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The sensitivity of the *cis*/*trans*-isomerization of enalapril and enalaprilat to solvent conditions

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Abstract

The *cis*- and *trans*-isomers of enalapril and enalaprilat can be resolved by HPLC and by capillary electrophoresis. The isomeric content of enalapril is perturbed by the ionization of both its carboxyl and amine groups, while the isomeric content of enalaprilat is only perturbed by the ionization of its amine group. Increasing the hydrophobicity of the analyte solvent, as reflected in its molar polarization, increases the *Z* (*cis*) content of enalapril and markedly decreases the kinetics for isomerization. Far UV circular dichroic measurements suggest that the increase in *Z* (*cis*) content of enalapril is due to protonation of its carboxylate group. Taken together, the in-vitro properties of enalapril and enalaprilat suggest that the in-vivo transformation of the prodrug enalapril to the inhibitor enalaprilat and its delivery to angiotensin-converting enzyme should not be significantly limited by *cis*/*trans*-isomerization.

Introduction

A dipeptidyl carboxypeptidase called angiotensin-converting enzyme (ACE) catalyses the conversion of the relatively inactive decapeptide angiotensin I to the potent octapeptide angiotensin II via the reaction DRVYIHPFHL $\rightarrow$ DRVYIHPF + HL. Since ACE is a membrane glycoprotein located in the luminal face of vascular endothelial cells, this conversion must occur within or at the surface of a blood vessel wall (Jackson & Garrison 1995). The in-vivo activity of ACE can be modulated by the introduction of small peptides containing C-terminal proline residues functioning as competitive inhibitors. Among such inhibitors are enalaprilat and its prodrug form enalapril, whose structures are shown in Figure 1. Enalapril is hydrolysed to enalaprilat in vivo by an esterase located predominantly in the liver with some activity in the kidney but none reported in plasma or intestinal mucosa (Larmour et al 1985). Studies with perfused rat liver preparations indicate that this esterase is membrane bound (Abu-Zahra & Pang 2000). However, the stereochemical requirements of this hepatic esterase remain unknown.

According to the CIP rules (Cahn et al 1966), the proline tertiary amide bond in enalapril and enalaprilat can be either *Z*-(*cis*) or *E*-(*trans*) with rotation around the proline peptide bond restricted due to the partial double bond character of the tertiary amide, as illustrated in Figure 1. However, confusion can arise as many authors (e.g. Brandts et al 1975; Grathwohl & Wuthrich 1976, 1981; Rabenstein & Isab 1982; Thorsett et al 1986; Sakamoto et al 1990; Qin et al 1992; Saran et al 1994; Moore & Jorgenson 1995; Kocijan et al 2001) use the protein convention, in which the *Z*-(*cis*)-isomer is termed the *trans*-isomer, based on the polypeptide chain entering and exiting opposite corners of the rectangle formed by the planar peptide bond atoms. In this report, we will exclusively employ the CIP rules as encoded in IUPAC.

Molecular modelling studies (Thorsett et al 1986) proposed, and a recent crystallographic study (Natesh et al 2004) confirmed, that ACE inhibitors including enalaprilat are all bound to ACE as the *Z*-(*cis*)-isomers. Since the interconversion between proline isomers is relatively slow on the biological time-scale, and since the ratio of *cis*- to *trans*-isomers in solution appears to depend on both pH and hydrophobicity (Stellwagen & Ledger 2003), the efficacy of a drug containing proline peptide bonds may be dependent on the equilibrium concentration of a particular isomer.

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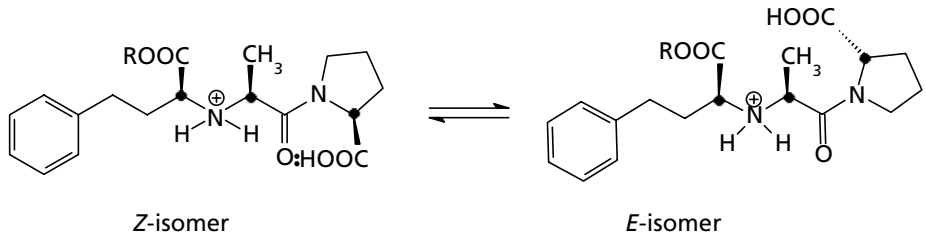

Figure 1 *Z*/*E*-(*cis*/*trans*)-isomerism of the cationic forms of enalaprilat (S-1-[N-[1-carboxy-3-phenylpropyl]-L-alanyl]-L-proline, R = H) and enalapril (S-1-[N-[1-ethoxycarbonyl-3-phenylpropyl]-L-alanyl]-L-proline, $R = C_2H_5$). •, indicate the locations of the chiral atoms. A putative intramolecular proline carboxyl:carbonyl hydrogen bond is shown by a colon in the *Z*-(*cis*)-isomer.

This paper reports the use of HPLC, capillary electrophoresis (CE) and circular dichroic spectroscopy (CD) to measure the equilibrium and kinetic properties of the *cis*/*trans*-isomerization of enalapril and enalaprilat in model solvents. The aprotic solvent acetonitrile (methyl cyanide) was used to mimic the membranous environments of the hepatic esterase and of ACE. Acetonitrile was employed because traditional apolar solvents used to mimic membranes, such as octanol, chloroform or dioxan, are immiscible with water, have a high UV absorbance, and/or a high viscosity at low temperatures, properties inappropriate for analysis using HPLC and CE.

Materials and Methods

Materials

Stock solutions of 4 mM enalapril maleate (Sigma, St Louis, MO) and 5 mM enalaprilat (Merck, Sharpe and Dohme, Quimica de Puerto Rico Inc, Barcelona, Puerto Rico) were prepared in deionized water, unless stated otherwise. Aliquots of stock solution were diluted 10-fold in various buffered solutions prior to electrophoretic measurements, 25-fold prior to chromatographic measurements and 100-fold prior to dichroic measurements, unless stated otherwise.

Chromatography

HPLC was performed using an LC-10AT pump (Shimadzu, Kyoto, Japan) connected to a UV detector (SPD-10A, Shimadzu) set at 245 nm. Samples were injected from a 100 μL loop onto a water-jacketed Phenomenex LC18 (5 μm) column (150 × 4.6 mm) with an LC18 guard column (4 × 3 mm). The column was maintained at 8°C and equilibrated with a mobile phase of 10 mM ammonium acetate, pH 6.0, in 30% acetonitrile at a flow rate of 1 mL min^{-1} unless reported otherwise. The relative concentrations of *trans*- and *cis*-enalapril were calculated from either their peak heights or peak areas since these two parameters gave a linear correlation having an r^2 value of at least 0.94. The percentage *Z*-(*cis*)-isomer values obtained from HPLC measurements were all multiplied by 1.33 to equate HPLC and CE measurements. This factor presumably accounts for the differential absorbance of the isomers at 214 and 245 nm.

Electrophoresis

Polyacrylamide-coated fused silica capillaries having an inner diameter of 100 μm were purchased from Beckman/Coulter. Each capillary was trimmed such that its total length, L_T, was 40.0 cm and the length to the detection window, L_D, was 29.8 cm. The trimmed capillary was mounted in a thermostatted cassette, filled with deionized water and placed in a Beckman/Coulter P/ACE MDQ Molecular Characterization System. The capillary was thermostatted at 15.0°C, unless noted otherwise, and rinsed for at least 1 min with capillary buffer at 25 psi prior to injection of a sample. Samples were pressure injected for 3 s at 0.5 psi. Electrophoresis was done at a constant voltage, V, selected not to exceed a current of 35 μA. Absorbance was constantly monitored using a 214 nm filter.

Electrophoretic mobility, μ, having the units cm^2 Vs^{-1}, was calculated from the observed peak migration time, t in seconds, using the equation $\mu = L_T L_D/Vt$. Peak areas were obtained using the integration routines encoded in the 32 Karat software of the instrument. Selected electropherograms were additionally analysed by computer simulation with ChromWin software (Trapp & Schurig 2001) using a modified stochastic model (SM+) and the advanced method 'Find Isomerization Barrier II'. In test electropherograms, the percentage *Z*-(*cis*)-isomer injected into the capillary determined using ChromWin simulation and the percentage *Z*-(*cis*)-isomer in the electropherogram determined using Beckman/Coulter 32 Karat area analysis gave comparable values.

Circular dichroism

Circular dichroic spectra at equilibrium and 25°C were obtained using an Aviv Model 62DS circular dichroism spectrometer (Aviv Associates, Lakewood, NJ), 10 mm rectangular quartz cuvettes, a scan step of 1 nm and a dwell time of 5 s. Each spectrum represents an average of at least five repeat scans corrected for chromatic aberrations due to cell and solvent. Averaged dichroic spectra were smoothed using SigmaPlot 8.0. Ellipticity was expressed in the units deg.cm^2 mmol^{-1}.

Results

Chromatographic measurements

Reversed-phase adsorption chromatography (HPLC) elution profiles of enalapril maleate obtained after dissolution in the chromatographic mobile phase at zero time and maintained at 8°C exhibited three components. The major component, having an elution time of less than 2 min, represents the enalapril co-ion maleate ((*Z*)-butenedioate). The identity of this component was established by injection of a solution of sodium maleate in the same solvent. The area of the maleate peak was independent of the residence time of the sample in the chromatographic solvent. By contrast, the relative areas of the remaining two components in the elution profiles changed with increasing residence time of enalapril in the chromatographic mobile solvent prior to injection. Comparative analysis of successive chromatograms indicated a net transfer of material from the slower component, having an elution time of about 4 min, to the minor faster component, having an elution time of about 2.5 min. Raising the temperature of the chromatographic column markedly hastened the interconversion of these two components and destroyed their baseline resolution. Such behaviour is consistent with that expected for the *cis*/*trans*-isomerization of a prolyl peptide bond (Brandts et al 1975; Grathwohl & Wuthrich 1981).

The slower component in the chromatograms is assumed to represent the *Z*-(*cis*)-peptide isomer of enalapril for two reasons. Firstly, enalapril maleate is known to crystallize as the *Z*-(*cis*)-isomer (In et al 1986) and would be expected to be the predominant isomer immediately upon dissolution. Secondly, the *Z*-(*cis*)-isomer of enalapril has a larger contiguous apolar surface than does the *E*-(*trans*)-isomer (In et al 1986) and would be expected to be bound more strongly to an adsorption matrix, resulting in a slower elution. By contrast, some authors (Qin et al 1992; Kocijan et al 2001) have concluded that the major component in HPLC elution profiles and in electropherograms of enalapril maleate is the *E*-(*trans*)-isomer. Their conclusions are ultimately based on the statement that *E*-(*trans*)-alanylproline has a greater apolar surface area than its *Z*-(*cis*)-isomer. While this statement is correct, it misses out the contribution of the phenylbutryl moiety to the apolar surface of enalapril. Inspection of the molecular conformation of the crystallographic structure of the *Z*-(*cis*)-isomer of the enalapril maleate (In et al 1986) clearly indicates that the apolar surface of the *Z*-(*cis*)-isomer is more contiguous than would be the case for the surface of the *E*-(*trans*)-isomer.

The baseline resolution of the isomers in the elution profiles indicates that no significant change in the isomeric content occurs *during* chromatographic analysis at 8°C. The relative area of the faster *E*-(*trans*)- and slower *Z*-(*cis*)-components therefore indicates the relative isomeric content of enalapril just *prior to* chromatographic analysis. The isomerization of enalapril maleate in water (pH 4.2) at 8°C was found to occur in a single kinetic phase having a half-time of 58 min with 52% in the *Z*-(*cis*)-isomer at equilibrium ($r^2 = 0.99$). The isomerization half-time and equilibrium composition in acetonitrile and in ethanol were < 2 min and 65% *Z* (*cis*) and < 5 min and 60% *Z* (*cis*), respectively.

Solutions of enalapril maleate at equilibrium were lyophilized and reanalysed within 10 min of redissolving in the mobile phase. Results indicated that lyophilization of aqueous solutions of enalapril maleate returned the sample to the all-*Z*-(*cis*)-isomer whereas evaporation of organic solvent maintains an isomeric ratio close to the equilibrium composition in that solvent.

Unfortunately, the *cis*- and *trans*-isomers of enalaprilat are very poorly resolved in the chromatographic mobile solvent employed for the analysis of enalapril. This may result from either faster exchange kinetics or, more likely, a decrease in the apolar surface, which facilitates resolution.

Electrophoretic measurements

We have previously shown (Stellwagen & Ledger 2003) that the enalapril maleate isomers can be resolved with essentially baseline resolution by capillary electrophoresis in selected capillary buffers at 15°C. Such measurements facilitate evaluation of the effect of pH and the presence of aprotic solvents on the isomer equilibrium of enalapril maleate prior to electrophoresis. As a representative example, the dependence of the isomeric content of enalapril on the pH of the sample buffer prior to electrophoretic analysis is illustrated by the open circles in Figure 2. The isomeric contents of enalapril measured in a variety of solvents by this procedure are consistent with previous values obtained using NMR measurements (Wyvratt et al 1984; Sakamoto et 1990; Saran et al 1994; Trabelsi et al 2000) and with the HPLC measurements described above.

Enalaprilat was subjected to the same electrophoretic protocols as enalapril. The electropherogram observed

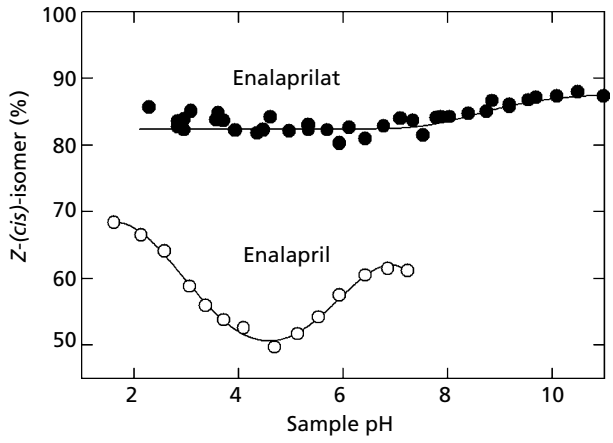

Figure 2 Dependence of the fractional concentration of the *Z*-(*cis*)-isomer on the pH of the sample buffer at 23 °C. ○, indicate measurements obtained using enalapril maleate (Stellwagen & Ledger 2003) and ●, enalaprilat. Experimental values were obtained by analysis of electropherograms using either the 32 Karat software or ChromWin.

following injection of enalaprilat into a capillary containing 20 mM cacodylate buffer, pH 6.87, at 15°C exhibited two peaks of unequal area having near-baseline resolution. Increasing the capillary temperature stepwise to 60°C caused these two peaks to coalesce, consistent with their being *cis*/*trans*-isomers. The dependence of the relative area of the predominant peak of enalaprilat on the pH of the equilibrated sample prior to injection is shown by the filled circles in Figure 2. Making the traditional assumption that the predominant isomer at low pH is the *Z*-(*cis*)-isomer (Evans & Rabenstein 1974; Grathwohl & Wuthrich 1976), this isomer is seen to predominate throughout the observed pH range. The illustrated fractional compositions of the *Z*-(*cis*)-isomer at pH values of 2.5, 7.5 and 9.3 are similar to the fractional compositions previously reported at pH 2.5 (80%) and 7.5 (88%) using NMR measurements (Wyvratt et al 1984) and at pH 9.3 (82%) using CE measurements (Trapp et al 2004).

The dependence of the mobility of each isomer of enalaprilat on the pH of the capillary buffer is shown in Figure 3. Analysis of these dependencies using sigmoidal transitions ($r^2 = 0.99$) indicates that both carboxyl groups in enalaprilat have an apparent pK of 2.9 and that this value is the same in both isomers. By contrast, the apparent pK of the single amine group is different in the two isomers, having a value of 8.2 in the *Z*-(*cis*)-isomer and of 8.7 in the *E*-(*trans*)-isomer. Given these apparent pK values, it is not surprising that the isomeric content of enalaprilat is insensitive to the protonation of the carboxyl groups but is sensitive to the protonation of the amine group, as shown in Figure 2. The latter sensitivity suggests the preferential formation of an amine:peptide hydrogen bond in the *E*-(*trans*)-isomer of enalaprilat.

Our assignment of the pK values in enalaprilat are at odds with a previous publication (Qin et al 1992) which reports pK values of 3.3 and 7.6 for the two carboxyl groups and no pK value for the amine in an unresolved isomeric mixture. The relative spans of the mobility increments observed for enalaprilat in Figure 3 suggest that the lower reported pK value more likely represents both carboxyl groups and that the higher reported pK value then represents that of the missing amine group.

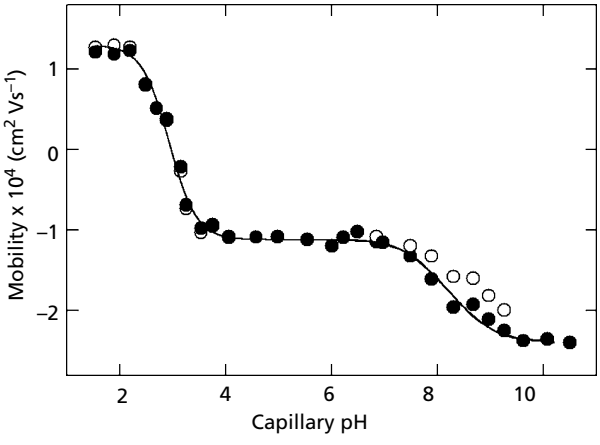

Figure 3 Dependence of electrophoretic mobility of enalaprilat on the pH of the capillary buffer. The symbols indicate the experimental values obtained following injection of enalaprilat into capillaries equilibrated with malonate, formate, cacodylate or tris acetate buffers of the indicated pH values. •, indicate the mobility of the major component, the *Z*-(*cis*)-isomer, and ○, indicate the mobility of the minor component, the *E*-(*trans*)-isomer, in each electropherogram. The line represents sigmoidal fits to the filled circles.

Dichroic measurements

Both enalapril and enalaprilat contain three chiral centres whose locations are indicated by the filled circles in Figure 1. Changes in the ionic environment about each chiral centre should be reflected by changes in their circular dichroic spectra, particularly in the far UV. As shown in Figure 4, the far UV spectrum of both enalapril and enalaprilat is markedly dependent on the pH of the aqueous buffer. The pH dependence of the ellipticity at 215 nm correlates rather well with corresponding changes in the electrophoretic mobility for enalapril and for enalaprilat, as shown in Figure 5. Thus the state of protonation of both the carboxyl and the amine groups is important to the dichroic spectrum.

Dissolution of enalapril in the aprotic solvent acetonitrile instead of water markedly alters its dichroic spectrum, as shown in Figure 6. The dichroic spectrum in acetonitrile is similar to that observed for enalapril in

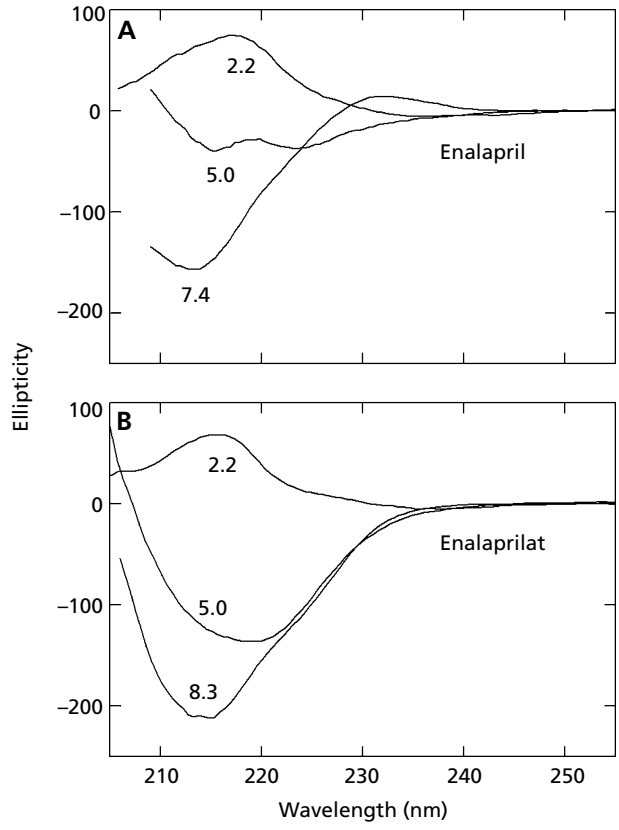

Figure 4 The effect of pH on the dichroic spectra of a 48 μM solution of enalapril maleate (A) and 50 μM enalaprilat (B). The spectra were obtained at equilibrium at the indicated pH values.

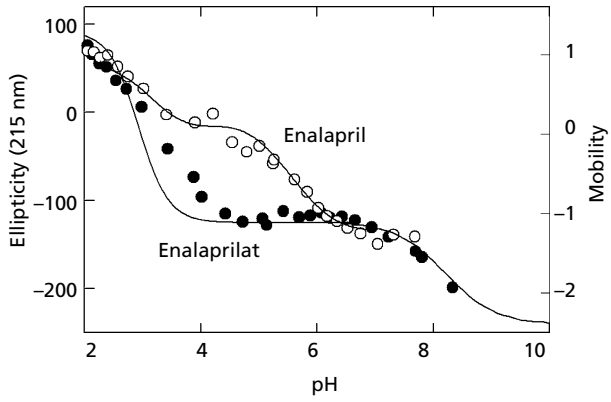

Figure 5 Comparison of the effect of pH on the dichroism and mobility of enalapril maleate and enalaprilat. The symbols represent the ellipticity of enalapril maleate, ○, and enalaprilat, ●, measured at 215 nm. The lines represent the pH dependence of the electrophoretic mobility of enalapril maleate (Stellwagen & Ledger 2003) and of enalaprilat (Figure 3).

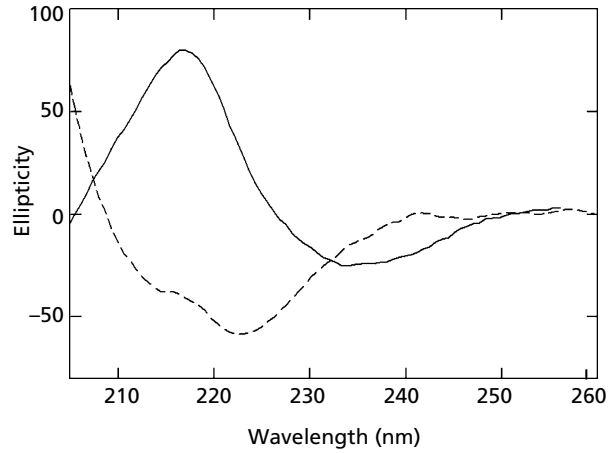

Figure 6 The effect of acetonitrile on the dichroic spectrum of a 31 μM solution of enalapril maleate equilibrated at 25°C. The dashed line indicates the spectrum observed in water and the solid line the spectrum observed in neat acetonitrile.

water at pH 2.2 (Figure 4A), suggesting that dissolution of enalapril in acetonitrile increases the *Z*-(*cis*)-isomeric content by protonation of the proline carboxylate.

Solvent polarization

Dissolution of enalapril in mixed or neat solvents less polar than water, including aprotic solvents, increases the *Z*-(*cis*)-isomeric content. As shown in Figure 7, the increase in *Z* (*cis*) content is proportional ($r^2 = 0.70$) to the hydrophobicity of the solvent expressed as its molar polarization (Marcus 2002). This proportionality predicts that *Z* (*cis*) would be the predominant isomer in biological membranes.

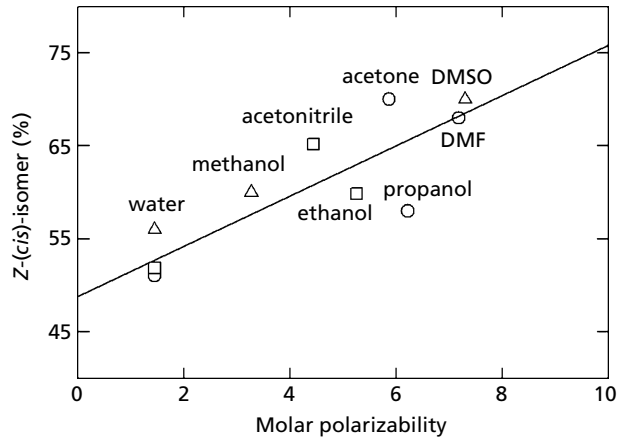

Figure 7 The effect of solvent polarity on the isomerization of enalapril maleate. Solvent polarity is expressed as its molar polarization, P. The molar polarization of a mixed solvent (Marcus 2002) is essentially the sum of the products of the volume fractions, f, and molar polarization, P, of each solvent component, $P = f_A P_A + f_B P_B$. The ○, □ and △ indicate the percentage *Z*-(*cis*)-isomer values determined by capillary electrophoresis (Stellwagen & Ledger 2003), chromatographic and NMR (Wyvratt et al 1984; Sakamoto et al 1990; Saran et al 1994; Trabelsi et al 2000) measurements, respectively.

Discussion

Since ACE selectively binds the *Z*-(*cis*)-isomer of enalaprilat (Natesh et al 2004), it is important that conditions maximize the *Z*-(*cis*)-isomeric content of both enalaprilat and its prodrug, enalapril maleate. This extends backwards to prodrug formulation. We observe that lyophilization of aqueous solutions of the enalapril maleate returns the sample to the all-*Z*-(*cis*)-isomer whereas evaporation of organic solvent maintains an isomeric ratio close to the equilibrium composition in that solvent. This is an important consideration in drug formulation where techniques such as spray drying are used. Nonetheless a significant fraction of enalapril would be expected (Figure 2) to be in the *E* (*trans*) form at equilibrium under the physiological conditions of the plasma and may at times approach 50% in the stomach, duodenum or ileum. Accordingly, care should be exercised if using dissolution testing as a prognostic tool (Dressman et al 1998) for enalapril absorption. On the other hand the concentration of *E*-(*trans*)-enalaprilat should not exceed 20% isomer in any tissue fluid.

The esterase, which hydrolyses the prodrug enalapril to the ACE inhibitor enalaprilat, is considered to be a hepatic membrane protein (Larmour et al 1985; Abu-Zahra & Pang 2000). We used our HPLC method to confirm that there is an enalapril esterase in hepatic microsomes but not in plasma nor in homogenates of intestinal mucosa. While it is unclear whether the catalytic site of the membranous esterase is exposed to the aqueous environment of the cytoplasm or plasma, enalapril should in any case be able to penetrate a lipid bilayer since enalapril is quite soluble in aprotic solvents. If an aprotic solvent can

mimic a membranous environment, then the *Z*-(*cis*)-isomer of enalapril should be more predominant (Figure 7) in the membrane and the isomerization kinetics should be markedly increased. Although we were unable to determine whether the hepatic esterase displays any stereoselectivity between the *cis*- and *trans*-isomers, we can safely assume that hydrolysis of enalapril yields predominantly the *Z*-(*cis*)-isomer of enalaprilat.

The fractional content of the bioactive *Z*-(*cis*)-isomer of enalaprilat would be expected to be significantly higher in the plasma at equilibrium than the fractional content of the *Z*-(*cis*)-isomer of the prodrug, enalapril maleate (Figure 2). Furthermore, when the freshly generated inhibitor enalaprilat is returned to the plasma on its trip to its target enzyme, the ACE in the vascular walls, the isomeric forms will have the opportunity to re-equilibrate. Indeed, using the temperature dependence of the *E* (*trans*) $\rightarrow$ *Z* (*cis*) reaction of enalaprilat reported by Trapp et al (2004), the predicted half-time for re-equilibration should be only about 0.6 min at 37°C. Finally, since ACE is a membranous enzyme like the esterase, we would anticipate that the *Z* (*cis*) content of enalaprilat and its isomerization rate would be increased in the hydrophobic environment of the membrane, as observed for enalapril (Figure 7). Accordingly, we propose that *cis*/*trans* isomerization of these drugs is unlikely to be of consequence for the inhibition of the ACE in the vascular endothelium or for activity in the epithelial endopeptidases of the lower intestine.

Conclusions

Conversion of the prodrug enalapril to the inhibitor enalaprilat and transference of the latter to its membranous target protein increases the fractional content of the bioactive *Z*-(*cis*)-isomer and markedly decreases the half-time for its isomerization. Accordingly, we propose that *cis*/*trans* isomerization is unlikely to be of consequence for the inhibition by enalaprilat of the ACE of the vascular endothelium or for the activation of enalapril by the epithelial endopeptidases of the lower intestine.

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