ENZYME INHIBITORS FOR RENIN ASSAY IN RAT PLASMA

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Summary
1. Degradation of 125I-labelled [Ile51-angiotensin I and unlabelled angiotensin I was studied during incubation of rat plasma treated with disodium ethylenediaminetetra-acetate (disodium EDTA) at 37°C, pH 6.5, for 1 h with 8-hydroxyquinoline, phenylmethylsulphonyl fluoride or di-isopropyl fluorophosphate.

2. Isoelectric focusing showed approximately 100, 59–66 and 10–31% preservation of 125I-labelled angiotensin I during incubation with 8-hydroxyquinoline (5.0 mmol/l), phenylmethylsulphonyl fluoride (15 mmol/l) and di-isopropyl fluorophosphate (1.09 mmol/l) respectively. Mean recovery of unlabelled angiotensin I was 102, 77 and 32% with 8-hydroxyquinoline, phenylmethylsulphonyl fluoride and di-isopropyl fluorophosphate respectively.

3. 8-Hydroxyquinoline appears the preferable enzyme inhibitor for renin assay in rat plasma.

Key words: enzyme inhibitors, renin.

Introduction
Plasma renin activity is generally measured by radioimmunoassay of angiotensin I generated in vitro in the presence of inhibitors of angiotensin I-degrading enzymes (Sealey & Laragh, 1973; Oparil, 1976). Most assay methods were originally developed for human plasma and modified for rat plasma (Menard & Catt, 1972; Carvalho, Shapiro, Hopper & Page, 1975; Katz & Roper, 1977). Incubation conditions during angiotensin I generation have been studied in detail in human plasma, with special attention being paid to an optimal inhibitor system (Fleming, Stewart & Hutchinson, 1974; Kodish & Katz, 1974; Oparil, Koerner & Haber, 1974; Delorme, Guyene, Corvol & Menard, 1976).

Having tested the optimal inhibitor for rat plasma, we report great differences in efficacy of three commonly used enzyme inhibitors for rat plasma renin activity assay.

Methods
Blood from female Wistar or Sprague–Dawley rats was used as pooled disodium EDTA-plasma (6 g of disodium EDTA/l of blood).

To study the stability of 125I-labelled angiotensin I during incubation 200μl of thawed plasma was pipetted into tubes in ice/water. 8-Hydroxyquinoline (Merck, no. 7098; final concentration 3.5–5.0 mmol/l of ethanol), phenylmethylsulphonyl fluoride (Sigma Chemical Co, no. p-7626; 7.5–30 mmol/l of ethanol) and di-isopropyl fluorophosphate (Koch-Light Laboratories Ltd no. 2359p; 1.09–9.0 mmol/l of propan-2-ol) were tested as inhibitors of angiotensin I-degrading enzymes. pH was adjusted to 6.5, the optimal pH for angiotensin I generation in rat plasma (Menard & Catt, 1972), by a single addition of hydrochloric acid (0.1 mmol/l). Then 0.4 pmol of 125I-monoiodo-labelled angiotensin I, specific radioactivity 400 kCi/mol, prepared as described by Fyhrquist, Soveri, Puutula & Stenman (1976), was added to each tube to a total volume of 280 μl. Half of the tubes were immediately transferred to −20°C, the other half were incubated at 37°C for 1 h, then rapidly cooled and stored at −20°C until isoelectric focusing was performed.

Isolelectric focusing in polyacrylamide gel was
FIG. 1. Results with isoelectric focusing of $^{125}$I-labelled angiotensin I before (controls) and after incubation with rat plasma treated with disodium EDTA at 37°C for 1 h with 8-hydroxyquinoline (5-0 mmol/l) (8-OH-quinoline), phenylmethylsulphonyl fluoride (15 mmol/l) (PMSF) or di-isopropylfluorophosphate (1-09 mmol/l) (DFP). The range of pH covered is 3-10. The isoelectric point of sheep methaemoglobin (7-3) is indicated by arrows.

performed on Ampholine/polyacrylamide-gel plates (LKB Aminkemi, Sweden) as described by Fyhrquist & Puutula (1978).

To test the stability of unlabelled angiotensin I during incubation, plasma samples (240 μl) of known dilution containing 125-1000 pg of [Ile$^5$]angiotensin I (Schwarz–Mann) were incubated at pH 6-5 for 1 h at 37°C. The reaction was stopped by addition of 1-76 ml of ice-cold assay buffer and the angiotensin I content was determined by radioimmunoassay (Fyhrquist et al., 1976).

Results

8-Hydroxyquinoline was superior to the other two enzyme inhibitors tested (Fig. 1). Combination of 8-hydroxyquinoline (5-0 mmol/l) and disodium EDTA resulted in close to 100% preservation of $^{125}$I-labelled angiotensin I. All radioactivity was focused at pH 7-3, corresponding to the isoelectric point of $^{125}$I-labelled angiotensin I. For complete enzyme inhibition 5-0 mmol of 8-hydroxyquinoline/l was the lowest effective concentration.

After incubation with phenylmethylsulphonyl fluoride (15 mmol/l), at pH 6-5, 34-41% of the total radioactivity could be detected at a pH other than 7-3, indicating incomplete inhibition of angiotensin I-degrading enzymes. Higher concentrations of phenylmethylsulphonyl fluoride did not improve the results.

Di-isopropyl fluorophosphate (1-09 mmol/l) proved to be ineffective. Most of the added radioactivity focused at acidic pH and only 10-31% as unaltered $^{125}$I-labelled angiotensin I. Even in non-incubated control samples some degradation took place, presumably during the focusing procedure. At higher concentrations only slightly better results were achieved. After incubation with di-isopropyl fluorophosphate (5-0 and 9-0 mmol/l) only 35 and 38% of added $^{125}$I-labelled angiotensin I respectively was undegraded.

When unlabelled angiotensin I was added to plasma (pH 6-5) the following recovery percentages, determined by radioimmunoassay after incubation, were achieved: 102 ± 5-3 (mean ± SEM, n = 6), 77 ± 2-7 (n = 5) and 32 ± 17 (n = 3) with 8-hydroxyquinoline (5-0 mmol/l), phenylmethylsulphonyl fluoride (15 mmol/l) and di-isopropyl fluorophosphate (1-09 mmol/l) respectively.

Discussion

Our results showed great differences in stability of both labelled and unlabelled angiotensin I during incubation of plasma treated with disodium EDTA at 37°C, pH 6-5, for 1 h with the enzyme inhibitors used. 8-Hydroxyquinoline was superior to phenylmethylsulphonyl fluoride and di-isopropyl fluorophosphate as demonstrated by a close to 100% preservation of $^{125}$I-labelled angio-
tensin I during incubation and excellent recovery of unlabelled angiotensin in contrast to partial inhibition only of angiotensin I-degrading enzymes by phenylmethylsulphonyl fluoride and di-isopropyl fluorophosphate. In this regard our results are in accordance with those of Kodish & Katz (1974) and Oparil et al. (1974), who have reported particularly di-isopropyl fluorophosphate, but also phenylmethylsulphonyl fluoride, to be relatively ineffective at a higher pH. According to our results, this seems particularly to apply to rat plasma.

It should be noted that electrofocusing per se may, in theory, separate enzyme inhibitors from degrading enzymes and angiotensin I, thus modifying the apparent degree of enzyme inhibition. The non-incubated control samples remained, however, mostly unaltered during isoelectric focusing, indicating that degradation took place during incubation at 37°C. This was also supported by the results obtained with unlabelled angiotensin I, which were parallel to the results with 125I-labelled angiotensin I.

For a reliable assay method for plasma renin activity a nearly complete protection of angiotensin I during assay procedure is essential (Sealey & Laragh, 1973). Thus phenylmethylsulphonyl fluoride and di-isopropyl fluorophosphate, without an additional enzyme inhibitor, appear not to satisfy this requirement in rat plasma at pH 6.5. Caution is required when a method for human plasma is employed for rat plasma.

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References