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SEPARATION AND QUANTIFICATION OF ANGIOTENSINS AND SOME RELATED PEPTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance chromatographic technique for the separation of angiotensins and some related peptides is described. Complete separation of angiotensin I, angiotensin II, tetradecapeptide and the tetrapeptide Leu-Val-Tyr-Ser is achieved in a single step, using reversed-phase high-performance liquid chromatography. The application of this technique for the detection of renin activity in crude biological samples, employing the artificial renin substrate tetradecapeptide, is demonstrated.

INTRODUCTION

Extensive literature exists on the involvement of the peripheral renin—angiotensin system (RAS) in the regulation of blood pressure and aldosterone secretion [1]. Following the discovery of pharmacological effects of centrally administered components of the RAS, as well as the presence of RAS components and angiotensin receptor sites in the central nervous system [2], evidence has been given for the physiological role of a central RAS [3]. To further characterize the significance of a central RAS, the identification and subsequent quantification of all RAS components is important. Radioimmunoassay systems for angiotensins I and II (ANG I and ANG II), as well as radiochemical and fluorescence assays for renin have been described [4—7]. Dependent on the specificity of the antisera used in a radioimmunoassay, however, separation of angiotensins is essential in order to determine specifically the individual peptides. In addition, radiochemical and fluorescence assays for renin [5—7] require more or less extensive purification of the enzyme since crude biological

preparations contain additional proteolytic activities which interfere with the renin activity in these assays.

In the present study the high resolving power of reversed-phase high-performance liquid chromatography (HPLC) is used to separate angiotensins and some related peptides [ANG I, ANG II, tetradecapeptide (TDP) and tetrapeptide (TP); see Fig. 1]. Analysis by HPLC appears to be a promising method for assaying renin activity. Using TDP as artificial renin substrate, the accumulation of the various reaction products can be easily quantified by measuring peak heights in the UV absorbance profile of the HPLC eluate.

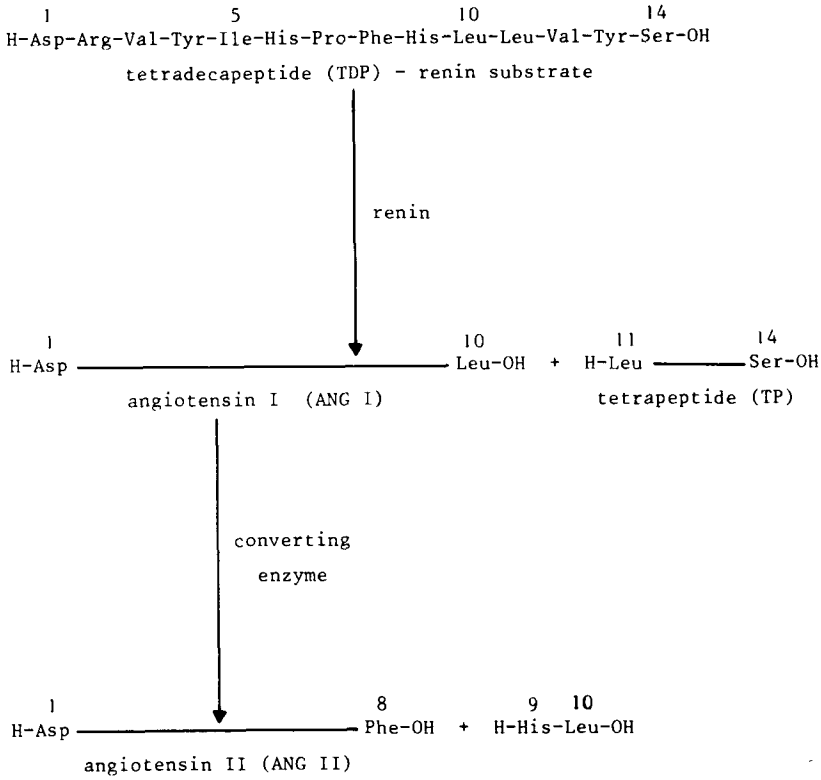

Fig. 1. Pathway of angiotensin II formation from tetradecapeptide (artificial renin substrate) by renin and converting enzyme.

MATERIALS

Synthetic ANG I (peptide content 88%) and ANG II (86%) were purchased from UCB (Brussels, Belgium). TDP (85%) was obtained from Beckman Bioproducts (Geneva, Switzerland), TP (81%) from Bachem Feinchemikalien (Bubendorf, G.F.R.), EDTA (disodium salt) from BDH Chemicals (Poole, Great Britain), 8-hydroxyquinoline (8-HQ) from Merck (Darmstadt, G.F.R.) and methanol (HPLC grade) from Baker (Deventer, The Netherlands). All other chemicals were reagent grade.

METHODS

HPLC of angiotensins and some related peptides

Separation of peptides was performed using HPLC equipment as described previously by Loeber et al. [8]. Samples were applied to a reversed-phase μBondapak C_{18} column (30 cm × 0.39 cm I.D.; 10 μm particle size; Waters Assoc., Milford, MA, U.S.A.). Elution (flow-rate 2 ml/min) was carried out at room temperature with a 45-min linear gradient of 0.01 *M* ammonium acetate, (pH 4.15) (*X*) and methanol containing 1.5 ml of acetic acid per liter (*Y*); initial conditions *X*/*Y* = 4:1, final conditions *X*/*Y* = 1:3. Eluted peptides were detected by UV absorbance which was monitored continuously at 210 nm.

Purification of renin

Hog renin, purified according to the method of Corvol et al. [9], was kindly provided by Dr. J. Menard (Paris, France). The specific activity was 280 GU*/mg protein.

Rat renin was partially purified following a method described by De Jong et al. [10]. The specific activity of the renin preparation thus obtained amounted to 2 GU/mg protein.

Renin assay

Synthetic TDP renin substrate (14.2 nmoles) was incubated with 20 mGU of renin for 0, 30 and 90 min at 37°C in 500 μl of 20 m*M* citrate—phosphate buffer (pH 6.0). The reaction was terminated by heating at 100°C for 10 min. Subsequently the incubation mixtures were centrifuged for 20 min at 20,000 *g* and the supernatants were subjected to HPLC fractionation as described above. Quantification of the peptides generated was done by measuring the peak heights above background in the HPLC profile. Control incubations (0 min) were carried out by boiling the reaction mixture immediately. Incubations with hog renin were done in the absence of angiotensinase inhibitors and incubations with the partially purified rat renin preparation without or with 10.0 m*M* EDTA and 4.3 m*M* 8-HQ to inhibit angiotensinase activity.

RESULTS

Separation and quantification of angiotensins and some related peptides

A typical elution pattern of a mixture of synthetic TP, ANG II, ANG I and TDP using HPLC is shown in Fig. 2. All peptides gave sharp, well-separated peaks. The retention times of the peptides were 6.6, 23.0, 30.2 and 37.2 min, respectively. The position of the various peaks in the elution pattern was not affected by the amount of peptides (up to 10 μg of each peptide) nor the sample volume (up to 1 ml) loaded on the column. In addition, application of the peptides in acetate, citrate or phosphate buffer did not affect the HPLC profile.

For each peptide tested a linear correlation exists between the amount of peptide (0.5—10.0 μg) applied to the column and the UV absorbance at 210 nm of the eluted peptides, as measured by peak heights.

*GU = Goldblatt Unit.

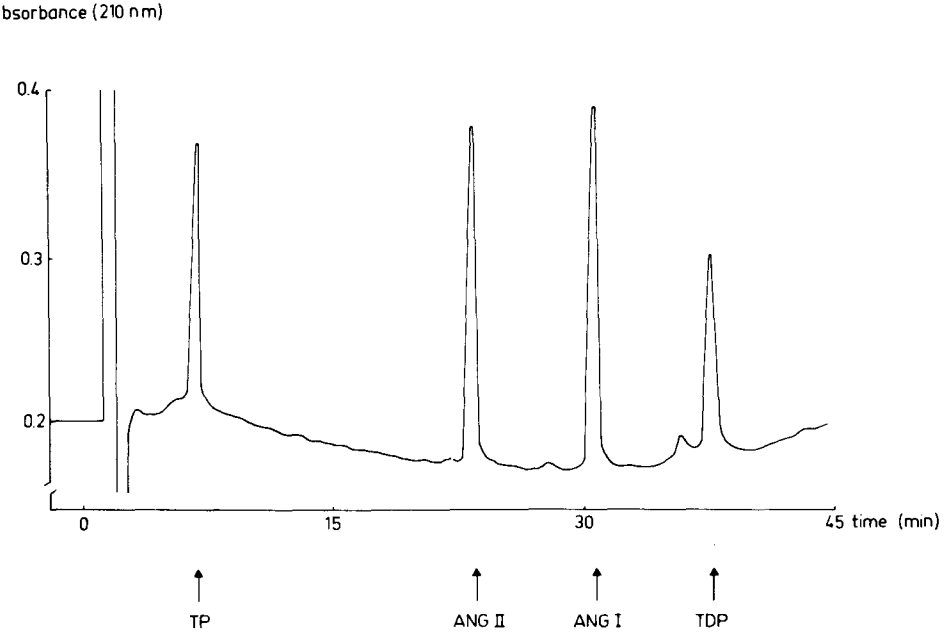

Fig. 2. UV absorbance profile of an HPLC eluate, showing the separation of a mixture containing synthetic ANG I, ANG II, TDP and TP (10 μg of each peptide) on a reversed-phase μBondapak C_{18} column. UV absorbance was monitored continuously at 210 nm.

Determination of renin activity using HPLC

Incubation of TDP (artificial renin substrate) with pure hog renin for 30 or 90 min resulted in the partial disappearance of this peptide, and the equimolar formation of ANG I and TP (Fig. 3B and Table I). Upon incubation for 30 min with partially purified rat renin, TDP was hydrolyzed completely, while the formation of ANG I and TP was not equimolar (Table I). A number of other, unidentified reaction products accumulated (Fig. 3C), suggesting non-specific enzymatic breakdown of peptides under these circumstances. Prolonged incubation up to 90 min resulted in further enzymatic breakdown of TP and ANG I (Table I). Addition of angiotensinase inhibitors (EDTA and 8-HQ) to the incubation mixture enhanced the accumulation of ANG I as expected, but also of some other digestion products (Fig. 3D and Table I).

DISCUSSION

Classical separation techniques like polyacrylamide gel electrophoresis, isoelectric focusing [11], paper chromatography [6, 12] and thin-layer chromatography [13] are generally used to separate ANG I and ANG II. Although the angiotensins have different mobilities and R_F values in these systems, separation of a peptide mixture will often result in an overlap of the components. A good separation of TDP and angiotensins by polyacrylamide gel electropheresis, isoelectric focusing or ion-exchange chromatography is hampered by close physicochemical similarities (unpublished results). Paper chromatography can be used for the separation of TDP, ANG I and TP [6], but is not useful to sepa-

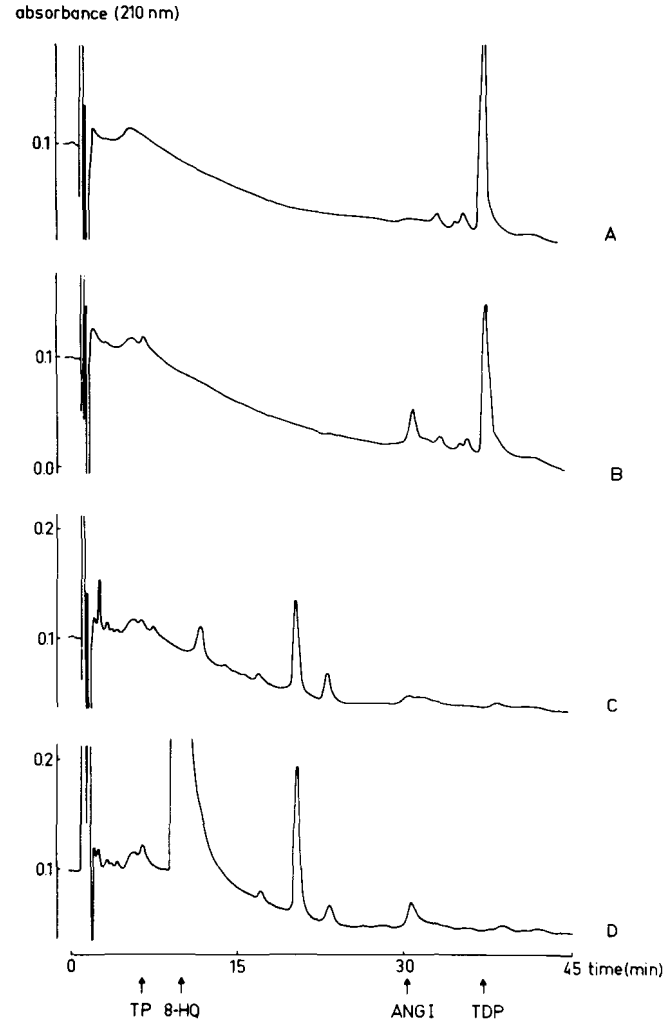

Fig. 3. UV absorbance profiles of HPLC separation of the reaction products of renin—TDP incubations. Incubations of 14.2 nmoles of TDP with 20 mGU of renin were made for 0 min (control incubation) or 30 min at 37°C in 500 µl of 20 m*M* citrate-phosphate buffer (pH 6.0). EDTA (10.0 m*M*) and 8-HQ (4.3 m*M*) were used as angiotensinase inhibitors. Conditions: control incubation (A), incubation with pure hog renin (B), partially purified rat renin (C), and rat renin in combination with angiotensinase inhibitors (D). The two small peaks preceding the TDP peak (see condition A) are caused by impurities in the TDP preparation.

rate ANG I and ANG II. Reversed-phase HPLC offers the possibility to separate complex mixtures, and to analyse the components qualitatively and quantitatively in a single and rapid step [14]. Retention on the reversed-phase columns is highly dependent on both molecular weight and polarity of the applied compounds. Application of this technique for various peptide mxitures has been described [8, 15]. In this study we demonstrate a complete separation of angiotensins and some related peptides by HPLC.

As expected from the small chain length, TP has a very short retention time on the reversed-phase column. Due to the apolar C-terminal dipeptide His-Leu,

TABLE I

TETRADECAPEPTIDE HYDROLYSIS BY HOG RENIN OR RAT RENIN

Incubation was carried out of 14.2 nmoles of TDP with 20 mGU of renin at 37°C in 500 µl of 20 m*M* citrate—phosphate buffer (pH 6.0). Rat renin was incubated in the absence and presence of angiotensinase inhibitors (10.0 m*M* EDTA/4.3 m*M* 8-HQ). Products were analysed by HPLC fractionation.

Enzyme	Incubation time (min)	Amount of peptides formed (nmoles)	
		TP	ANG I
Hog renin	0	–*	–
	30	1.4	1.3
	90	1.5	1.4
Rat renin	0	–	–
	30	0.9	0.3
	90	0.7	–
Rat renin + inhibitors	0	–	–
	30	2.2	1.1
	90	2.2	0.4

*Not detectable by UV monitoring of the HPLC eluate.

ANG I is less polar than ANG II. This, together with the difference in molecular weight, explains the complete separation of these peptides. Lengthening of ANG I with the apolar tetrapeptide Leu-Val-Tyr-Ser, yielding TDP, results in an increased retention time. Thus, with reversed-phase HPLC complete resolution of a mixture ANG I, ANG II, TP and TDP could be achieved. The elution pattern was very reproducible and independent of sample volume up to 1 ml, or of application buffers tested. The UV absorbance of the separated components in the eluate appeared to be linear with the amount of peptide applied to the column and thus enabled simple quantification in the microgram range. Thus, it appears that the procedure described here provides a simple and reliable method for the purification and quantification of angiotensins and related peptides in samples of various composition. Combination of appropriate radioimmunoassays with HPLC fractionation will increase the sensitivity of the peptide assay. Such a combination will also benefit the specificity of the radioimmunoassays since HPLC fractionation implicates separation of cross-reacting compounds (see ref. 8).

The determination of renin in biological samples without purification of the enzyme is problematic because these samples generally contain additional proteolytic activities. Radiochemical and fluorescence assays for renin [5—7] cannot distinguish these activities since the artificial renin substrates used in these assays will be hydrolyzed at several sites by the peptidases. This leads to the formation of multiple peptides, thereby causing serious interference in the assay. The use of these methods therefore requires extensive purification of renin. HPLC, on the other hand, offers the possibility to study directly the accumulation of all reaction products upon TDP incubation with unpurified

renin preparations. By this technique we have observed the formation of ANG I and TP as the only digestion products of TDP hydrolysis by pure hog renin. Following incubation of TDP with partially purified rat renin, in which other proteolytic enzymes are still present, the accumulation of other peptides could also be demonstrated. HPLC profiles also gave direct information about the inhibition of these additional proteolytic activities by angiotensinase inhibitors. Similarly, HPLC can prove to be a useful technique for studying the metabolism of angiotensins in biological systems (plasma, cerebrospinal fluid, tissue homogenates). In comparison to radiochemical and fluorescence enzyme assays, analysis of enzymatic reactions by HPLC fractionation enables a more specific detection of the reaction products, while possible accompanying co-products can be analysed properly.

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