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Short communication

Determination of enzyme (angiotensin convertase) inhibitors based on enzymatic reaction followed by HPLC

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Abstract

For determination of levels of plasmatic inhibitor of ACE (angiotensin convertase) a simple method was used based on a combination of enzymatic reaction followed by an HPLC determination of its product. The inhibitor (e.g. enalaprilat) was at first separated from the biological material by deproteination (methanol). Then, an aliquot of the sample was added to the reaction mixture containing a commercial ACE enzyme, its specific substrate FAPGG (*N*-(3-[2-furyl]acryloyl)-Phe-Gly-Gly) and buffer (Tris–HCl, pH 7.5). Degree of inhibition of the conversion of this substrate to FAP (desGlyGlyFAPGG) by the inhibitor present in the sample is related to its amount by a simple dose–response relationship. The amount of the FAP was determined by an HPLC on a RP-18 column with an acetonitril–nonylamine buffer (pH 2.4, adjusted with phosphoric acid) as a mobile phase with detection at 305 nm. Alternatively, the activity of the endogenous ACE present in the plasma was measured. The substrate FAPGG was added to the plasmatic sample containing both the inhibitor and endogenous ACE (as the sample was not deproteinized in this case) and the reaction product was determined as above. Inhibitor concentration has been obtained from a dose–response curve expressing the interaction with inhibitor with an ACE enzyme.

Keywords: Angiotensin converting enzyme; Enzyme assay; Reversed phase-HPLC; Angiotensin I convertase enzyme-inhibitors; Enalapril; Lisinopril

1. Introduction

Determination of a drug in biological fluids is often difficult e.g. when the absorption characteristics are poor because of low values of absorption coefficients or of lack of well-defined maxima.

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This is also the case of enalapril or lisinopril. Enalapril ((S)-1-[N-(1-(ethoxycarbonyl)-3-phenylpropyl]-L-alanyl)-L-proline) is a prodrug which is hydrolysed in the liver to its active form (diacid) enalaprilat; lisinopril differs from enalapril in that it is not an ester prodrug and thus does not require bioactivation by the liver.

Enalaprilat (and related compounds) is a highly active inhibitor of angiotensin I – converting enzyme (ACE), a dipeptidase hydrolyzing angiotensin I to the vasopressor octapeptide, angiotensin II [1]. It is used for treatment of essential and renovascular hypertension as it reduces the plasma levels of angiotensin II and aldosterone and increases endogenous bradykinin concentrations [1–3]. Determination of levels of active form, enalaprilat, in biological tissues is based either on radioimmunoassay (using antilisinopril antiserum [4–6]) or, undirectly, on assays of enzymatic activity of ACE (more precisely, on the inhibition of ACE activity by enalaprilat [6–8]). Enzymatic activity of enalaprilat is usually determined with suitable substrates by spectrophotometry [6] or fluorometry [8], or, by using a radiolabelled substrate [7].

The assay can be realized in two ways: the enzyme may be either endogenous, i.e. inherently present in the biological fluid (as plasma [4,7]) or exogenous [5,6] added to biological sample after removal of endogenous protein. This approach has been shown to work in principle with rat, dog and monkey ACE [7,8] from both plasma and urine. On the other hand, the results with human samples are controversial: the use of inhibition of endogenous ACE for quantification of enalaprilat has not been recommended as the relationship between enalaprilat concentration and degree of ACE inhibition (a dose-response curve) has seemed not to be suitable for determination of enalaprilat as it was rather steep and not sensitive [6].

The aim of this work is (i) to improve the determination of the degree of ACE inhibition by using a sensitive and stable substrate, 2-furylacryloyl-L-phenylalanyl-glycylglycine (FAPGG, which is converted to FAP, 2-furylacryloyl-L-phenylalanine), which has been used previously for spectrophotometric measurement of ACE activity [9] with a newly developed HPLC method and (ii) to quantitate the enalaprilat levels by using a model of inhibition based on sigmoidal character of the response curve (i.e. of the dependence of the degree of inhibition on the enalaprilat concentration). An appropriate model of inhibitor:ACE interaction should in theory express the binding of ACE inhibitor to two sites as there are two (active) binding sites for low-molecular weight substrates known to be present at the ACE molecule (1) with a dose-response (inhibition) curve analogous to the known Hill equation describing the effect of a drug which is capable of completely abolishing an effect [5,10].

The method developed here makes it possible to get the levels of an ACE inhibitor without using a sophisticated equipment or radiolabelled compounds. It is based on a combination of enzymatic reaction with an HPLC determination of the inhibition of the enzyme by analyzing the levels of the substrate (FAPGG) and product formed (FAP). As the degree of inhibition depends on the level of the ACE inhibitor (by a dose-response relationship), the respective concentrations of the inhibitor can be reasonably estimated.

2. Experimental

2.1. Materials

Inhibitors of ACE (enalapril maleate, lisinopril) were obtained from Sigma (St.Louis, MO, USA); enalaprilat was a gift from Dr. T. Vontor from this Institute. ACE (angiotensin converting enzyme, EC 3.4.15.1) from rabbit lung was purchased from Fluka BioChemika (Buchs, Switzerland), substrate FAPGG (N-(3-[2-furyl]acryloyl)-Phe–Gly–Gly), product FAP (des-GlyGlyFAPGG) and all other reagents were from Sigma (St.Louis, MO). Water, acetonitril and methanol were HPLC grade. Minipig (Goettingen minipig, Institute of Veterinary Medicine, Brno) plasma was obtained from freshly taken blood at desired intervals; samples of human plasma were obtained from laboratory, Faculty Hospital, Hradec Králové. The experiment was approved

by the local ethics committee. Plasma samples were kept at −70°C until used.

2.2. Methods

2.2.1. Determination of inhibitors with purified commercial ACE

To 100 μl of plasma containing an inhibitor of ACE, 200 μl of methanol was added and after shaking, the denaturated protein was removed by centrifugation (5000 × g for 10 min). Aliquots of supernatant (10–50 μl) were pipetted into 4 ml glass tubes and evaporated to dryness under nitrogen stream at 37°C. To the dry residue, 150 μl of buffer (50 mM Tris–HCl pH 7.5 containing 0.3 M NaCl) and 5 μl of enzyme (ACE from rabbit lung, 3.1 U/mg protein diluted to 250 μg of protein/ml) were added and the mixture was preincubated for 3 min. The reaction was started by addition of 50 μl of substrate solution (2×10^{-4} M FAPGG in the same buffer); after 25 min, the reaction was stopped by 500 μl of methanol. Reaction mixture was kept at 37°C in a water bath. The precipitate was removed by centrifugation (5000 × g for 10 min); supernatant was analyzed by HPLC.

2.2.2. Determination of inhibitors using endogenous plasmatic ACE

To 500 μl of plasma containing endogenous ACE and its inhibitor 150 μl of substrate (2×10^{-4} M FAPGG in water) was added. After 60 min incubation at 37°C in a water bath, the enzymatic reaction was stopped by 2 ml of methanol. Precipitate was removed by centrifugation (5000 × g for 10 min) and supernatant was analyzed by HPLC.

2.2.3. Chromatographic conditions

Chromatographic separation of FAP and FAPGG was carried out at ambient temperature on the chromatographic system (Thermo Separation Products, USA) consisting of a P 4000 quaternary pump, SCM 400 degasser, Spectra Focus UV detector and an AS 3500 autosampler with a 100 μl loop. The HPLC analysis was performed on a LiChrospher 100 RP-18 endcapped column (Merck, Darmstadt, Germany), (250 × 4.6 mm i.d.), particle size 5 μm. The compounds were separated isocratically with a mobile phase consisting of a mixture of 0.02 M nonylamine (adjusted to pH 2.4 with H_3PO_4) – acetonitrile (67.5:32.5 v/v). The nonylamine was used to get sharp peaks with the LiChrospher 100 RP-18 (endcapped) stationary phase. The flow rate was 1 ml min^{-1}. The injection volume was 100 μl; eluted analytes were detected at 305 nm.

2.2.4. ACE inhibition, dose-response relationship

ACE inhibition (in %) was calculated as inhibition $= 100(c_0 - c)/c_0$ where c_0 was the FAP (product of the enzymatic reaction) concentration when no ACE inhibitor was present and c the FAP concentration in the presence of the respective inhibitor (e.g. enalaprilat or lisinopril).

To get concentrations of the ACE inhibitor, a dose-response curve was first constructed from experimental data obtained with known amounts of inhibitor added to blank plasma; the further procedure followed the one described for determination of activity with either endogenous or exogenous ACE. The experimental data were then evaluated using a computer program (Table Curve 2D, SPSS Software, Chicago, USA) for fitting by an equation $y = a/[1 + (x/b)^n]$, which is a general form of a dose-response relationship (Hill equation, [10]). Here, the y has the meaning of the inhibition (in %), x is the inhibitor concentration, a is the height of the transition i.e. the extent of inhibition in %, b is the center of transition (dose of enalaprilat corresponding to half transition) and n is the power term describing the steepness of the transition (or, the number of the binding sites). A fit of experimental data was taken as satisfactory when the value of the respective coefficient of determination was $r^2 > 0.990$.

3. Results and discussion

The method presented here comprises de facto two parts: (i) evaluation of ACE inhibition based on amount substrate FAPGG converted to the respective product, FAP and (ii) determination of the levels of the inhibitor using the dose-response curve.

Before application to human samples, the method for determination of FAP has been validated with minipig plasma. A typical chromatogram of the reaction mixture with endogenous ACE is shown in Fig. 1. The method is specific which is apparently thanks to the detection wavelength used (305 nm) as there is little absorption of other compounds present. Calibration line for FAP was obtained (six calibration levels from 10^{-8} to 10^{-6} mol/l, six replicates) by the least squares method. The calibration was linear ($r^2 = 0.999$), precision (CV 0.5–1.5%) and accuracy (95–110%) was determined at all calibration levels with ranges given in parentheses. Limit of quantification was 5 ng FAP/ml of reaction mixture. The chromatograms were of the same quality with either endogenous or exogenous ACE used.

An example of the dose-response (calibration) relationship showing the dependence of the extent of the ACE inhibition on the amount of enalaprilat added to minipig plasma is shown in Fig. 2. Here, the method with endogenous ACE has been applied. The relationship follows the theoretical curve with goodness of fir expressed by coefficient of determination $r^2 = 0.998$; the parameter n expressing the steepness of the curve (and, in Hill's interpretation, the number of binding sites) is equal to 1.89. This value shows that the curve well expresses the properties of the ACE enzyme as it is known to possess two binding sites for low-molecular weight inhibitors [1]. This curve has been used to quantitate the levels of enalaprilat in minipig plasma (Fig. 3A) after application of 3 mg of enalapril. In the same figure, the results obtained in a parallel experiment using exogenous, commercial ACE added to deproteinated

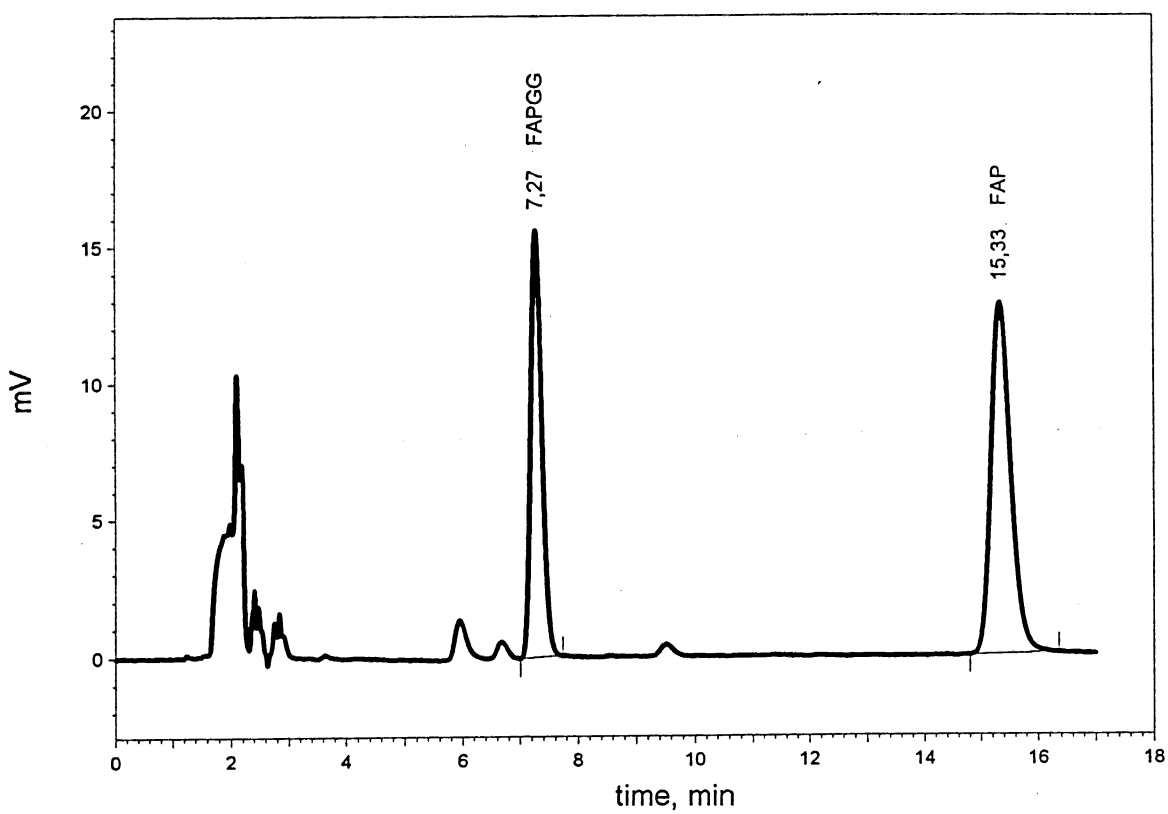

Fig. 1. Typical HPLC chromatogram of reaction mixture with endogenous ACE (present in the sample). Amount of substrate FAPGG added, 30 nmol. Detection at 305 nm; for further experimental details, see text.

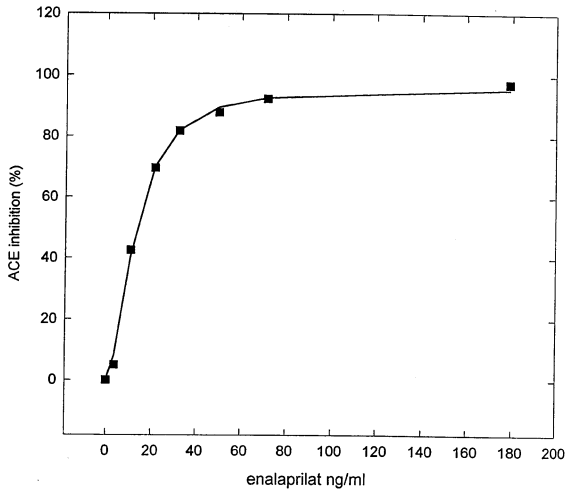

Fig. 2. Dose-response relationship. Dependence of the extent of inhibition of ACE enzyme (in %) on enalaprilat concentration.

plasma are displayed showing applicability of both approaches (i.e. with the endogenous as well as with the exogenous ACE).

In Fig. 3B, a result of a pilot experiment with human plasma is shown again after application of 10 mg of enalapril (Enap, Krka, Slovenia). The levels of enalaprilat follow a typical dependence with maximum of 65 ng/ml of plasma reached at about 4 h, which correspond well to kinetic parameters found in pharmacokinetic studies [5,6] when the maximal levels of 50–80 ng/ml of plasma have been attained 4 h after drug intake.

The results presented here show that the determination of ACE inhibition may be performed with a precise and reliable HPLC method giving data which may be further used for quantitation of inhibitor levels in plasma. The dose-response relationship used for obtaining the inhibitor levels reflects the properties of the ACE enzyme used in a particular experiment. These properties (as of any enzyme) may differ; however, the course of the dose-response curve is a general property of these systems [10] and the experimental curves obtained fitted well to the model equation. The fact that higher inhibitor concentration in the sample would almost completely inhibit ACE activity and that in this concentration range the determination of the actual inhibitor level would not be sensitive enough [6] can be easily overcome by diluting the sample to reach the inhibitor concentrations where the dose-response relationship is well applicable. This has to be evaluated in each experiment; on the other hand, the procedure is sufficiently easy and quick. Also, the amount of plasma is relatively small (100 or 500 μl) which is apparently another advantage of this method.

4. Conclusion

The method described here has been shown to be applicable to determination of inhibition of

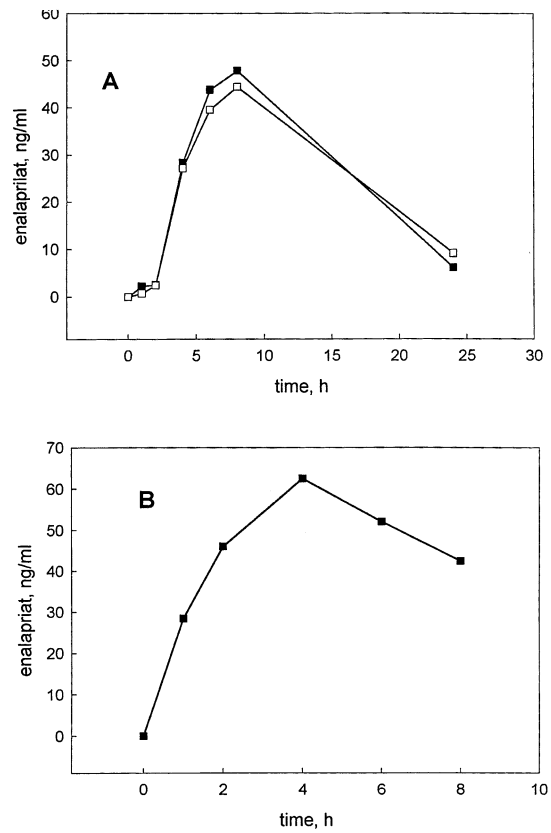

Fig. 3. Time course of the enalaprilat levels in the plasma after intake of 10 mg of enalapril. A, minipig samples (full squares, determination with exogenous enzyme, open squares, endogenous enzyme used), B, human plasma (exogenous ACE added).

ACE and of the levels of the respective inhibitors in plasma samples of minipig and man. Similar results were obtained in this laboratory with other experimental animals (rat, dog) and with various ACE inhibitors (e.g. lisinopril) showing wide applicability of this approach.

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