A Simple Radioassay for Angiotensin-Converting Enzyme

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(Received 1 August 1977)

Angiotensin-converting enzyme can be measured by the rate of release of ^3H -labelled hippurate from p -[^3H]benzoylglycylglycylglycine. The product is separable from the substrate by extraction of acidified reaction mixtures with ethyl acetate. Assay results for human serum angiotensin-converting enzyme can be obtained within 1.5 h of receipt of serum samples. Within the limits tested, the assay appears to be specific. However, interference by hitherto unrecognized enzymes of abnormal sera must be ruled out.

Several methods have been developed to measure angiotensin-converting enzyme (also known as kininase II), but none is at once simple, rapid and specific. The enzyme acts as a dipeptidyl carboxypeptidase (Skeggs *et al.*, 1956; Dorer *et al.*, 1974a) of no specificity, but of great selectivity. Thus the enzyme hydrolyses bradykinin more rapidly than any of its higher homologues (e.g. lysyl-bradykinin; Dorer *et al.*, 1974b) and has a greater affinity for des-Asp¹-angiotensin I than for angiotensin I (Tsai *et al.*, 1975; Chiu *et al.*, 1976).

The most commonly used assay method measures angiotensin-converting enzyme activity in terms of the rate of release of hippurate from hippuryl-histidyl-leucine (Cushman & Cheung, 1971). Reactions are terminated by acidification and then hippurate is separated from unhydrolysed substrate by extraction with ethyl acetate. Hippurate is quantified by measuring its A_{228} . Ethyl acetate absorbs strongly at 228 nm and all traces must be removed by evaporation. The residue must be dissolved in water or in 0.9% NaCl before quantification of hippurate (cf. Lieberman, 1975). Thus the assay is tedious, time-consuming and subject to a number of artifacts. Lipaemic or haemolysed serum samples cannot be used. Similarly, detergents such as Triton X-100 interfere with absorbance readings.

Assays using hippurylglycylglycine have been developed, the best characterized of which measures the release of glycylglycine with a quantitative ninhydrin reaction (e.g. see Dorer *et al.*, 1976). Hippurylglycylglycine appears to be somewhat superior to hippurylhistidyl-leucine in that it is less susceptible to hydrolysis by carboxypeptidase enzymes. However, the assay method of Dorer *et al.* (1976) cannot be used without prior dialysis of plasma or tissue homogenates.

In the present study, we have developed an assay method in which p -[^3H]benzoylglycylglycylglycine (^3H -labelled hippurylglycylglycine) is used as substrate for angiotensin-converting enzyme. Enzyme activity is measured in terms of the rate of release

of ^3H -labelled hippurate. Thus prior dialysis of serum samples and tissue homogenates is not required. As in the assay method of Cushman & Cheung (1971), hippurate is separated from unhydrolysed substrate by extraction of acidified reaction mixtures with ethyl acetate. However, ethyl acetate need not be removed as the hippurate content of the ethyl acetate extract can be quantified directly by liquid-scintillation counting.

Materials and Methods

Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] was from Calbiochem, La Jolla, CA, U.S.A. Glycylglycylglycine, glycylglycine and Triton X-100 were from Sigma Chemical Co., St. Louis, MO, U.S.A.; benzoic acid and p -iodobenzoic acid were from Eastman Organic Chemicals, Rochester, NY, U.S.A.; hippurylhistidyl-leucine was from Research Plus, Denville, NJ, U.S.A. Benzoylglycylglycylglycine was synthesized as described by Dorer *et al.* (1972) by reacting benzoyl chloride with glycylglycylglycine. Synthesis of benzoylglycylglycine used glycylglycine in reaction with benzoyl chloride. The p -iodobenzoylglycylglycylglycine was synthesized by reacting the N -hydroxysuccinimide ester of p -iodobenzoic acid with glycylglycylglycine. The ester was synthesized by mixing 10.25 mmol of p -iodobenzoic acid and 10.25 mmol of N -hydroxysuccinimide in dimethylformamide at 0°C with 11.28 mmol of dicyclohexylcarbodi-imide in dimethylformamide (added dropwise). After 2 h at 0°C, the reaction mixture was stirred at room temperature (24°C) for 16 h. The solvent was removed by rotary evaporation at approx. 40°C, a step that yielded white crystals. Dicyclohexylurea was dissolved and removed by washing the white solid with hot propan-2-ol. The N -hydroxysuccinimide ester of p -iodobenzoic acid was obtained by crystallization from ethyl acetate (m.p. 236–237°C, decomp.); elemental analysis: found: C, 37.8; H, 2.2; N, 4.0; calc. for $\text{C}_{11}\text{H}_8\text{N}_2\text{O}_4$ (mol.wt. 331.095): C, 38.3; H, 2.3; N, 4.1%. The ester

(9.79 mmol in 60 ml of dimethylformamide) was added dropwise with vigorous stirring to a cold solution (0°C) of glycylglycylglycine (10 mmol) and Na₂CO₃ (10 mmol) in 60 ml of water and dimethylformamide (2:1, v/v). The reaction was continued for 1 h at 0°C and then overnight at room temperature. Solvent was removed by rotary evaporation and the residue was dissolved in water. The solution was washed with ethyl acetate and then a white precipitate was formed by acidification with 2M-HCl to pH2. The precipitate was collected by filtration, washed with dilute HCl and water and then dried. The residue was washed three times with hot propan-2-ol, three times with hot ethyl acetate and finally with anhydrous diethyl ether. The product, dried under vacuum, was obtained in a yield of 73.8% (m.p. 243–245°C, decomp.); elemental analysis: found: C, 37.2; H, 3.4; N, 10.1; calc. for C₁₃H₁₄N₃O₅ (mol.wt. 419.186): C, 37.3; H, 3.4; N, 10.0%.

The *p*-iodobenzoylglycylglycylglycine was dehalogenated (by New England Nuclear, Boston, MA, U.S.A.) in ³H₂ gas to yield *p*-[³H]benzoylglycylglycylglycine ([³H]hippurylglycylglycine): 3.78 mg of the starting material was dissolved in 0.5 ml of 0.1% (w/v) acetic acid, to which 5 mg of 10% Pd/C catalyst was added. The mixture was treated with 5 Ci of ³H₂ gas at 53 kPa (400 torr) for 3 h at room temperature. Solvent was removed by evaporation under vacuum, and the product was dissolved in methanol/water (1:1, v/v) and separated from the catalyst by filtration. The solvent was removed under vacuum, and the product was dissolved in 3 ml of water and then freeze-dried. The product (128 mCi, specific radioactivity 25 Ci/mmol) was dissolved in 7.11 ml of water. The product behaved as a single substance, not distinguishable from authentic hippurylglycylglycine, on paper electrophoresis at pH2.0 and 5.0 (Ryan *et al.*, 1971) and on t.l.c. [silica-gel plates (New England Nuclear); solvent systems: (a) butan-1-ol/acetic acid/water (4:1:5, by vol); (b) butan-1-ol/ethyl acetate/acetic acid/water (1:1:1:1, by vol.).

Results and Discussion

The [³H]hippurylglycylglycine can be used without carrier hippurylglycylglycine to yield a reaction with angiotensin-converting enzyme that obeys first-order enzyme kinetics. However, to facilitate comparison with the assay described by Dorer *et al.* (1976), we used hippurylglycylglycine at a final concentration of 8 mM (apparent *K_m* = 5 mM) and [³H]hippurylglycylglycine at a final specific radioactivity of 0.125 Ci/mmol.

The conditions selected for assay of angiotensin-converting enzyme were those suggested by data of

Dorer *et al.* (1976). Typically, reactions were conducted in a final volume of 100 μl; 50 μl of buffered substrate (16 mM-[³H]hippurylglycylglycine, 0.125 mCi/mmol, in 0.05M-Hepes buffer, pH8.0, containing 0.1M-NaCl and 0.6M-Na₂SO₄) is delivered to the bottom of a glass test tube (11 mm × 75 mm). The reaction is started by adding 50 μl of enzyme in the same buffer. For assay of human serum angiotensin-converting enzyme, 50 μl of serum is added to 200 μl of buffer, and 50 μl of the resulting solution is used in the final reaction mixture. Guinea-pig serum has much larger quantities of enzyme (Yang *et al.*, 1971) and must be diluted 4–10-fold more than human serum. Each assay required a blank, which is composed of 50 μl of buffered substrate and 50 μl of buffer. All incubation mixtures are incubated at 37°C for time intervals such that no more than 10% of substrate is used. For human serum, reaction mixtures are incubated for 60 min. Reactions are linear with time when normal serum is used in dilutions of 1:4–1:20 (equivalent to 12.5 to 2.5 μl of serum in the final reaction mixture). In addition, the reactions are linear with time so long as no more than 10% of the substrate is used.

Reactions are terminated by adding 1.0 ml of 0.1M-HCl. Hippurate is extracted with 1.0 ml of ethyl acetate. The two phases are mixed by vortexing (approx. 15 s), and the layers are separated by centrifugation (2000g for 10 min). Approx. 91% of hippuric acid enters the organic phase, and less than 7% of substrate is partitioned into ethyl acetate. A sample of the ethyl acetate layer, usually 500 μl, is transferred to a scintillation vial containing 10 ml of a scintillation fluid containing detergent [e.g. Riafluor (New England Nuclear) or Hydromix (Yorktown Research, South Hackensack, NJ, U.S.A.)]. To measure the starting substrate concentration, 50 μl of the buffered substrate is added directly to a scintillation vial. The radioactivity of each vial is counted for 1–2 min. Although the substrate is added to counting vials as an aqueous solution, quenching different from that of the vials containing ethyl acetate extracts has not been encountered.

Enzyme activity is computed in munits/ml (1 unit equals the hydrolysis of 1 μmol of substrate/min at 37°C; Dorer *et al.*, 1976) by using the formula:

$$\text{munits/ml} = \frac{2 \times \frac{\text{test c.p.m.} - \text{blank c.p.m.}}{\text{substrate c.p.m.}} \times 800 \text{ nmol}}{\text{time (min)} \times \text{vol. of enzyme (ml)}} \times \frac{1}{1 - \frac{\text{blank c.p.m.}}{\text{substrate c.p.m.}}}$$

where 800 nmol is the quantity of starting substrate. The factor of 2 is to correct the c.p.m. of 1.0 ml of ethyl acetate. The expression:

$$\frac{1}{1 - \frac{\text{blank c.p.m.}}{\text{substrate c.p.m.}}}$$

is to correct for the fraction of substrate that is partitioned into ethyl acetate. For human serum enzyme activity, the equation can be simplified to:

$$\begin{aligned} \text{munits/ml} &= 2 \times \frac{\text{test c.p.m.} - \text{blank c.p.m.}}{\text{substrate c.p.m.}} \times 1333 \\ &\times \frac{1}{1 - \frac{\text{blank c.p.m.}}{\text{substrate c.p.m.}}} \end{aligned}$$

as time of incubation is 60min and the volume of serum in the assay is 0.01ml. Except for clearly haemolysed assay samples, differential quenching has not been encountered. Where quenching varies, the above computations should be revised to use d.p.m. rather than c.p.m.

We have used the assay to measure angiotensin-converting enzyme activity in sera of man, rat and guinea pig and in homogenates of rat kidney and guinea-pig lung (Table 1). The enzymic activity of the sera and tissue homogenates tested is indistinguishable from angiotensin-converting enzyme. The activity is inhibited (50%) by: 0.1mM-EDTA; 2.8nM-bradykinin-potentiating peptide 9α; 1μM-bradykinin-potentiating peptide 5α; 12μM-angiotensin I; 6μM-bradykinin. Bradykinin-potentiating peptides 9α and 5α [derived from the venom of *Bothrops jararaca* (South American pit viper)] are reported to be specific inhibitors of converting enzyme (for review see Bakhle, 1974). The human serum enzyme as measured by our assay shared characteristics of pure pig lung angiotensin-converting enzyme (Dorer *et al.*, 1976). It required Cl⁻ and was most active at pH8.0.

Further to test the specificity of the assay, serum samples (from healthy volunteers, patients with active

sarcoidosis and guinea pigs) were examined for their ability to form radioactive products other than hippurate. After incubation of the sera with substrate, the ethyl acetate extracts were evaporated (to approx. 0.1ml) and were then applied to silica-gel thin-layer plates. The plates were developed in solvent system (b). In each experiment, all radioactivity was associated with hippurate (*R_F* 0.71) and unhydrolysed substrate (*R_F* 0.49). None was associated with benzoic acid (*R_F* 0.82) or hippurylglycine (*R_F* 0.59). These results, together with those of the inhibition studies, suggest that enzymes other than angiotensin-converting enzyme do not interfere. However, we cannot rule out the possibility that hitherto unrecognized enzymes (e.g. in abnormal sera not yet tested) may hydrolyse the substrate.

Lieberman (1975) has shown that angiotensin-converting enzyme concentrations are increased in sera of some patients with active sarcoidosis, a finding confirmed by a number of independent investigators (e.g. Silverstein *et al.*, 1976; Zorn *et al.*, 1977). Most of the clinical studies have used hippurylhistidyl-leucine as the assay substrate and have measured the rate of release of hippurate by the method of Cushman & Cheung (1971) or the rate of release of histidyl-leucine (e.g. Friedland & Silverstein, 1976). The latter product forms a strong fluorophor on reaction with *o*-phthaldialdehyde (Piquilloud *et al.*, 1970). To compare our assay method with that of Cushman & Cheung (as modified by Lieberman, 1975, 1976), we used both assays to measure angiotensin-converting enzyme activity of 94 human sera. A linear correlation was obtained (*r*=0.891, *P*<0.001). One unit of enzyme activity as defined by Lieberman (1976) is equivalent to 3.6munits of enzyme activity as measured by our assay.

Table 1. Angiotensin-converting enzyme activity of sera and tissue homogenates

Assays were performed as described in the text, taking care to limit substrate utilization to 10% or less. The results are means±s.d. for the numbers of determinations in parentheses. Specific activities for sera are expressed in munits/ml and specific activities for homogenates are expressed in munits/g wet wt. of tissue. The mean value (±s.d.) for one human serum sample (from a patient with active sarcoidosis) assayed 28 times over 14 days was 179±7munits/ml. The coefficient of variation was 3.6%.

Enzyme source	Specific activity
Human sera (20)	103 ± 32
Rat sera (22)	191 ± 22
Guinea-pig sera (20)	1410 ± 212
Rat kidney (20)	370 ± 153
Guinea-pig lung (15)	5740 ± 2070

Table 2. Effects of an intravenous injection of bradykinin-potentiating peptide 9α on arterial plasma angiotensin-converting enzyme activity of guinea pigs

Guinea pigs were anaesthetized with pentobarbital (30mg/kg intraperitoneally) and then were injected with heparin (1000units/kg intravenously). Cannulas were placed in an external jugular vein and in a common carotid artery. Arterial blood samples were collected immediately before and at timed intervals after intravenous injection of 0.5ml of 0.9% NaCl (guinea pig B) or bradykinin-potentiating peptide 9α (2mg/kg of body wt.) in 0.5ml of 0.9% NaCl (guinea pig A). The values in parentheses are percentages of control values.

	Angiotensin-converting enzyme activity (munits/ml)	
	Guinea pig A	Guinea pig B
Before injection	1350 (100)	1430 (100)
10min after injection	47 (3.5)	1360 (95.5)
30min after injection	322 (23.9)	1490 (104.7)

Gavras *et al.* (1974) have shown that bradykinin-potentiating peptide 9 α (also known as SQ 20881) can be used to treat renin-related hypertensive cardiovascular disease. At present, it is not known what degree of inhibition of angiotensin-converting enzyme is required to obtain a therapeutic effect. To improve understanding of the clinical use of bradykinin-potentiating peptide 9 α and other inhibitors of angiotensin-converting enzyme, we measured arterial plasma enzyme activities of anaesthetized guinea pigs before and after a single intravenous injection of bradykinin-potentiating peptide. The results shown in Table 2 suggest that serial measurements of serum angiotensin-converting enzyme may be useful for monitoring treatment of patients with bradykinin-potentiating peptide 9 α and other inhibitors of angiotensin-converting enzyme (e.g. SQ 14225; Ondetti *et al.*, 1977).

The major advantages of our assay over those used previously are simplicity and the shorter time required to obtain assay results. Prior dialysis of enzyme source is not required and there is no need to evaporate organic solvents before quantifying hippurate. Quenching problems have not been encountered except on assay of haemolysed serum samples. Triton X-100 in concentrations up to 0.05% (v/v) in the final reaction mixture does not disturb the apparent partition coefficients of either hippurate or hippurylglycylglycine. Lipaemic serum samples sometimes cause the formation of stable emulsions on extraction with ethyl acetate, but the emulsions can be broken by centrifugation, repeated if necessary. The major disadvantage of our assay is that it requires a liquid-scintillation counter.

This work was supported by a grant from the Council for Tobacco Research, U.S.A., Inc.

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