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Short Communication

Determination of angiotensins by capillary electrophoresis

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Abstract

A protocol to separate ten peptides of the angiotensin family by capillary electrophoresis was described. The experiment was carried out using the Waters Quanta 4000 Electrophoresis system and the steps taken to determine the optimum electrophoretic conditions include (i) the use of different electrolytes, (ii) variation of ionic composition and pH of the electrolytes, (iii) variation of applied voltage and the wavelength of ultraviolet detection. Successful separation of the ten angiotensin peptides was obtained using a voltage of 10 kV, 0.1 *M* phosphoric acid (pH 1.95) as electrolyte and ultraviolet detection at 185 nm. The protocol was then used to follow the metabolism of exogenous angiotensin I (ANG I) in rat lung homogenate and the separation and identification of an angiotensin peptide in human plasma. In addition to these two applications, the protocol can be use to separate and identify angiotensins and other peptides for which specific antibodies have yet to be developed.

1. Introduction

The separation of angiotensins by high-performance liquid chromatography and the quantitation of the separated peptides by radioimmunoassay, currently used by most investigators, is a laborious multi-step procedure. Although this method is satisfactory in terms of sensitivity, many angiotensins cross-react with antibodies which are supposed to be specific to a particular angiotensin. Antibodies raised against angiotensin I (ANG I) have been shown to have 100% cross-reactivity with ANG-(2-10) and ANG-(3-10) [1], and antibodies specific for ANG II also cross-react with ANG III, ANG-(3-8) and ANG-(4-8) [2]. Because of such cross-reactivities, complete separation of the angiotensins by HPLC is a necessary step in their quantitation. Hence for tissue angiotensins that are present in concentrations below the UV detection limit of the HPLC system, precalibration of the chromatographic column with standard angiotensins and collection of "undetected" samples for assay with radioimmunoassay are part of the laborious routine.

In the present paper we report the simultaneous separation and identification of ten angiotensins in a one-step analysis by capillary electrophoresis. The protocol developed was then applied to study the degradation of ANG I in lung homogenate of rat and the isolation of an angiotensin peptide in human plasma.

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2. Experimental

2.1. Chemicals

Porcine renin substrate tetradecapeptide, human angiotensin I and angiotensin II were purchased from Sigma (St. Louis, MO, USA). Human angiotensin III, des-asp-Angiotensin I and Angiotensin-(1-7) were purchased from Bachem (Bubendorf, Switzerland). Angiotensin-(1-9), -(2-9), -(3-10) and -(3-8) were synthesized by the Biotechnology Unit, National University of Singapore. Orthophosphoric acid was purchased from Merck (Darmstadt, Germany). Enalaprilat was a gift from Merck Sharp and Dohme (West Point, PA, USA).

2.2. Instrumentation

Capillary electrophoresis was conducted using the Waters Quanta 4000 Electrophoresis system (Milford, MA, USA) mounted with a mercury lamp and a 185-nm filter. The silica capillary tubing employed for analysis was uncoated, with an internal diameter of 75 μm and a length of 60 cm.

2.3. Standard solutions

Standard solutions (10^{-3} *M*) of the angiotensins were prepared in distilled water. Electrolyte (either phosphoric acid or phosphate buffer) was used for dilution.

2.4. Preparation of lung extract and human plasma

Sprague–Dawley rats (250–280 g) were killed by decapitation. The lungs were immediately removed and minced. One gram of the minced tissue was homogenised in 5 volumes of 0.1 *M* phosphate buffer pH 7.5 using a Potter–Elvejhem tissue grinder immersed in crushed ice. A 5-ml volume of lung homogenate was then introduced into a dialysing tubing with a 10 000 molecular mass cut-off. It was then dialysed at 4°C with 3 changes of 500 ml phosphate buffer at 30-min intervals to remove endogenous peptides.

From a healthy human male volunteer a 20-ml blood sample was obtained in a heparinised tube containing 100 m*M* EDTA and enalaprilat (0.4 mg/ml of blood). Plasma was immediately obtained from each blood sample by centrifugation at 6000 *g* for 15 min at 4°C. The plasma was acidified with HCl (to a concentration of 0.05 *M*) and centrifuged further at 400 000 *g* for 60 min. Each 3-ml supernatant was then passed through a C_{18} cartridge (Sep-Pak, Waters Associates). The cartridge was washed twice with 1 ml of 5% methanol and 2 ml of 100% methanol were used to elute the angiotensins. The eluate was then filtered through 0.45-μm filters and vacuum dried. The dried sample was reconstituted in 75 μl of phosphate buffer and centrifuged at 400 000 *g* for 60 min prior to analysis by electrophoresis.

2.5. Determination of angiotensins

Preliminary experiments carried out to determine the optimum electrophoretic conditions for the separation of the 10 angiotensins include (i) the use of (0.05–0.2 *M*) phosphoric acid (pH from 1.5–2) and potassium phosphate buffer (pH from 6.5–7.5) as electrolytes, (ii) varying the applied voltage from 7 to 12 kV, and (iii) detection of the angiotensins at 185 and 254 nm, using a mercury lamp as the source of ultraviolet light, and 214 nm using a zinc lamp as the source of ultraviolet light. The derived optimum conditions are given in the legend of Fig. 1. The individual electropherogram of each standard peptide was verified by "spiking" with the known peptide.

2.6. Accuracy of determination

A solution of 10^{-5} *M* ANG II (prepared by diluting 10^{-3} *M* standard with 0.1 *M* phosphoric acid) was assayed on five successive days using the above-described electrophoresis. Five

aliquots of the same ANG II solution were also assayed on the same day. From this data, the inter-day and intra-day coefficient of variation was found to be 5.6 and 4.4% respectively. In addition, the limit of detection of ANG-(1-14) which has the lowest absorbance at 185 nm, was found to be 10^{-6} *M* or 1 nmol/ml (at a signal-to-noise ratio of >4).

2.7. Metabolism of ANG I in lung extract

Dialysed lung homogenate (100 μl) was added to 200 μl of a solution of ANG-I in 0.1 *M* potassium phosphate buffer pH 7.5 containing 300 μM ANG-I and 0.1 *M* NaCl in a final volume of 300 μl. Incubation was carried out at 37°C and three sequential aliquots of 90 μl of this incubation were pipetted into three separate vials containing 10 μl of 5 *M* perchloric acid at 0, 10, 30 min, respectively. These three solutions were then centrifuged at 400 000 *g* and 4°C (Beckman TL100) for 40 min. The angiotensins in each supernatant were then separated by capillary electrophoresis (see legend of Fig. 2).

2.8. Separation of plasma angiotensins

The endogenous angiotensins and other peptides in the plasma were separated by capillary electrophoresis as described in the legend of Fig. 3. Peak X, which was identified by spiking to be ANG-(2-10), was collected. One hundred collections were obtained in a single vial from 100 electrophoretic runs. The collected sample was then analyzed using the flow-injection analysis method on the Perkin-Elmer API *III* LC–MS–MS System. Because the sample was collected in 0.05 *M* phosphate buffer it (20 μl) was introduced into the System through a 15 cm × 320 μm I.D. capillary column packed with Poros R/H perfusion material (to desalt the sample) and eluted by a gradient of 100% water to 100% acetonitrile over a 20-min period with 0.1% formic acid. The flow-rate was 40 μl/min. Fig. 4 shows the mass spectrum of the standard ANG-(2-10) and the collected sample.

3. Results and discussion

Fig. 1 shows the electropherogram of angiotensinogen (ANG-(1-14)) and 9 peptides of the angiotensin family. This is the first demonstration of a successful separation of 9 angiotensin peptides by a single-step analytical technique. Part of the success is due to the fact that in capillary electrophoresis, UV detection of peptides at sub-200 nm wavelengths is feasible. Two factors that limit the use of sub-200 nm wavelengths in HPLC are partly ameliorated by the features of capillary electrophoresis. The first is the low intensity of most light sources in this spectral range coupled with low photodetector response. This is partly overcome by the use of a mercury lamp (lamps are interchangeable) as the UV source and a fixed wavelength (185 nm) filter, features available in the Waters Quanta 4000 Electrophoresis system. The second factor,

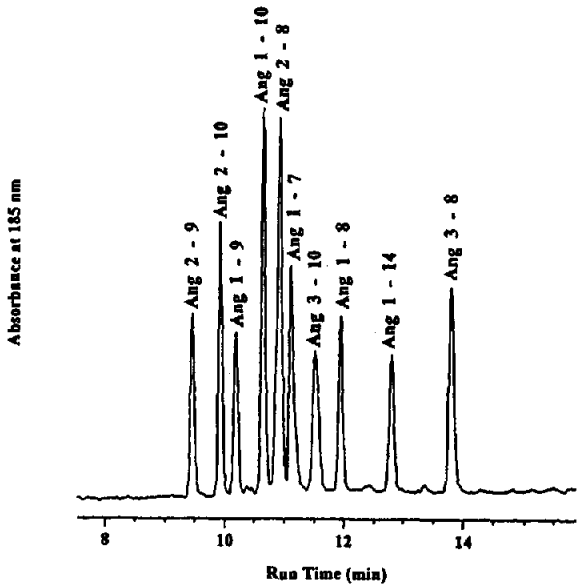

Fig. 1. Capillary electropherogram of 10 angiotensin fragments on a Waters Quanta 4000 Electrophoresis System. An aliquot of a standard solution containing 10^{-5} *M* of each angiotensin was hydrostatically sampled (for 4 s, approximately 20 nl) and subjected to electrophoresis at 10 kV for a period of 20 min in 0.1 *M* phosphoric acid, pH 1.95. The separated angiotensins were detected at 185 nm using a mercury lamp as the UV light source.

the low transmission of most organic solvent in this range, is overcome by the use of aqueous separation media (electrolyte) and the small pathlength used for detection. The advantage of using sub-200 nm wavelengths is the dramatic increase in molar absorptivity of many analytes including peptides [3] since the peak UV absorption of the peptide bond occurs in the 185–190 nm range [4].

Fig. 2 shows the time-dependent formation of ANG II and ANG III in a homogenate of rat lung. Fig. 3 shows the electropherogram of peptides extracted from human plasma and Fig. 4 shows the mass spectrum of standard ANG-(2-10) and a electrophoretic sample containing

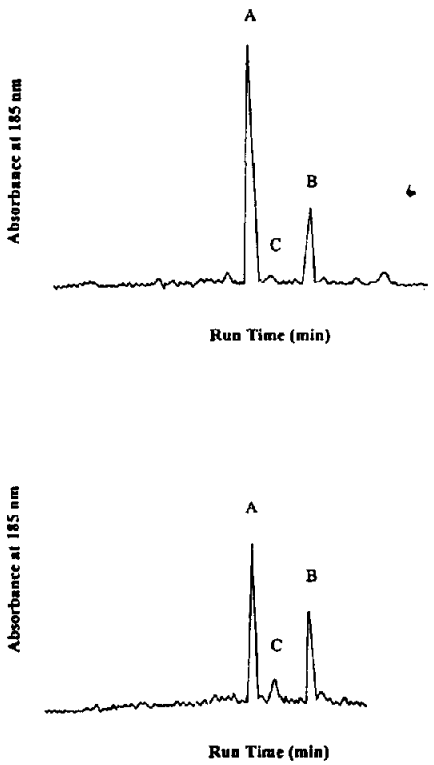

Fig. 2. Degradation of exogenous ANG I to ANG II and ANG III in rat lung homogenate at 5 (upper electropherogram) and 10 (lower electropherogram) min of incubation. Electrophoresis was carried out at 10 kV for a period of 20 min in 0.1 *M* phosphoric acid, pH 1.95 (A = ANG I, B = ANG II, and C = ANG III). The separated angiotensins were detected at 185 nm using the mercury lamp and the UV light source.

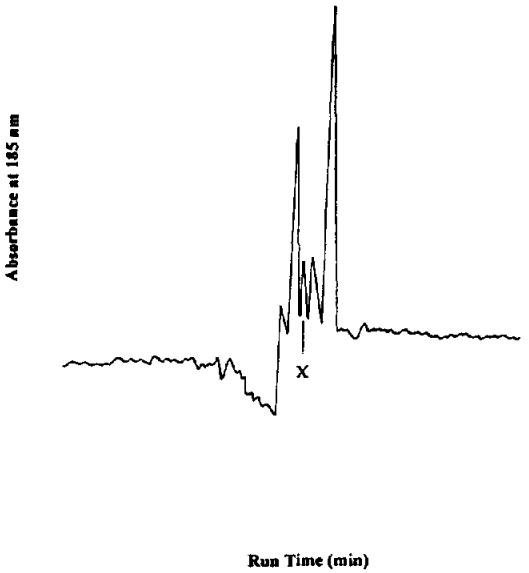

Fig. 3. Capillary electropherogram of human plasma extract. Electrophoresis was carried out at 10 kV in 0.05 *M* phosphate buffer, pH 7.32. The angiotensins were detected at 185 nm using the mercury as the UV light source. Peak X was identified by spiking to be ANG-(2-10). It was then collected (100 collections) and analyzed by mass spectrometry (see Fig. 4 for details).

ANG-(2-10). The confirmation of peak X as ANG-(2-10) by mass spectrometry supports the presence of ANG-(2-10) as a major angiotensin peptide in human plasma [1]. The protocol described can thus be used to detect and separate endogenous or exogenous angiotensins in small tissue samples by a simple one-step analysis with a 20-min run-time. In addition, the metabolism of any particular angiotensin in tissue extracts can be conveniently and reliably studied. With this protocol, we have found that ANG I is mainly degraded to ANG-(2-10) instead of ANG II in rat aortic homogenate, and determined the activity of the aminopeptidase responsible for the degradation [5]. A similar study carried out by Johnson and Drummer using HPLC and UV detection at 214 nm failed to decipher the breakdown products of ANG I in crude homogenates of rat lung and aorta [6]. Using the protocol described in the present paper we have also shown that the formation of ANG-(2-10) from ANG I in the hypothalamus of

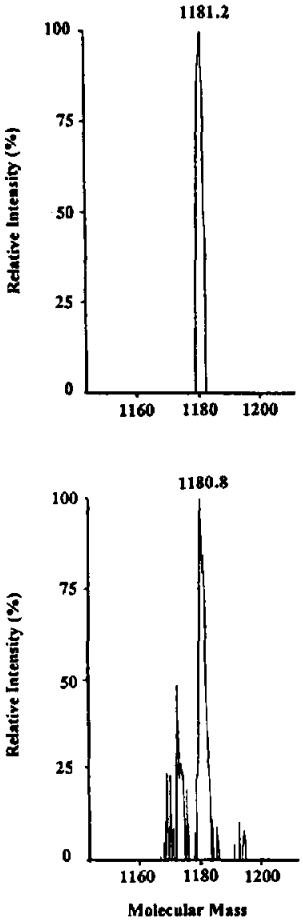

Fig. 4. Mass spectrum of the standard angiotensin, ANG-(2-10) (upper spectrum) and a pooled sample of the electrophoretic peak X (lower spectrum). An 20-μl aliquot of the standard ANG-(2-10) was fed directly into a stream of 50% acetonitrile, 50% water plus 0.1% formic acid running at 10 μl/min via the Rheodyne 8125 sample injector of the Perkin-Elmer API III LC–MS System. Because sample X was collected in 0.05 *M* phosphate buffer, it (20 μl) was fed into the System through a 15 cm × 320 μm I.D. capillary column packed with Poros R/H perfusion material (to desalt the sample) and eluted by a gradient of 100% water to 100% acetonitrile over a 20-min period with 0.1% formic acid. The flow-rate was 40 μl/min.

the two models of hypertensive rats was significantly greater than in normotensive control rats [7]. The protocol also enables one to separate angiotensins, *e.g.* ANG-(1-9), -(2-9), and other peptides for which specific antibodies have yet to be developed. With the automated sample collection available in the more recent electrophoresis systems, collection of peaks from multiple runs can be conveniently carried out. Unknown peaks can then be identified by their mass spectrum or by more specific techniques.

4. Acknowledgements

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5. References

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