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Poly(ethylene glycol) derivatized prodrugs through mixed disulfide bond formation: preliminary report on captopril

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Abstract

Whether or not functionalized PEGs may be useful in the case of low molecular weight drugs has not yet been examined so far. For that purpose captopril, a thiol containing drug, was converted into a prodrug, captopril-S-S-PEG, which does not inhibit ACE activity. The inhibitory action of captopril-SH can be regenerated by chemical reduction (gluthatione, DTT or $NaBH_4$) of that prodrug. Yet, as those chemical reagents themselves partially inhibit ACE activity, the inhibitory effect of the freed captopril-SH alone was shown to be fully recovered after coulometric reduction.

Keywords: Captopril; ACE; PEG; Coulometry; Polarography

1. Introduction

Polyoxy(ethylene glycols) (PEGs) are hydrophilic polymers which induce few adverse reactions in humans and which have, as a consequence, in the last decade found many applications as pharmaceutical additives (Pang, 1994). More recently, the chemical modification of drugs with PEGs has been attempted as a means to enhance their therapeutical efficacy (Burnham, 1994; Duncan and Spreafico, 1994). The success encountered with PEG-protein adducts, so far the most widely studied conjugates, has already resulted in their use into clinical practice. On the other hand, most of the PEG-drug conjugates containing low molecular weight drugs are still at an early stage of development.

The low aqueous solubility of a drug may be a major handicap affecting both its delivery and its bioavailability. Also, the low permeability of cell membranes to highly hydrophilic bioactive molecules will severely limit their cellular uptake. PEG being highly soluble both in water as well as a wide variety of organic solvents, it is thus expected that the covalent attachment of PEGs to

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otherwise poorly soluble drugs may contribute in improving their therapeutic index (Carini et al., 1990; Jaschke et al., 1993; Shorr, 1994). Such an improvement may further result from the modification of the pattern of the drug biodistribution which could depend upon the molecular weight of the PEG moiety in the conjugates (Yamaoka et al., 1994). PEGs being commercially available in a wide range of molecular weights (from several hundreds Daltons up to 20 kDa), theoretically, the organ and the subcellular distribution can be indefinitely modified thereby facilitating drug targeting.

Unfortunately, as compared to 'free' drugs, their conjugates based on high molecular weight PEGs generally show a reduced intrinsic bioactivity. As an example, one may cite the case of polymeric doxorubicin adducts prepared by linking mPEG (5 kDa) to the doxorubicin primary amino group, using an amino acid (Gly, Phe, or Trp) or the tripeptide Gly-Val-Phe as spacer arms (Caliceti et al., 1993). Towards Ehrlich solid tumor in mice, the glycine-spaced derivative was completely devoid of activity, whereas the phenylalanine and the tryptophan derivatives were 20 and 16% active respectively. With respect to free doxorubicin, the tripeptide-spaced derivative showed 50% activity (Caliceti et al., 1993). Similarly, a mPEG-pepstatin derivative, prepared by coupling amino-PEG (5 kDa) to the carboxylic acid function of pepstatin A through an amide bond, inhibited the aspartic proteinase pepsin but it exhibited a 400-fold decreased affinity towards the proteinase as compared to free pepstatin (Brygier et al., 1994)

Such observations have emphasized the usefullness of linking the PEG and the drug moieties through chemical bonds able, when required, to be degraded under physiological conditions. Ester bonds which may fall into this category have been examined in this context as illustrated by the examples of PEG-α-tocopherol (Carini et al., 1990) and PEG-taxoid (Shorr, 1994) conjugates. Another possibility exploits the susceptibility to hydrolysis by specific enzymes such as provided after having incorporated L-amino acid residues between the PEG chain and the drug (Caliceti et al., 1993; Ulbrich et al., 1986).

New activated forms of PEG have also been recently synthesized to reversibly modify thiol-containing enzymes through the formation of mixed disulfide bonds (Woghiren et al., 1993; Kuan et al., 1994; Benhar et al., 1994; Musu et al., 1994; Paul et al., 1994; Musu et al., 1995; Vincentelli et al., 1995). Whether or not such functionalized PEGs may be useful in the case of low molecular weight drugs has not yet been examined and constitutes the object of the present communication. Captopril was used as the thiol containing drug.

2. Materials and methods

Aldrich-Chemie (Steinheim, Germany) provided 2,2′-dipyridyldisulfide, 3-(*N*-morpholino)propane sulfonic acid (MOPS), dithiothreitol (DTT), and $NaBH_4$. Captopril (*N*-(*S*-3-mercapto-2-methylpropionyl)-L-proline) and reduced glutathione were supplied by Fluka Chemie AG (Buchs, Switzerland) and Boehringer Mannheim, respectively. Titration of the captopril SH group, using 2,2′-dipyridyldisulfide as the titrant, indicates a 98% purity grade. Sigma Chemical Co (St. Louis, MO) provided L-cysteine, 2-mercaptoethanol, angiotensin converting enzyme (ACE; lot No. 38F96103) and *N*-(3-(2-furyl)acryloyl)-Phe-Gly-Gly (FAPGG; lot No. 82H5810). Fractogel TSK HW-40 was purchased from Merck (Darmstadt, Germany). mPEG-S-S-Py (the structure of which is shown in Fig. 1) used here to derivatize captopril was synthesized and purified as described previously (Musu et al., 1994). Distilled water was further purified using the MilliQ water purification system (Millipore SA, B-1130, Brussels). All the other reagents used were of the highest grade available.

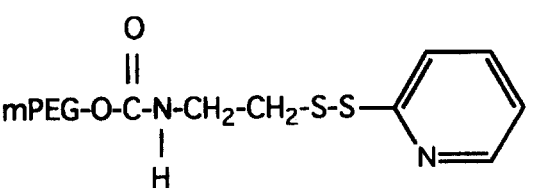

Fig. 1. Chemical structure of mPEG-S-S-Py.

2.1. Coulometric and polarographic measurements

Controlled potential coulometry was performed using a PAR 173 potentiostat and a PAR 179 integrator. A 5-ml capacity cell was equipped with a mercury pool of 1.55 cm^2 area as the working electrode and a platinum gauze auxiliary electrode immersed in a central compartment separated from the solution by a sintered glass disk. The potentials were imposed versus a calomel-saturated KCl reference electrode through a bridge containing the supporting electrolyte.

Polarographic measurements were performed using a PAR 174 A three-electrode polarograph coupled with a PAR SMDE 303 stand and a PAR RE0074 X-Y recorder. The potentials were recorded in this case versus the Ag/AgCl/saturated KCl reference electrode. Unless otherwise indicated, the following parameters have been selected: d.c. polarography: drop time, 1 s; scan rate, 5 $mV \cdot s^{-1}$; drop size, medium; d.p. polarography: drop time, 1 s; scan rate, 5 $mV \cdot s^{-1}$; drop size, medium; pulse amplitude, 50 mV. The solutions were deaerated for 15 min by passing pure nitrogen through the solution before starting the coulometric or polarographic experiments. During the coulometric experiments, stirring was accomplished by nitrogen bubbling. Reduction was achieved within 5 h. At the end of the reaction, samples were removed under a nitrogen atmosphere to avoid any reoxidation.

2.2. ACE activity measurements

ACE activities were measured spectrophotometrically at 24.5°C using the N-blocked tripeptide FAPGG as the substate (Shapiro and Riordan, 1984). Each test tube contained 15.4 nM ACE, 38.5 μM substrate and 0 or 1.6 μM inhibitor (e.g.: captopril) in 50 mM MOPS buffer containing 300 mM NaCl and 1 μM $ZnCl_2$ (required for enzyme activity) at pH 7.50. Reactions were always started by adding the substrate and absorbance decreases at 328 nm accompanying peptide hydrolyses were recorded continuously with a Cary spectrophotometer model 118 equipped with a thermostated cuvette holding.

When an inhibitor was present, it was first preincubated with the enzyme at least for 2 h at 24.5°C before the assay was initiated.

K_i values were determined by the Henderson method in which a plot of $[I_t]/(1 - V_i/V_c)$ versus V_c/V_i was drawn. I_t was the total inhibitor concentration and V_c and V_i were the steady-state velocities measured, respectively, in the absence and in the presence of inhibitor. The slope of the line provided the apparent K_i (Henderson, 1972).

2.3. Other analytical methods

The concentrations of ACE, FAPGG, mPEG-S-S-Py and 2-thiopyridone were determined spectrophotometrically using, respectively, ε_{280} = 225 000 $M^{-1} \cdot cm^{-1}$ (Shapiro and Riordan, 1984), ε_{328} = 12 000 $M^{-1} \cdot cm^{-1}$ (Holmquist et al., 1979), ε_{281} = 4850 $M^{-1} \cdot cm^{-1}$ (Vincentelli et al., 1995) and ε_{343} = 7700 $M^{-1} \cdot cm^{-1}$ (Shipton and Brockelhurst, 1978). Thiol titrations were carried out using 2,2′-dipyridyldisulfide as the titrant (Shipton and Brockelhurst, 1978).

3. Results and discussion

3.1. Synthesis and purification of captopril-S-S-PEG

Captopril, 2.44 × 10^{-5} mol of captopril were added to 15 ml of a 1.63 × 10^{-3} M mPEG-S-S-Py in water. The pH was maintained above 8. The reaction mixture was fractionated on a 3.5 × 110 cm column of Fractogel TSK HW-40 preequilibrated and eluted with MilliQ water. Fig. 2 displays the absorption spectra of mPEG-S-S-Py, captopril-SH and captopril-S-S-PEG. A ε_{260nm} of 360 $M^{-1} \cdot cm^{-1}$ was calculated for captopril-S-S-PEG.

3.2. ACE activity assay

Experiments were performed in MOPS buffer because phosphate or TRIS buffers inhibit ACE (Bünning et al., 1983).

All reagents: ACE, captopril and FAPGG are stable in MOPS buffer at room temperature over the experiment time.

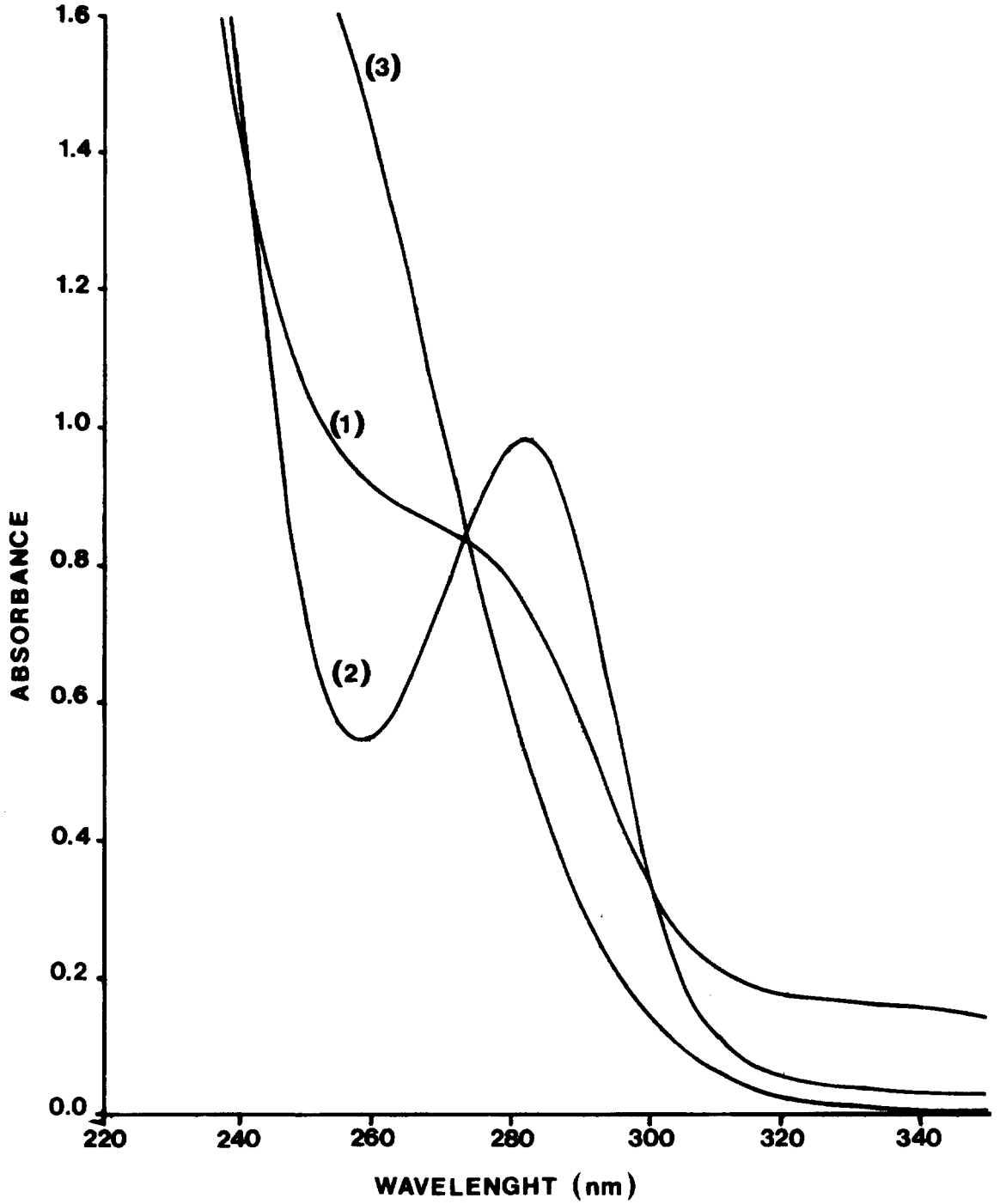

Fig. 2. Absorption spectra of: (1) captopril-S-S-PEG, (2) PEG-S-S-Py, (3) captopril.

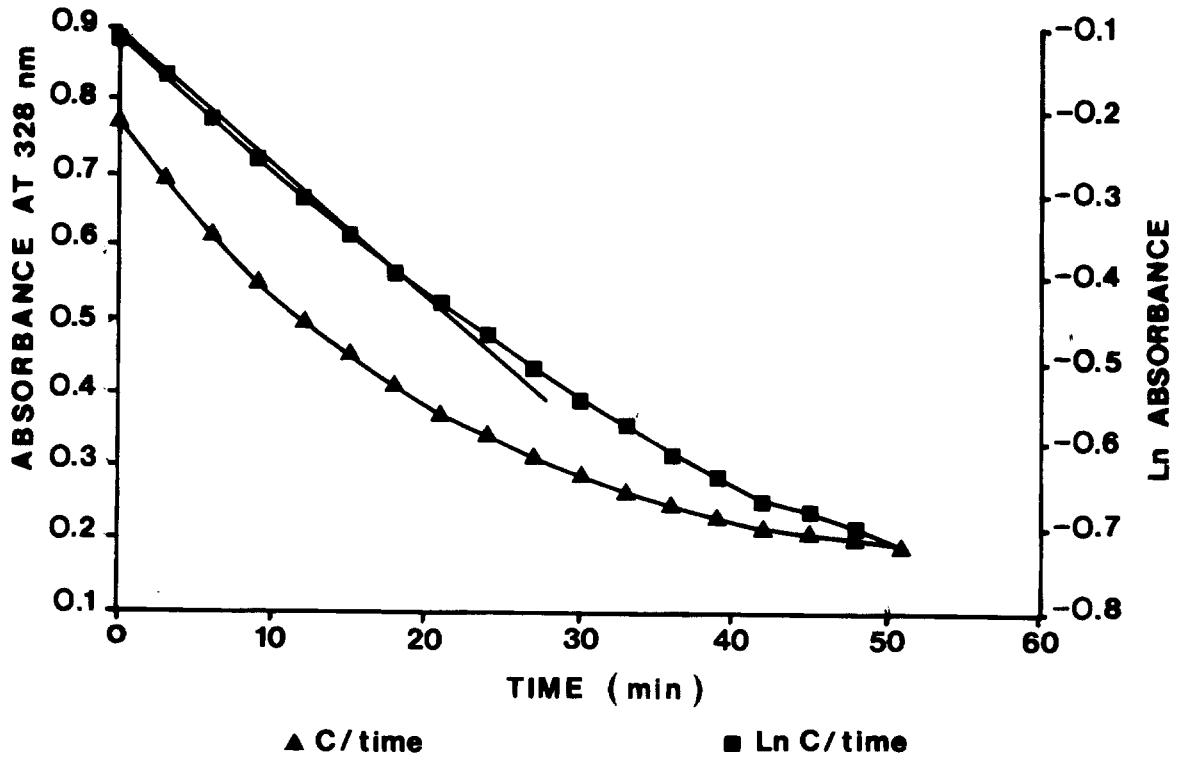

Fig. 3. Kinetics of the reaction between ACE and FAPGG in 50 mM MOPS buffer, 300 mM NaCl, 1μM $ZnCl_2$, pH 7.5. (▲) Concentration of FAPGG versus time. (■) The ln plot of the concentration of FAPGG versus time show an apparent first-order during the first 20 min.

Fig. 3 shows, as reference, kinetic data of the reaction between ACE, first incubated in MOPS buffer for 2 h, and FAPGG. Reactions follow an apparent first-order during the first 20 min.

3.3. Inhibition of ACE activity by captopril-SH obtained by chemical reduction of captopril-S-S-PEG

Fig. 4 (curve e) shows that captopril-SH regenerated by chemical reduction of captopril-S-S-PEG either by gluthatione, by DTT or by $NaBH_4$ completely inhibits ACE activity. MOPS buffer alone or MOPS buffer containing PEG-5000 or captopril-S-S-PEG (curve a) have no effect on ACE activity. However the three reducing agents used: gluthatione (curve d), DTT (curve c) and $NaBH_4$ (curve b) partially inhibit ACE activity. It is the reason why coulometry has been applied to regenerate the captopril-SH.

3.4. Coulometric reduction of captopril-S-S-PEG

Before starting the coulometric reduction of the captopril-S-S-PEG complex, polarograms of this compound were recorded in order to determine the potential to be imposed to reduce the disulfide bond. These polarograms were compared with those obtained for captopril alone and recorded in the same conditions. All measurements have been performed using a 0.05 M sodium chloride supporting electrolyte, this medium being required for the enzymatic reactions.

Since a 1.6×10^{-3} M captopril-S-S-PEG solution will be used through this study, the same concentration of captopril was investigated first.

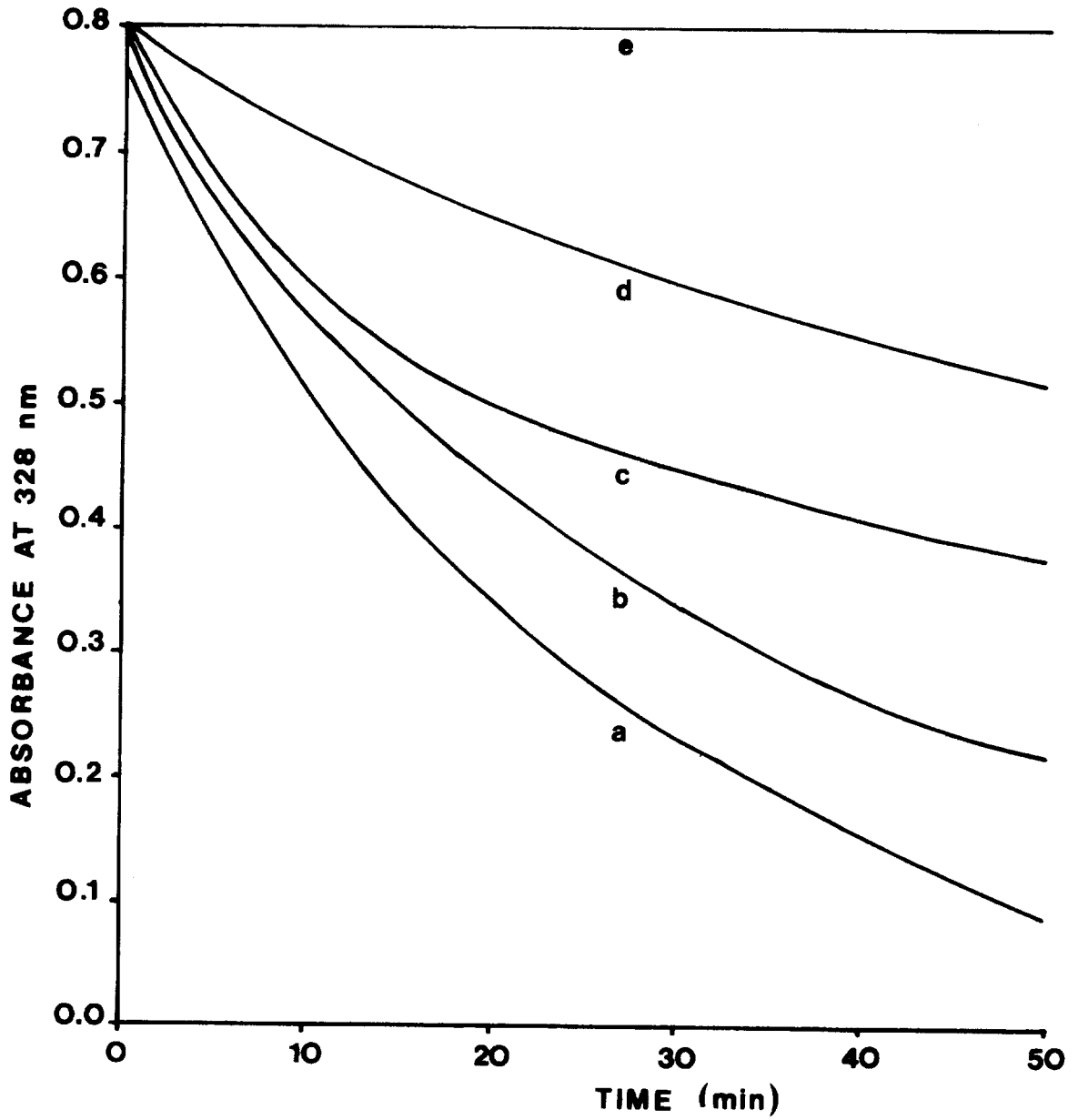

Fig. 4. Kinetics of the reaction between ACE and FAPGG. (For experimental details, see text). (a) In MOPS buffer or in MOPS buffer containing PEG-5000 or captopril-S-S-PEG. (b–d) In MOPS buffer containing respectively: $NaBH_4$, DTT or gluthatione. (e) In MOPS buffer containing captopril.

This compound exhibits an anodic wave at -0.28 V, followed by a small anodic wave at -0.50 V. This process corresponds to the formation of the mercury thiolate accompanied by adsorption phenomena as demonstrated elsewhere (Passamonti et al., 1987; Passamonti et al., 1991). A well defined cathodic wave appears at -1.67 V. Decreasing the concentration to 1.6×10^{-4} M provokes the splitting of the anodic wave into two waves of similar intensity (the wave at -0.28 V decreases while that at -0.50 V increases).

Exposure of the solution to air for 48 h in the

presence of metallic mercury provokes the disappearance of the cathodic wave while the anodic waves become cathodic. This last phenomenon corresponds to the slow formation of the mercury derivative by an oxidation process by air in the presence of the thiol group. Simultaneously a small irreversible wave appears at − 1.10 V, corresponding to the reduction of oxidized captopril disulfide concurrently formed.

A similar oxidation of captopril solutions by air without metallic mercury gives rise to a slower process. The intensity of the anodic waves decreases, as well as that of the cathodic wave at − 1.67 V while the irreversible wave of the disulfide bond increases at − 1.10 V.

Polarography of captopril in a buffered medium exhibits the same anodic waves but the reduction wave at − 1.67 V no longer exists. This latter is probably due to a further reduction of captopril favoured by a protonation at the electrode surface by the protons released by the thiol group during the formation of the disulfide bond or the mercury derivative since these compounds do not exhibit this wave.

The reduction of captopril-S-S-PEG was investigated using d.p. polarography owing to the ill-defined response provided by this very large molecule (M_r > 5000). In the 0.05 M NaCl supporting electrolyte, a 1.6×10^{-3} M solution of captopril-S-S-PEG exibits a very small peak at − 0.50 V, probably due to free thiol groups remaining in the complex or in the PEG moiety, and a large broad peak at − 1.25 V attributed to the reduction of the disulfide bond. No peak appears at − 1.67 V.

Since the aim is to release free captopril from the captopril-S-S-PEG complex, coulometric experiments were performed at − 1.55 V in order to break the disulfide bond and to regenerate the thiol form of captopril. Two millilitres of the solution were introduced to the coulometric cell, corresponding to 3.2×10^{-6} mole of captopril-S-S-PEG. Assuming a two-electron transfer for the reduction of the S-S bond, 0.62 C will be necessary to complete the reduction process. Polarographic control of the electrolyzed solution demonstrated the full reduction of the disulfide bond: the peak at − 1.25 V disappeared while a very high, well-defined peak developed at − 0.50 V, corresponding to the formation of the mercury derivative of free captopril.

This electrolyzed solution is not stable and cannot be conserved in the presence of air. After 48 h in such conditions (without any contact with mercury), d.p. polarography reveals a marked decrease of the peak at − 0.50 V (reoxidation of the thiol form) and the appearance of two overlapped reduction peaks of similar intensity, the first at − 1.10 V, the second at − 1.27 V. Both captopril-S-S-captopril and captopril-S-S-PEG are thus formed during this reoxidation process but the latter in larger amounts since, for a similar intensity of the peaks, the diffusion coefficient of captopril-S-S-PEG is much lower than that of captopril-S-S-captopril. This means that the captopril-S-S-PEG derivative is formed preferentially and is more stable than the captopril-S-S-captopril compound.

3.5. Inhibition of ACE activity by captopril-SH obtained by coulometric reduction of captopril-S-S-PEG

Captopril-SH obtained by coulometric reduction of captopril-S-S-PEG inhibits completely ACE activity (Fig. 4; curve e). However as was shown by d.p. polarography, captopril-SH is reoxidized by air. Reduced captopril looses its inhibitor action if it is left in the presence of air for 24 h but conserves its inhibitor function for at least 3 weeks if it is kept frozen in solution.

3.6. Determination of apparent inhibition constant, K_i app

The strength of binding of captopril to ACE is due to the interaction of its sulphydryl group with the zinc atom of the active site of the enzyme. As captopril is a tight binding inhibitor, in the experimental conditions used (enzyme concentration, Cl^- concentration, pH), K_i app can be determined by the Henderson method (Henderson, 1972). From the slope of the line on Fig. 5, a K_i app of 0.7 nM ($\pm$ 0.1) is calculated for the substrate concentration used. This value is in good agreement with the results from the literature (Shapiro and Riordan, 1984).

4. Concluding comments

The pharmacological activity of captopril is thought to be associated with the ability of its free thiol group to bind to the Zn atom present in the active site of ACE (Shapiro and Riordan, 1984). Affinity of captopril for the active site of ACE is high and thus, it is expected that stoichiometric amounts of this drug might be sufficient to completely saturate and inhibit the target metalloproteinase. Nevertheless, when administered in vivo, high doses of captopril are required to be therapeutically effective. The reversible chemical modification by mPEG theoretically offers one of several possibilities for improving bioavailability of captopril (Carini et al., 1990; Jaschke et al., 993; Shorr, 1994; Yamaoka et al., 1994).

The mPEG-S-S-captopril which is readily synthesized, as shown here, exibits some properties expected for a prodrug. As a conjugate, captopril is completely unable to inhibit ACE. However, intracellular concentrations of glutathione are likely to be high enough to be able to regenerate fully active captopril from the conjugate as suggested by the in vitro results obtained in the course of the present work.

Of the several possibilities resulting from the simultaneous presence of a thiol group and of a carboxylic acid function in the model drug, we choose to covalently link PEG to captopril through a mixed disulfide bond. This choice was dictated by the following considerations: (1) ACE is mainly located in the endothelial cells of the cardiovascular system. It is generally recognized that the low ACE concentration which is present in plasma only plays a minor role in the conversion of angiotensin I to angiotensin II, (Brunner et al., 1993). (2) Disulfide links have been reported to be chemically stable in plasma (Bickel et al., 1995).

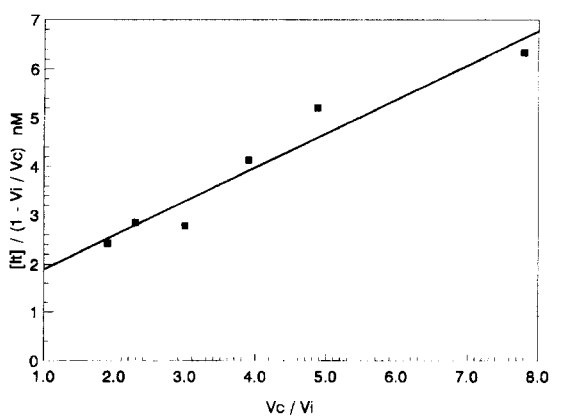

Fig. 5. Henderson plot to determine the apparent K_i for the binding of captopril to ACE.

Thus, one may reasonably speculate that reduction of the mPEG-S-S-captopril will occur only after the incorporation of the conjugate into the endothelial cells of the cardiovascular system.

Derivatization of free thiol groups in drugs may have other technological advantages such as to afford protection against inactivation through γ-ray sterilization (Volland et al., 1994). Further work should examine the membranotropic properties of the mPEG-S-S-captopril conjugate.

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