly increased sensitivity, and decreased analysis time [14,15]. However, mass spectrometry is neither appropriate for peptide quantification, nor yet available in most laboratories. Many HPLC methods have been developed to identify and quantify RAS peptides and their fragments [16,17]. Some of these HPLC procedures were combined with radioimmunoassay [18,19], or with pre-column derivatization [20] to increase sensitivity. However, the HPLC methods reported are mostly used to identify some of the RAS peptides and related fragments [16,19,21].

A HPLC system capable of separating all RAS peptides, most of their fragments, and peptidase inhibitors, can be a powerful tool in studying RAS peptides and their metabolism in vitro. To address this issue, we have developed and applied a new HPLC procedure for the separation of renin substrates, the angiotensins, peptides derived from angiotensins, and some peptidase inhibitors.

2. Experimental

2.1. Equipment

The liquid chromatograph used consisted of: two Model 6000A pumps, a Model 660 programmer (both from Waters Associates, Milford, MA, USA), and an LDC Analytical Model SM4000 absorbance detector set at 214 nm wavelength. A Rheodyne injector Model 7161 fitted with a 100-μl loop was used. The detector output was either displayed on a 10 mV recorder (RB201, ECB, São Paulo, Brazil) at a chart speed of 1 cm/min, or collected using the LCTalk software, version 2.03.02 (LDC Analytical), with 1 V full scale.

2.2. Standards and reagents

Human renin substrate tridecapeptide and porcine renin substrate tetradecapeptide, angiotensins I, II, and III {[Des-Asp^1]-AII}, [Des-Asp^1]-Ang I, [Des-Phe^8-Ang II], Ang II (1–4), Ang II (3–8), Ang II (4–8) (Ang V), Ang II (5–8), Ang I (4–10), Ang I (5–10), saralasin (Sar^1–Val^5–Ala^8–Ang II), pepstatin, amastatin and bestatin were from Peninsula Labs. (Belmont, CA, USA). *N*-Benzyloxycarbonyl-prolyl-prolinal (*Z*-Pro-prolinal) was synthesized according to Wilk and Orlowski [22]. The amino acid composition and concentration of peptide solutions were determined by amino acid analysis using PC-6A resin (Pierce, Rockford, IL, USA) after acid hydrolysis with constant boiling HCl in evacuated sealed tubes, at 110 °C for 22 h. Chemicals and amino acid standards used were from Pierce. Acetonitrile (LiChrosolv grade, for chromatography) and phosphoric acid were from Merck (Darmstadt, Germany). Lisinopril {*N*-[(*S*)-1-carboxy-3-phenyl-propyl]-L-Lys-L-Pro} was a gift from Dr. Martin Hitchens, Merck, Sharp and Dohme Research Labs. (West Point, PA, USA). *N*-[1-(*R*,*S*)-carboxy-2-phenylethyl]-L-Ala-L-Ala-L-Phe-*p*-aminobenzoate (c-f-A-A-F-pAB) was kindly provided by Dr. M. Orlowski, Mount Sinai School of Medicine (New York, NY, USA). Water was purified using a Milli-Q apparatus (Millipore, Bedford, MA, USA).

2.3. Peptide synthesis

An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu) was used for the solid-phase synthesis of Ang II (1–7), Phe–His–Leu, Leu–Val–Tyr–Ser and His–Leu by the Fmoc-procedure. The final peptides were deprotected in TFA and purified by semipreparative HPLC using an Econosil C_{18} column (10 μm, 250×22.5 mm) and a two-solvent

system: (A) trifluoroacetic acid (TFA)–water (1:1000) and (B) TFA–acetonitrile (ACN)–water (1:900:100). The column was eluted at a flow-rate of 5 ml/min with a 10 (or 30)–50 (or 60)% gradient of solvent B over 30 or 45 min. Analytical HPLC was performed using a binary HPLC system from Shimadzu with an SPD-10AV Shimadzu UV–Vis detector, coupled to an Ultrasphere C_{18} column (5 μm, 150×4.6 mm) which was eluted with solvent systems A_1 (H_3PO_4–water, 1:1000) and B_1 (ACN–water–H_3PO_4, 900:100:1) at a flow-rate of 1.0 ml/min and a 10–80% gradient of B_1 over 20 min. The HPLC column eluates were monitored by their absorbance at 220 nm. Ang II (1–7) was homogeneous on the basis of matrix assisted laser desorption ionization time-of-flight mass spectrometry analysis (TofSpec-E, Micromass). All peptides synthesized were homogeneous on the basis of amino acid composition, gave a single peak on analytical HPLC, and were used as peptide standards.

2.4. Mobile phase preparation

Two gradient-based chromatographic systems were used, each one based in one pair of HPLC solvents. Solvent A of system I was prepared by adding 200 μl 15 *M* H_3PO_4 to each 100 ml of 100 m*M* Na_2HPO_4. Solvent A of system II was prepared by adding 200 μl 15 *M* H_3PO_4 to each 100 ml of 50 m*M* Na_2HPO_4. Solvent B was the same for both systems, and was prepared by mixing: 90 ml acetonitrile and 10 ml aqueous 0.15 *M* H_3PO_4. Mobile phases were filtered through a 0.45-μm membrane (Millipore) and degassed by sonication under reduced pressure before use.

2.5. Chromatographic conditions

Peptides, peptide fragments, and peptidase inhibitors were separated by gradient elution chromatography. Linear gradients (curve 6) used were: from 20 to 40% B in 20 min for system I, and from 10 to 30% B in 10 min for system II. After the end of the gradient, these systems were held in isocratic final conditions for 10 and 15 min, respectively. Chromatography was carried out at a flow-rate of 0.5 ml/min, at 25 °C, using a 150 mm×3.9 mm stainless steel, 4 μm Nova Pak C_{18} column (Waters Associates) for both systems. A Nova Pak C_{18} precolumn (Sentry Guard, Waters) was placed between the injector and column. Absorbance at 214 nm was used to monitor the effluent. At the end of each run, initial conditions were restored by running a reversed gradient in 5 min, at 0.5 ml/min. Then, the column was re-equilibrated in about 10 min. This procedure was necessary to maintain an optimal column performance. The time of elution of anthracene in methanol was taken as t_0.

2.6. Angiotensin converting enzyme (ACE) purification

ACE was purified from rabbit lung using an established procedure [23]. The enzyme preparation obtained: (1) was homogeneous by polyacrylamide gel electrophoresis under reducing, denaturing conditions, (2) was recognized by a goat serum anti-rabbit lung ACE [24] after immunoblotting, and (3) exhibited a specific activity of 3.93 μmol His–Leu released/min/mg protein, at pH 7.5 and 25 °C, when assayed [25] with Hip–His–Leu as the substrate. The hydrolysis of Hip–His–Leu by this ACE preparation was fully blocked by 1 μ*M* lisinopril.

2.7. Conversion of angiotensin I

Angiotensin I (13 nmol) was incubated with 3.74 μg homogeneous rabbit lung ACE in 100 μl 100 m*M* Tris–HCl buffer, pH 7.5, containing 300 m*M* NaCl, at 25 °C. The reaction was stopped after 0, 15, 30, 60 and 120 min by the addition of 10 μl 1 *M* H_3PO_4. The reaction medium was centrifuged for 5 min at 10 000 rpm, and 90 μl supernatant was collected and injected into the column.

3. Results and discussion

3.1. Analytical system

To establish the initial chromatographic conditions, the effects of column types and reagents on retention times and peak shapes of the RAS peptides were tested using acetonitrile as the organic modifier. The objective was to obtain symmetrical peaks and column capacity factors (k') in the range 1–15.

Better separations were achieved using Nova Pak C_{18} (150×4.9 mm, 4 μm spherical packing material, Waters) than μBondapak C_{18} (300×4.9 mm, 10 μm irregular packing material, Waters), probably due to differences of particle size and shape between the columns. Pronounced tailing was obtained when the mobile phase contained trifluoroacetic acid in acetonitrile. The best resolution of the RAS peptides, most of their fragments, and several peptidase inhibitors have been obtained using the solvent systems I and II.

Sharp and well-resolved HPLC peaks (Fig. 1) were obtained for porcine tetradecapeptide and human tridecapeptide renin substrates, angiotensins I, II, III, IV, V, some RAS peptide fragments and peptidase inhibitors, which included amastatin, bestatin, pepstatin and the renin inhibitor Pro–His–Pro–Phe–His–Phe–Phe–Val–Tyr–Lys. Lisinopril, Z-Pro-prolinal and cf-AAF-pAB were also separated using system I (data not shown). Other RAS peptides were separated using system II (Fig. 2). This was the case for His–Leu, well separated from materials eluting at t_0, Ang II (1–7), Ang II (1–4), Leu–Val–Tyr–Ser, Ang II (5–8), Phe–His–Leu, Ang II (4–8), and for the Ang II antagonist saralasin.

The variation of retention times was less than 2% for all peptides, except for that of Ang II (1–4), which was 6%. The column capacity factors were in the range 0.6 to 10.76. The k' values for lisinopril, amastatin, phosphoramidon, bestatin, Z-Pro-prolinal, cf-AAF-pAB, Pro–His–Pro–Phe–His–Phe–Phe–Val–Tyr–Lys, and pepstatin were: 0.7, 2.12, 4.56, 5.64, 6.24, 7.94, 10.6, and 11.36 (system I), and the k' for amastatin, phosphoramidon, bestatin, Z-Pro-prolinal and saralasin were 3.63, 3.74, 4.21, 4.82 and 5.64 (system II), respectively. All peptides were eluted within 32 or 22 min using solvent systems I and II, respectively.

Changes of sodium phosphate concentration in the mobile phase can lead to non-linear changes of k', and these changes can be used to improve separation or to produce an advantageous inversion of the

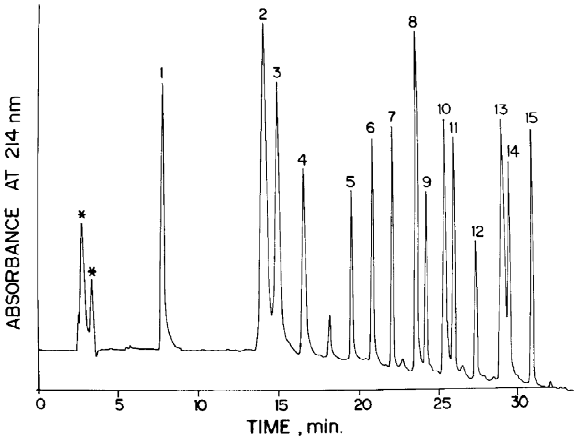

Fig. 1. Reversed-phase HPLC of authentic RAS peptide standards and peptidase inhibitors using chromatographic system I described here. The effluent was monitored at 214 nm, 0.05 AUFS. Chromatogram of a 50 μl sample containing: 1, 2.7 nmol amastatin; 2, 1 pmol Ang II (5–8); 3, 248 pmol Phe–His–Leu; 4, 1.1 nmol bestatin; 5, 80.6 pmol Ang II; 6, 63.8 pmol Ang III; 7, 83.8 pmol Ang II (3–8); 8; 237.7 pmol Ang I (5–10); 9, 82.5 pmol Ang I; 10, 110 pmol [Des-Asp^1]-Ang I; 11, 115.8 pmol Ang I (4–10); 12, 121.6 pmol human renin tridecapeptide substrate; 13, 420 pmol renin inhibitor (Pro–His–Pro–Phe–His–Phe–Phe–Val–Tyr–Lys); 14, 170 pmol porcine renin substrate tetradecapeptide; 15, 1.7 nmol pepstatin; *, materials eluting at or near the void volume.

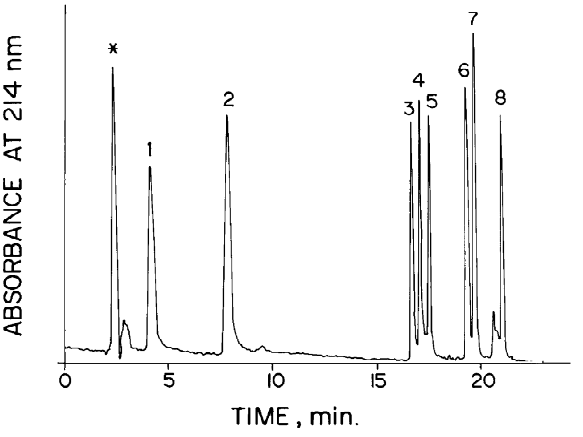

Fig. 2. Reversed-phase HPLC of authentic peptide standards using chromatographic system II as described here. The effluent was monitored at 214 nm, 0.05 AUFS. Chromatogram of a 50 μl sample containing: 1, 7 pmol His–Leu; 2, 6 pmol Ang II (1–4); 3, 133.3 pmol saralasin; 4, 2 pmol Leu–Val–Tyr–Ser; 5, 2 pmol [Des Phe^8]-Ang II; 6, 194.7 pmol Ang II (5–8); 7, 3 pmol Phe–His–Leu; 8, 86.2 pmol Ang II (4–8); *, materials eluting at or near the void volume.

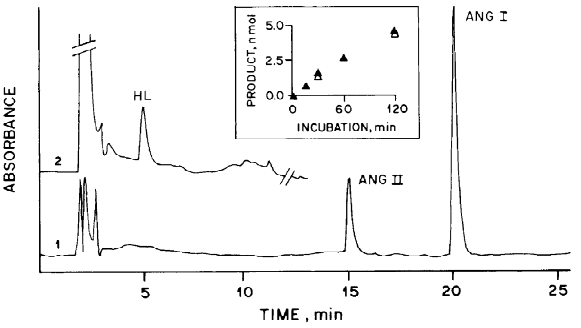

Fig. 3. Conversion of Ang I by purified ACE. Ang I (13 nmol) was incubated with 3.74 μg homogeneous ACE in 100 μl 0.1 *M* Tris–HCl buffer, pH 7.5, containing 0.3 *M* NaCl, at 37 °C for 0, 15, 30, 60 and 120 min. The reaction was stopped adding 10 μl 1 *M* phosphoric acid. The incubation medium was centrifuged at 8000 *g* for 5 min, and the supernatant was analyzed. Twenty- and 40-μl volumes of the supernatant were injected to measure angiotensins I and II (chromatographic system I, chromatogram 1), and His–Leu (chromatographic system II, chromatogram 2), respectively. The inset shows that Ang II and His–Leu were formed in nearly equimolar amounts, which were linearly related to incubation time. The regression lines [$y=(a\pm\text{SD})x+(b\pm\text{SD})$] for Ang II and His–Leu were: $y=(0.0381\pm0.0027)x+(0.2709\pm0.1671)$ and $y=(0.0368\pm0.0023)x+(0.1996\pm0.1474)$. The r^2 values for Ang II and His–Leu were 0.9851 and 0.9876, respectively.

elution position of two compounds difficult to resolve from each other [26]. We have used this approach to resolve RAS peptides from each other. Solvent systems I and II used here differed by: (1) both sodium phosphate concentration (100 and 50 m*M*, respectively) and pH (6.8 and 6.2, respectively) in solvent A, (2) the rate of linear decrease of phosphate buffer concentration and the rate of linear increase of acetonitrile concentration, and (3) the acetonitrile concentration itself in the mobile phase during gradient elution. Its use allowed the separation of all RAS peptides, many of their fragments and several peptidase inhibitors. Even His–Leu, which usually elutes at or near the void volume, was resolved from void eluting materials (Figs. 2 and 3).

3.2. Linearity of standard curves

There was a linear relationship between peak height and concentration for all the RAS peptides, which were separated using systems I (Table 1), and II (Table 2). The lower limits of quantification determined here were between 7.8 pmol for Ang II (Table 1) and 228.8 pmol for His–Leu (Table 2), for signal-to-noise ratios of 17:1 and 8:1, respectively.

Table 1
Relationship between the amount of peptide injected and the corresponding peak height, as determined using chromatographic system I

Peptide	t_R (min)±SD	k'	Pmol range	$a\pm$SD	$b\pm$SD	r^2
Ang II (5–8)	13.8±0.6	4.60	74.9–1497	0.013±0.0004	−0.32±0.29	0.9846
Phe–His–Leu	14.8±0.5	5.00	9.5–190.8	0.094±0.0025	−0.36±0.22	0.9887
Ang II	19.6±0.1	6.80	7.8–155	0.065±0.0004	0.03±0.03	0.9994
Ang III	20.8±0.0	7.32	12.3–245.2	0.056±0.0003	−0.05±0.04	0.9994
Ang II (3–8)	21.9±0.1	7.80	112.8–2256	0.007±0.0001	−0.04±0.03	0.9997
Ang I (5–10)	23.5±0.1	8.40	32–640	0.033±0.0003	−0.17±0.07	0.9990
Ang I	24.1±0.1	8.64	7.9–158.6	0.073±0.0006	−0.08±0.04	0.9989
[des-Asp^1]-Ang I	25.3±0.1	9.12	15.9–317.3	0.052±0.0006	−0.19±0.08	0.9981
Ang I (4–10)	25.9±0.1	9.36	15.4–308	0.048±0.0003	−0.06±0.05	0.9992
H (1–13)	27.2±0.1	9.88	9.4–187.1	0.047±0.0005	−0.06±0.05	0.9978
P (1–14)	29.3±0.1	10.76	13.1–262.2	0.049±0.0015	0.04±0.19	0.9847

The regression lines [peak height=$(a\pm\text{SD})x$(peptide amount)+$(b\pm\text{SD})$] were determined by the least squares method using the GraphPad Software, from triplicate determinations; *a*, angular coefficient; *b*, linear coefficient and r^2=regression factor. Column capacity factors (k') and retention times (t_R) for the peptides are also given ($n=15$). H (1–13) and P (1–14), human tridecapeptide and porcine tetradecapeptide renin substrate, respectively.

Table 2
Relationship between the amount of peptide injected and the corresponding peak height, as determined using chromatographic system II

Peptide	t_R (min)±SD	k'	Pmol range	a±SD	b±SD	r^2
His–Leu	4.0±0.08	0.60	228.8–1830	0.004±0.0002	0.12±0.16	0.9758
Ang II (1–4)	7.8±0.29	2.12	206.3–1650	0.006±0.0003	−0.08±0.02	0.9660
Leu–Val–Tyr–Ser	17.1±0.06	5.84	72.5–580	0.019±0.0004	0.03±0.01	0.9922
Ang II (1–7)	17.5±0.08	6.04	47.4–378.8	0.029±0.0004	0.06±0.08	0.9963
Ang II (5–8)	19.3±0.07	6.72	60.8–486.6	0.025±0.0005	−0.08±0.01	0.9932
Phe–His–Leu	19.7±0.11	6.84	82.6–661.3	0.024±0.0005	−0.48±0.16	0.9929
Ang II (4–8)	20.9±0.08	7.40	13.5–107.7	0.099±0.0027	0.17±0.14	0.9885

The regression lines [peak height=(a±SD)x(peptide amount)+(b±SD)] were determined by the least-squares method using the GraphPad Software, from triplicate determinations; a, angular coefficient; b, linear coefficient and r^2=regression factor. Column capacity factor (k') and retention time (t_R) for the peptides (n=15) are also given.

3.3. Analytical characteristics of the assay

Intra-assay variations for systems I and II were in the range 0.8 to 5.9 (n=6) and 2 to 4% (n=6), respectively. Inter-assay variations for systems I and II were in the range 2 to 9 (n=6) and 2.5 to 8% (n=6), respectively, except for the renin substrate tetradecapeptide (11%) and Phe–His–Leu (13%).

3.4. Hydrolysis of angiotensin I by ACE

The hydrolysis of angiotensin I by a homogeneous rabbit lung ACE preparation led to the formation of equimolar amounts of angiotensin II and its complementary peptide fragment His–Leu (Fig. 3). The yield of these products relative to hydrolyzed angiotensin I was 88–129%. There was a linear relationship between the amounts of Ang II and His–Leu formed and the incubation time (Fig. 3, inset). The formation of Ang II and His–Leu by purified ACE was fully blocked by 1 μ*M* lisinopril. Although the present method does not detect Ang (1–9) and free Leu, which could be formed by cleavage of the His^9–Leu^{10} peptide bond of Ang I by ACE2 [27,28], the above properties suggest that this ACE preparation is composed mainly or completely by ACE because: (1) the only detected cleavage of angiotensin I occurred at the Phe^8–His^9 bond, with high yield; (2) its activity was fully inhibited by lisinopril, which does not inhibit ACE2 [27]; (3) ACE purification included a lisinopril-Sepharose affinity column. In addition, ACE2 hydrolyzes angiotensin II, a product of Ang I cleavage by ACE, to [desPhe8]-Ang II, which has not been detected during Ang I hydrolysis to an extent of about 30% (Fig. 3).

4. Conclusions

We have described here a new HPLC system that permits the measurement of all known biologically active RAS peptides and many of its hydrolysis products in the presence of several peptidase inhibitors. This method additionally permits the detection and measurement of complementary peptide products resulting from the hydrolysis of a given peptide bond of some RAS peptides. Taken together, the method described and the use of a peptidase-inhibitor paradigm, in which the effects of selective peptidase inhibitors can be attributed to the blockade of a given peptide bond cleavage, can led to the identification of enzyme(s) involved in the metabolism of RAS peptides.

Both monoclonal and polyclonal anti-angiotensin II antibodies discriminate among Ang I, Ang II and [$DesPhe^8$]-Ang II, but cross-react with Ang III, Ang IV and Ang V [19]. The sensitivity of radioimmunoassay using anti-Ang II antibodies makes possible the measurement of angiotensins in body tissues and fluids, but does not discriminate among the members of the angiotensin family. In this context, the present method can also be useful to enhance the radioimmunoassay specificity of angiotensin peptides by separating them from each other before radioimmunoassay.

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