STEREOSELECTIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ELECTROPHORESIS ANALYSIS AND *IN VITRO* STUDY OF THE SERUM PROTEIN BINDING OF CARVEDILOL ENANTIOMERS

by

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ABSTRACT

Carvedilol, 1-(9H-carbazol-4-yloxy)-3-[[2-(2-methoxyphenoxy)ethyl]amino]-2-propanol, is a new antihypertensive drug which has recently been introduced on the market in Canada under the trade name, COREG. It contains a chiral centre in its structure and therefore exists as two enantiomers. The drug is marketed as the racemate, however, the two enantiomers possess different pharmacological actions. (-)-(S)-Carvedilol is a much more potent β_1-blocker than (+)-(R)-carvedilol, whereas both enantiomers exhibit the same α_1-adrenergic antagonism. This study consisted of the development of sensitive stereoselective assays for carvedilol using high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) and the determination of the stereoselective protein binding of carvedilol.

An attempt to derivatize carvedilol enantiomers with fluorogenic reagents was undertaken. The reaction with 2-naphthoyl chloride, 2-anthroyl chloride, and (+)-(S)-naproxen chloride resulted in incomplete derivatization. Mass spectrometric analysis of the reaction products revealed the formation of the mono-derivative of carvedilol with 2-naphthoyl chloride at the secondary amine group on the side chain and also the di-derivative at both the amine group and the hydroxyl group of carvedilol. The reaction with (+)-(S)-1-(1-naphthyl)ethyl isocyanate was also examined and was incomplete and produced multiple derivatives.

A direct chiral HPLC method was therefore developed, without the need for derivatization, using (S)-indoline-2-carboxylic acid and (R)-1-(α-naphthyl)ethylamine as the stationary phase. The assay was validated for carvedilol enantiomers in serum. A limit of quantitation (LOQ) of 1 ng/ml for both enantiomers was obtained.

A new stereoselective CE method was also developed for the analysis of the enantiomers of carvedilol in serum. Several types and concentrations of cyclodextrins were tested. Near baseline resolution was obtained using 10 mM hydroxypropyl-β-cyclodextrin as the chiral selector. The electrophoretic conditions were optimized. The chiral CE method for carvedilol was validated for the drug enantiomers in serum. An LOQ of 50 ng/ml per enantiomer was obtained.

The HPLC and the CE assays were compared by analyzing a series of serum samples containing racemic carvedilol in different concentrations using the two methods. The concentrations obtained by the two assays were not found to be significantly different.

The stereoselective binding of carvedilol enantiomers to serum proteins was investigated by equilibrium dialysis. Carvedilol is highly bound to serum proteins (> 98%). The free fractions obtained after dialysis of serum containing carvedilol were 0.6% for (+)-(R)-carvedilol and 0.9% for (-)-(S)-carvedilol with an R/S ratio of 0.67. The binding of the two enantiomers was found to be significantly different, with (-)-(S)-carvedilol being less bound. Binding to isolated serum proteins was also determined. Using 4% human serum albumin (HSA) in isotonic phosphate buffer, the unbound fractions obtained were 2.4% and 2.6% for (+)-(R)-and (-)-(S)-carvedilol, respectively, with an R/S ratio of 0.92. The stereoselective binding to HSA was not found to be significantly different. On the other hand, the binding of carvedilol to 100 mg% α_1-acid glycoprotein (AAG) in isotonic phosphate buffer was found to be highly stereoselective. The unbound fractions obtained were 1.5% for (+)-(R)-carvedilol and 2.5% for (-)-(S)-carvedilol, with an R/S ratio of 0.60. The results

suggest that AAG is the major protein responsible for the stereoselective binding of carvedilol enantiomers in serum.

Carvedilol enantiomers were not found to exhibit concentration-dependent binding in human serum over the concentration range of 0.5-4 μg/ml per enantiomer. However, binding to 4% HSA was found to be significantly different above 2 μg/ml for each enantiomer. The free fractions increased substantially above concentrations of 3 μg/ml of each enantiomer when the binding to 100 mg% AAG was tested suggesting that the AAG binding sites are saturable.

In order to simulate an increase in the levels of AAG as would occur in myocardial infarction or surgery, the binding of carvedilol enantiomers to 4% HSA combined with 100 mg% AAG or 400 mg% AAG was compared. A decrease in the free drug concentration was observed for both enantiomers when the levels of AAG increased from 100 to 400 mg% and the R/S ratio changed from 0.82 to 0.67.

In summary, comparative HPLC and CE assay methods were developed for the stereoselective analysis of carvedilol enantiomers. While the fluorescent detection used for the HPLC method allowed for lower detection limits required for determination of free (unbound) enantiomer concentrations in serum, the CE method, with a higher limit of quantitation, was used for the determination of total enantiomer concentrations (free and bound). However, the actual sensitivity of the CE method, considering the amount of sample injected, was greater by approximately 10 fold than the HPLC method. Using a combination of both methods, the stereoselective protein binding characteristics were established for the enantiomers of carvedilol using human serum and purified protein fractions of human serum.

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AAG	α_1-acid glycoprotein
ANOVA	analysis of variance
AUC	area under the plasma concentration-time curve
C_{max}	maximum plasma concentration
°C	degrees Celsius
CD	cyclodextrin
CE	capillary electrophoresis
Cl	clearance
CSP	chiral stationary phase
C.V.	coefficient of variation
EM	emission (fluorescence)
EOF	electroosmotic flow
EX	excitation (fluorescence)
f_u	post-dialysis free fraction
g	gravitational acceleration (centrifugation)
GITC	2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate
h	hours
HPLC	high-performance liquid chromatography
HSA	human serum albumin
I.D.	internal diameter
IS	internal standard

i.v.	intravenous
K_D	dissociation constant
kV	kilovolts
LC	liquid chromatography
LC/MS	liquid chromatography/mass spectrometry
LOQ	limit of quantitation
M	molarity of the solution
min	minutes
ml	millilitre
mM	millimolar
µg	microgram
µl	microlitre
µm	micrometer
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass to charge ratio
N	number of theoretical plates (capillary electrophoresis)
ng	nanogram
pH	-log $[H^+]$
r	correlation coefficient
r^2	coefficient of determination
R_f	solute migration distance/eluent migration distance (TLC)
R_s	resolution factor

s	seconds
s.d.	standard deviation
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEA	triethylamine
TFA	trifluoroacetic acid
TLC	thin layer chromatography
t_R	retention time (chromatography)
UV	ultraviolet
v	volume

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1. INTRODUCTION

1.1 Carvedilol

1.1.1 Chemistry

Carvedilol, 1-(9H-carbazol-4-yloxy)-3-[[2-(2-methoxyphenoxy)ethyl]amino]-2-propanol (Figure 1), is a new antihypertensive drug. It has been recently introduced on the market in Canada under the brand name COREG and is manufactured by SmithKline Beecham. It possesses a chiral centre in its structure and thus exists as two enantiomers. It is administered as the racemate. Carvedilol is a basic, highly lipophilic compound. It has a log octanol/pH 7.4 buffer partition coefficient (log P) of 3.3 (Varin *et al.*, 1986).

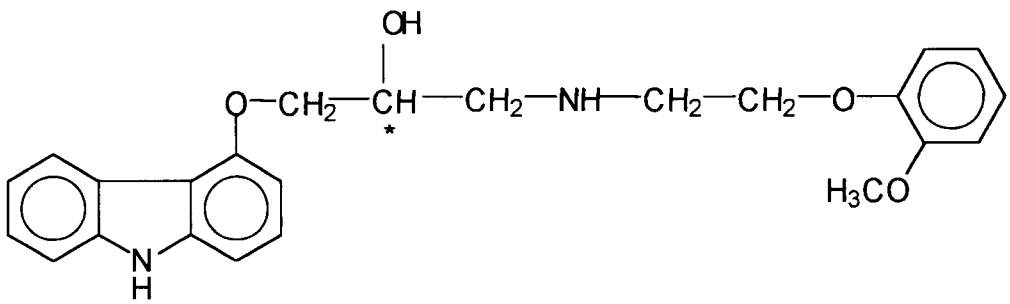

Figure 1. Chemical structure of carvedilol. The asterisk denotes the chiral centre.

1.1.2 Pharmacological Action

Carvedilol is a non-selective β-adrenergic receptor antagonist and an α_1-adrenoceptor blocker (Ruffolo Jr. *et al.*, 1990, Nichols *et al.*, 1991, de Mey *et al.*,

1994). The decrease in blood pressure produced by carvedilol is the result of its action at β_1- and α_1-adrenergic receptors. The β_1-blockade produces a decrease in heart rate and in the force of contraction of the cardiac muscle. The α_1-antagonism results in relaxation of the smooth muscle in the blood vessels causing a decrease in peripheral vascular resistance (Ruffolo Jr. *et al.*, 1990, Tomlinson *et al.*, 1992).

The combination of β-blockers with vasodilators has proven to be very useful in the treatment of hypertension (Tsukiyama *et al.*, 1988; Prichard, 1984). The two actions combined offer the advantage of reducing the incidence of side effects associated with individual therapy with either drug class. Therefore, with carvedilol administration, the reflex tachycardia commonly seen with vasodilators is avoided by the β_1-blockade, and vasoconstriction after β_1-blockade is counteracted by the α_1-antagonistic activity of the drug (Ruffolo Jr. *et al.*, 1991).

At higher concentrations (> 1 μM) carvedilol also exhibits calcium channel antagonist activity (Nichols *et al.*, 1991). Although this effect occurs at doses higher than the typical clinical dose, it is possible that it contributes to the vasodilatory action of carvedilol, at least in cutaneous and renal vascular beds (Ruffolo Jr. *et al.*, 1991, Lessem and Lukas, 1993).

An antihypertensive effect in patients is obtained with oral administration of a 25 mg dose of carvedilol given once daily. The drug is well tolerated with the most common adverse effects being vertigo, headache, bronchospasm, fatigue and skin reactions (Ruffolo Jr. *et al.*, 1991, McTavish *et al.*, 1993).

It has been shown that long-term treatment with β-blockers can improve cardiac function and reduce the symptoms of heart failure (Pfeffer and Stevenson, 1996). Clinical trials have shown recently that carvedilol decreased the risk of death and

hospitalization in patients with heart failure (Packer *et al.*, 1996). Carvedilol appears to be a promising drug for the therapy of congestive heart failure because it combines vasodilation and β-blocking properties (Ruffolo Jr. *et al.*, 1991).

It has also been reported that carvedilol exhibits cardioprotective effects, with a marked reduction of infarct size observed in animal models of acute myocardial infarction (Lysko *et al.*, 1995).

1.1.2.1 Enantioselectivity in Action

In vitro studies with guinea pig atrium have shown that the β_1-blocking activity of carvedilol is mainly due to the S-enantiomer. (-)-(S)-Carvedilol was 100 times more potent than (+)-(R)-carvedilol in inhibiting the response to isoprotenerenol with dissociation constants (K_D) at the β_1-adrenergic receptor of 0.4 nM and 45 nM for (-)-(S)- and (+)-(R)-carvedilol, respectively (Nichols *et al.*, 1989). At the α_1-receptor in rabbit aorta the activities of the two enantiomers were not significantly different with K_D values of 16 nM and 14 nM for (+)-(R)- and (-)-(S)-carvedilol, respectively (Nichols *et al.*, 1989). The larger antihypertensive effect of (-)-(S)-carvedilol, compared to (+)-(R)-carvedilol, was also observed *in vivo* in spontaneously hypertensive rats (Nichols *et al.*, 1989, Bartsch *et al.*, 1990).

The enantioselectivity in action can be explained by examining the chemical structure of carvedilol. Carvedilol contains two pharmacophores in its structure, namely the aryloxypropanolamine and the phenoxyalkylamine group. The aryloxypropanolamines are a general class of β-antagonists, an example of this group of compounds being propranolol (Nichols *et al.*, 1989, Fantucci *et al.*, 1992). In the case of carvedilol, this part of the molecule contains the chiral centre, which could

explain the stereoselective action of the drug at the β_1-adrenoceptors. It is also known that the absolute configuration of the hydroxyl group in the aryloxypropanolamine moiety should be S for the interaction with the β_1-adrenergic receptor (Fantucci *et al.*, 1992), which is the case for carvedilol. Phenoxyalkylamines are α_1-antagonists which can display stereoselectivity in action (Patil *et al.*, 1975, Ruffolo Jr., 1984). Therefore the absence of stereoselectivity in the interaction of carvedilol enantiomers with the α_1-receptors could be due to the absence of an asymmetric carbon in the phenoxyalkylamine part of the molecule (Nichols *et al.*, 1989).

Carvedilol is a rare example of drug in which the enantiomers not only differ in terms of potency but also have qualitatively different pharmacological profiles (Ruffolo Jr. *et al.*, 1991). This means that the clinical effect of the racemate cannot be obtained with the use of either enantiomer alone (Nichols *et al.*, 1989).

1.1.3 Pharmacokinetics of Carvedilol

1.1.3.1 Pharmacokinetics of the Racemate

1.1.3.1.1 Absorption and Distribution

Carvedilol is rapidly absorbed after oral administration. Maximum concentrations in plasma (C_{max}) were 128 ng/ml following a 50 mg dosing with a suspension formulation, and 66 ng/ml after administration of a 50 mg capsule formulation, and were attained within 1 to 2 hours (vonMöllendorf *et al.*, 1987). After a 12.5 mg intravenous injection, a C_{max} of 173 ng/ml was achieved at the end of the infusion (Neugebauer *et al.*, 1987). High intra- and inter-subject variability in AUC and

C_{max} for carvedilol was reported by Tenero *et al.* (1990). The bioavailability of the oral dosage form is very low (20-25%) due to extensive first-pass hepatic metabolism (Stahl *et al.*, 1990; Neugebauer *et al.*, 1987). The volume of distribution is large, about 1.5 to 2 l/kg in healthy volunteers, and is probably the result of extensive distribution into extravascular tissues due to the high lipophilicity of the drug (McTavish *et al.*, 1993).

1.1.3.1.2 Metabolism and Elimination

The terminal elimination half-life reported for carvedilol after intravenous administration was approximately 2.4 hours, whilst after oral dosage the half-life was much longer; about 6.4-7 hours for the capsules. The reason for this difference is not clear. It could be due to a continued reabsorption of the drug (Neugebauer *et al.*, 1987). The total body clearance of carvedilol was reported to be 589 ml/min and is mainly the result of hepatic clearance. Less than 2% of unaltered drug is recovered in urine (Neugebauer *et al.*, 1987). About 16% of the metabolites are eliminated in the urine while 60% are eliminated in the faeces (Neugebauer *et al.*, 1987).

Glucuronidation occurs to a high extent; 22% of the radioactivity detected in plasma after administration of ^{14}C-carvedilol was due to carvedilol-glucuronide (Neugebauer and Neubert, 1991). In urine, of the 16% excreted, 32.4% was identified as the carvedilol-glucuronide (Neugebauer and Neubert, 1991). Glucuronidation of carvedilol at the hydroxyl group and at the carbazol nitrogen (minor metabolite) was detected in rat bile as well as a carbamoyl glucuronide at the aliphatic amine group (Schaefer, 1992, Schaefer *et al.*, 1992).

Other major metabolites identified were aliphatic side-chain cleavage products (20% in plasma and 13% in urine) and aromatic ring hydroxylated and conjugated

metabolites (18% in urine) (Neugebauer *et al.*, 1987). Figure 2 shows the metabolites of carvedilol that have been identified in humans and in rats (Neugebauer *et al.*, 1987, Fujimaki and Hakusui, 1990, Fujimaki *et al.*, 1991, Neugebauer and Neubert, 1991, Fujimaki, 1994, Yue *et al.*, 1994, Zhou and Wood, 1995). Fujimaki and Hakusui (1990) reported the presence of two major metabolites in the rat bile, 1-hydroxycarvedilol-*O*-glucuronide (39% of the dose) and 8-hydroxycarvedilol-*O*-glucuronide (22% of the dose). Apparently *O*-desmethyl carvedilol possesses some pharmacological activity (Neugebauer *et al.*, 1987; Neugebauer and Neubert, 1991) but it is detected in human plasma in very low concentrations (< 1% of all metabolites). 4'-Hydroxycarvedilol has been shown to be equipotent to the parent drug as a β-blocker and exhibits 1/3 of the vasodilating potency and this may also be true for 5'-hydroxycarvedilol (Neugebauer and Neubert, 1991, Ruffolo Jr. *et al.*, 1991). Plasma concentrations of these two metabolites have not been determined yet but they may contribute to the pharmacological action of carvedilol (Neugebauer and Neubert, 1991). A major metabolite identified in urine, 3-hydroxycarvedilol, was found to possess weak β-blocking and similar α_1-antagonistic activity to carvedilol, and a potent antioxidant activity for inhibition of lipid peroxidation (Yue *et al.*, 1994). Although plasma levels have not been determined yet, it is possible that an effective level of 3-hydroxycarvedilol may be achieved with carvedilol administration, and that the metabolite contributes to the therapeutic effect of the parent drug (Yue *et al.*, 1994).

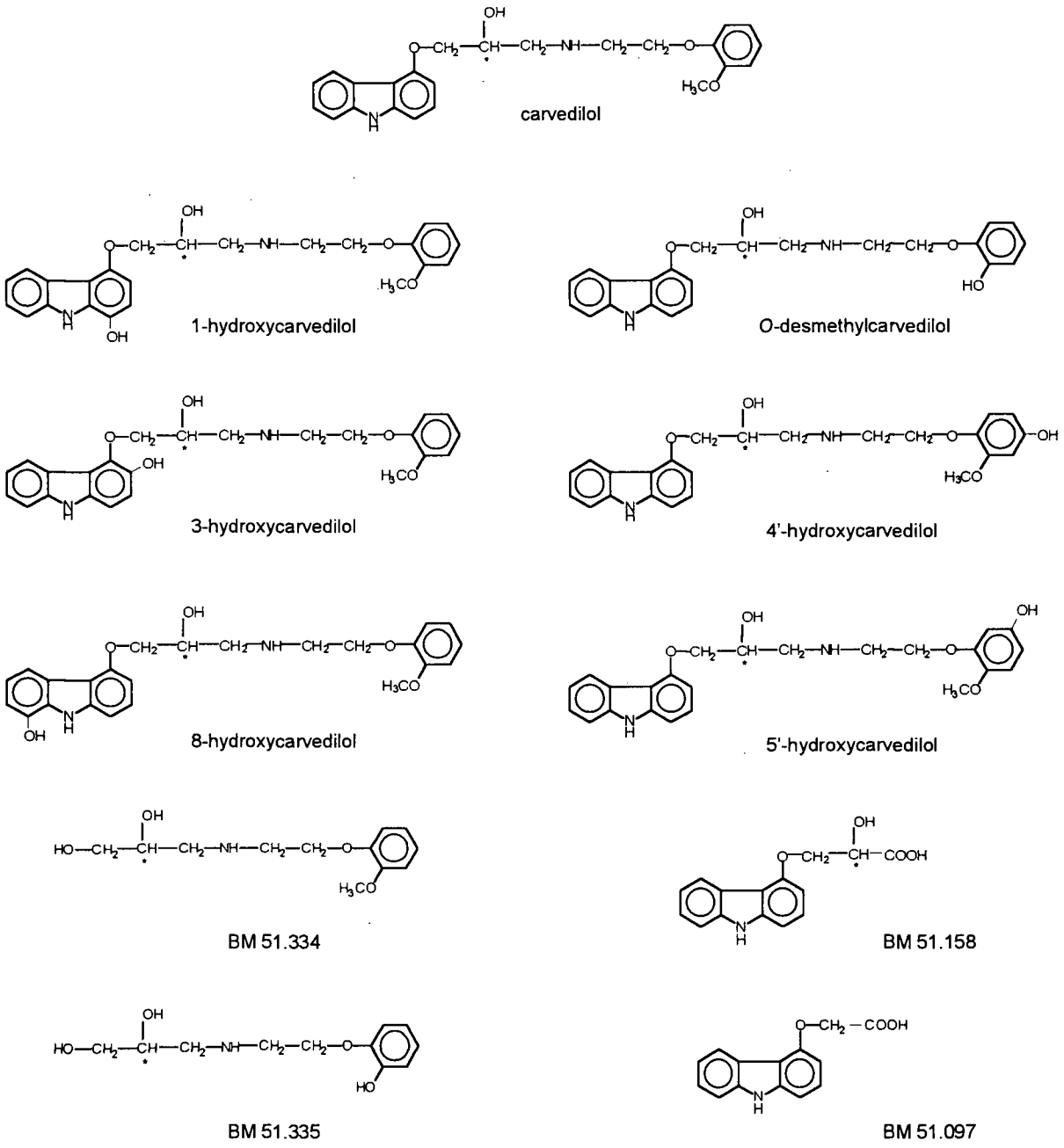

Figure 2. Metabolites of carvedilol identified in humans and in rats.

1.1.3.1.3 Protein Binding

Carvedilol was reported to be more than 95 % bound to plasma proteins, with albumin being the principal binding site (Ruffolo Jr. *et al.*, 1992). Unfortunately, the authors do not describe how this value was obtained. It has also been reported by this group that plasma protein binding of carvedilol is independent of concentration over the 50 to 1000 ng/ml concentration range (Ruffolo Jr. *et al.*, 1992), although the authors do not describe the experimental protocol nor the unbound fractions obtained.

1.1.3.2 Pharmacokinetics of Carvedilol Enantiomers

1.1.3.2.1 Absorption and Distribution

The enantiomers of carvedilol have been reported to exhibit stereoselective pharmacokinetics in humans (Neugebauer *et al.*, 1990). After intravenous administration in humans and in rats, the concentration-time curves in plasma were parallel but the (-)-(S)-enantiomer was detected at lower concentrations compared to its antipode (Neugebauer *et al.*, 1990, Stahl *et al.*, 1993b). After a 12.5 mg i.v. short infusion, Spahn *et al.* (1990) reported a C_{max} of 70 ng/ml and 89 ng/ml, for (-)-(S)- and (+)-(R)-carvedilol, respectively. The C_{max} values obtained by the same group, after oral administration of 50 mg of drug, were 21 ng/ml and 78 ng/ml, for (-)-(S)- and (+)-(R)-carvedilol, respectively (Spahn *et al.*, 1990). Similar values were also obtained by Neugebauer *et al.* (1990). The C_{max} values reported by Fujimaki *et al.* (1990) after an oral dose of 20 mg of carvedilol were much lower, only 10 ng/ml and 25 ng/ml, for (-)-(S)- and (+)-(R)-carvedilol, respectively. The ratio R/S of the areas under the curve (AUC) was 1.3 after intravenous administration and it was much higher (R/S = 2.7)

when the drug was administered orally (Neugebauer *et al.*, 1990). The volume of distribution at steady-state in rats was reported to be higher for (-)-(S)-carvedilol (3.32 l/kg compared to 2.21 l/kg for (+)-(R)-carvedilol) (Fujimaki, 1992). The oral bioavailability of (+)-(R)-carvedilol was 31% while for (-)-(S)-carvedilol it was 15% (Neugebauer *et al.*, 1990).

1.1.3.2.2 Metabolism and Elimination

The difference between bioavailabilities for the two enantiomers is indicative of stereoselective first-pass metabolism. Carvedilol is metabolized mainly by conjugation with glucuronic acid (Neugebauer and Neubert, 1991). However glucuronidation does not seem to be the enantiospecific metabolic reaction since the same enantiomeric ratio was found for carvedilol glucuronides and for the parent drug (Neugebauer *et al.*, 1990). Thus, other enantioselective metabolic pathways are probably involved. It has been reported that the quantities of 1-hydroxycarvedilol-*O*-glucuronide and 8-hydroxycarvedilol-*O*-glucuronide excreted in rat bile after administration of racemic carvedilol differ significantly with a 1-hydroxy/8-hydroxy ratio of 1.77 (Fujimaki and Hakusui, 1990). When the R-enantiomer was administered alone, 1-hydroxycarvedilol-*O*-glucuronide was formed in greater quantities (1-hydroxy/8-hydroxy = 4.58), whereas following administration of the S-enantiomer 8-hydroxycarvedilol-*O*-glucuronide was predominant (1-hydroxy/8-hydroxy = 0.61) (Fujimaki and Hakusui, 1990, Fujimaki *et al.*, 1991).

In vitro studies with rat liver microsomes showed that *O*-demethylation was slightly stereoselective for (+)-(R)-carvedilol and 4'-hydroxylation was stereoselective for the S-antipode (Fujimaki, 1994).

Systemic clearance was found to be 9.4% higher for (-)-(S)-carvedilol after intravenous administration (Neugebauer *et al.*, 1990). The elimination half-life after i.v. administration was reported by Neugebauer *et al.* (1990) to be 3.2 and 3.5 h for (-)-(S)- and (+)-(R)-carvedilol, respectively. Similar elimination half-lives were obtained by Spahn *et al.* (1990). The elimination half-life for (-)-(S)-carvedilol was significantly longer when carvedilol was administered orally (22 h, compared to 10 h for (+)-(R)-carvedilol) despite a higher clearance of (-)-(S)-carvedilol (2439 ml/min *versus* 1791 ml/min for (+)-(R)-carvedilol) (Neugebauer *et al.*, 1990). The authors admit that these results are difficult to explain, and that they could be due to sensitivity limitations of the analytical method, with determinations near the quantitation limit, or they could be the result of stereoselective enterohepatic recirculation of (-)-(S)-carvedilol (Neugebauer *et al.*, 1990). The $t_{1/2}$ values reported by Spahn *et al.* (1990) after oral administration of the same dose were different than those obtained by Neugebauer *et al.* (1990): 5.6 and 3.3 h for (-)-(S)- and (+)-(R)-carvedilol, respectively.

1.1.3.2.3 Protein Binding

The protein binding for carvedilol enantiomers was determined in human plasma by Fujimaki *et al.* (1990) using equilibrium dialysis. Using a single plasma concentration of 1 μg/ml dialyzed against phosphate buffer for 2 h, the free fraction in plasma obtained for (-)-(S)-carvedilol was 1.4 times greater than that for (+)-(R)-carvedilol. The same method was used by Stahl *et al.* (1993a, 1993c) using rat plasma to which 400 ng/ml of each enantiomer were added. The racemate was also tested at the same concentration. The binding of the R-enantiomer (99.3%) was higher than that of the S-antipode (98.9%) in control plasma and lower for both enantiomers in plasma

from rats with a portacaval shunt, a pharmacokinetic model for liver cirrhosis (R: 98.1% and S: 97.6%). The S/R ratio for unbound carvedilol to albumin was reported to be 0.79 which is lower than the unbound fraction ratio in plasma, 1.57, indicating that other proteins may be involved in binding (Stahl *et al.*, 1993a, Stahl *et al.*, 1993c).

1.1.3.3 Pharmacokinetics of Racemic Carvedilol in Disease States

1.1.3.3.1 Pharmacokinetics of Racemic Carvedilol in Liver Cirrhosis

In patients with liver cirrhosis the C_{max} of racemic carvedilol increased, and the systemic clearance was reduced by about 36% (Neugebauer *et al.*, 1988). The bioavailability increased from 18.6% in healthy subjects to 82.6% in these patients (Neugebauer *et al.*, 1988). The half-life obtained after intravenous dosage was about 3-fold longer than for the healthy volunteers, and no significant differences between half-lives after intravenous and oral administration were observed (Neugebauer *et al.*, 1988). The authors suggested that the dosage of carvedilol should be adjusted in this patient group. Although stereoselective metabolism was not abolished in cirrhotic patients (R/S ratios for the AUCs and C_{max} were greater than 1), the decrease in first-pass extraction eliminated the difference in bioavailability of the two enantiomers (Neugebauer *et al.*, 1992). No change in total protein binding was observed in patients with liver cirrhosis (Neugebauer *et al.*, 1988).

1.1.3.3.2 Pharmacokinetics of Racemic Carvedilol in Renal Failure

The pharmacokinetics of carvedilol are not substantially altered in patients with renal failure since the drug is eliminated primarily via the faeces. Although a decrease

in renal elimination of carvedilol and metabolites occurs due to renal impairment, no changes in the AUC, $t_{1/2}$, C_{max}, or t_{max} were observed (Krämer *et al.*, 1992). Renal clearance of carvedilol accounts for only 0.7% of the total body clearance and the dose does not need to be adjusted for this group of patients (Hakusui and Fujimaki, 1988, Krämer *et al.*, 1992).

1.1.3.4 Pharmacokinetics of Racemic Carvedilol in the Elderly

There appears to be no significant difference in the pharmacokinetic parameters of the racemate in elderly hypertensive patients and dosage adjustments are not necessary in this population (Ruffolo Jr. *et al.*, 1992). A slight increase in C_{max} values, 58 ng/ml in young adults compared to 69 ng/ml in older subjects, has been reported, but this difference is not likely to be clinically significant (Morgan *et al.*, 1990, Morgan, 1994).

1.1.4 Analysis of Carvedilol

1.1.4.1 Achiral Analysis of Racemic Carvedilol

The analytical methods for determination of racemic carvedilol in plasma and urine involve partition mode HPLC. The methods are sensitive and selective and consist of an extraction from the biological matrix with organic solvent, injection into the HPLC using an internal standard, and detection by fluorometry. Correlation between the detector response and carvedilol concentrations in serum was reported to be very good within the range of 0.25 to 150 ng/ml (Varin *et al.*, 1986). The detection limit obtained by Reiff (1987) was 0.38 ng/ml in plasma, and 0.82 ng/ml in urine, whereas

Varin *et al.* (1986) reported a sensitivity of 0.1 ng/ml in plasma. Louis *et al.* (1987) found a detection limit of 2-5 ng/ml for carvedilol in plasma, and Neugebauer *et al.* (1987) reported a detection limit of 2 ng/ml in plasma.

1.1.4.2 Stereoselective Analysis of Carvedilol Enantiomers

The methods reported in the literature for the analysis of the enantiomers of carvedilol consist basically of derivatization of the drug with optically pure derivatizing reagents to form diastereomers which are then analyzed by HPLC using either adsorption or partition mode with fluorometric detection. Stahl *et al.* (1993a) reported a detection limit below 1 ng/ml for each enantiomer after derivatization with (+)-(R)-phenylethyl isocyanate (PEI) and analysis using a silica column. The linearity of peak area-concentration was confirmed from 1 to 1500 ng/ml per enantiomer in the biological material. Another method used the chiral reagent 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) to form diastereomers with carvedilol enantiomers which were then separated using a C_{18} HPLC column (Eisenberg *et al.*, 1989, Fujimaki *et al.*, 1990, Neugebauer *et al.*, 1990, Zhou and Wood, 1995). The lower limit of quantitation as reported by Neugebauer *et al.* (1990) was 1 ng/ml for (-)-(S)-carvedilol, and 2 ng/ml for (+)-(R)-carvedilol. Eisenberg *et al.* (1989) reported a rapid and selective reaction of GITC with the secondary amine group of carvedilol in 30 min at room temperature. However, the chemical structure of the derivative was not confirmed. These investigators obtained a limit of quantitation of 2 ng/ml for both enantiomers. A limit of quantitation of 1.55 ng/ml per enantiomer using GITC derivatization was obtained by Fujimaki *et al.* (1990). After some modifications on the

method of Eisenberg *et al.* (1989), Zhou and Wood (1995) were able to improve the limit of quantitation of the assay to 0.5 ng/ml for each enantiomer.

Spahn (1988) proposed the use of (+)-(S)-naproxen chloride as a fluorescent chiral reagent for the derivatization of alcohols and amines. The reagent was used to determine the pharmacokinetics of carvedilol enantiomers in human plasma. The resulting fluorescent diastereomers were separated on a silica column. The limit of detection using this technique was 1 ng/ml per enantiomer in plasma with linearity between peak area and concentration of carvedilol in the range of 1-200 ng/ml of each enantiomer (Spahn *et al.*, 1990).

1.2 Chiral Separations Using High-Performance Liquid Chromatography

1.2.1 Diastereomer Formation

The formation of diastereomers, which possess different physico-chemical properties, is often used to obtain chiral discrimination using conventional HPLC columns (Taylor and Maher, 1992). The technique may consist of the formation of transient diastereomeric ion-pairs, through the introduction of a chiral counter-ion to the mobile phase such as (+)-10-camphorsulphonic acid or N-benzoxycarbonyl-glycyl-L-proline. The resulting diastereomeric complexes will exhibit different stability constants or distinct distribution properties between the mobile phase and the stationary phase and resolution will occur (Krstulovic, 1989, Lindsay, 1992).

Diastereomers can also be formed by derivatizing the enantiomers with a homochiral derivatizing reagent. The chiral derivatizing reagent must have high optical purity so that the correct R/S ratio of the compound of interest can be determined. This

can be a problem since many chiral derivatizing reagents tend to racemize during storage (Zief and Crane, 1988). Another requirement to obtain acceptable resolution is that the conformational differences must be pronounced, and that the chiral centres from the compound and from the reagent are close to each other in the resulting diastereomer (Zief and Crane, 1988). A potential problem in chiral derivatization is the possibility of different rates of reaction for each enantiomer, resulting in unequal quantities of the diastereomers formed (Krstulovic, 1989). Ideally, the derivatization should be quantitative for analytical purposes, while not completely necessary for preparative applications (Zief and Crane, 1988).

1.2.2 Chiral Stationary Phases

There are numerous types of chiral stationary phases (CSP) currently available on the market; however, their use is usually limited to a rather narrow range of chemical structures and mobile phases. Furthermore, the CSPs are often expensive compared to non-chiral HPLC columns. A very specific conformational fit between the enantiomers and the CSP has to exist in order for chiral discrimination to occur.

The most common type of CSP is the donor-acceptor type phase, or Pirkle type phase, where interactions with the solute are determined by hydrogen bonding, dipole-dipole interaction, charge transfer, and steric hindrance. A minimum of 3 simultaneous interactions should exist for chiral discrimination to occur and at least one of these interactions should be stereochemically dependent (Wainer and Doyle, 1984, Pirkle and Pochapsky, 1989).

Cyclodextrins can be bound to silica to form CSP (Taylor and Maher, 1992). Cyclodextrins have a cone-like structure, with a hydrophobic interior, and a polar outer

surface. Separation occurs by inclusion of the analyte in the cavity and interaction with the secondary hydroxyl groups at the surface (Armstrong *et al.*, 1986). Different stability constants for the cyclodextrin complexes will then produce diverse retention of the enantiomers, resulting in chiral separation (Fujimura *et al.*, 1990).

Cellulose esters and poly-(triphenylmethyl)methacrylates are also used as CSP. The separation involves the combination of chemical interactions and inclusion of the analyte in a chiral cavity. They usually require the use of non-polar solvents (Stevenson and Wilson, 1987).

Another type of stationary phase used in chiral HPLC consists of a protein, such as bovine serum albumin (for acidic drugs) or α_1-acid glycoprotein (for basic drugs), bound to silica. Separation occurs via hydrophobic and polar interactions. They can only be used with aqueous buffers since the proteins are not stable in organic solvents or at extremes of pH (Stevenson and Wilson, 1987).

Ligand exchange columns are used for the separation of amino acids and amino alcohols. They consist of proline or hydroxyproline bound to silica and treated with copper salts. The analyte must form a reversible complex with the copper ion (Stevenson and Wilson, 1987).

1.3 Capillary Electrophoresis

1.3.1 Fundamentals

Capillary Electrophoresis (CE) was first introduced by Jorgenson and Lukacs (1981). The technique is basically a modification of conventional electrophoresis in that it is performed in narrow-bore silica capillaries, typically 25 to 75 μm I.D., 10 to 100 cm length, filled with an appropriate buffer. The large surface area-to-volume ratio of the capillary allows a large electric potential to be applied across the capillary while maintaining effective heat dissipation (Fanali *et al.*, 1990, Landers, 1993).

A schematic of a CE instrument is shown in Figure 3. The two ends of the capillary and the electrodes are immersed in buffer solutions. During injection, the sample is introduced at one end of the capillary (usually the anodic end) and a large voltage is applied. Molecules will migrate at different velocities governed by their charge-to-mass ratios. Even neutral and negatively charged compounds will be carried towards the detector end (cathode) because of a phenomenon called electroosmotic flow (EOF). At the detector end, solutes will be passing the window in zones, therefore the name capillary zone electrophoresis (CZE) is also applied to this technique (Jorgenson and Lukacs, 1981).

Another feature of the CE instruments is the capability of reversing the polarity of the electrodes, from normal polarity (cathode is at the detector end) to reverse polarity, where the detector end is at the anode. This would be desirable when analyzing negative ions in the absence of EOF (Goodall *et al.*, 1990).

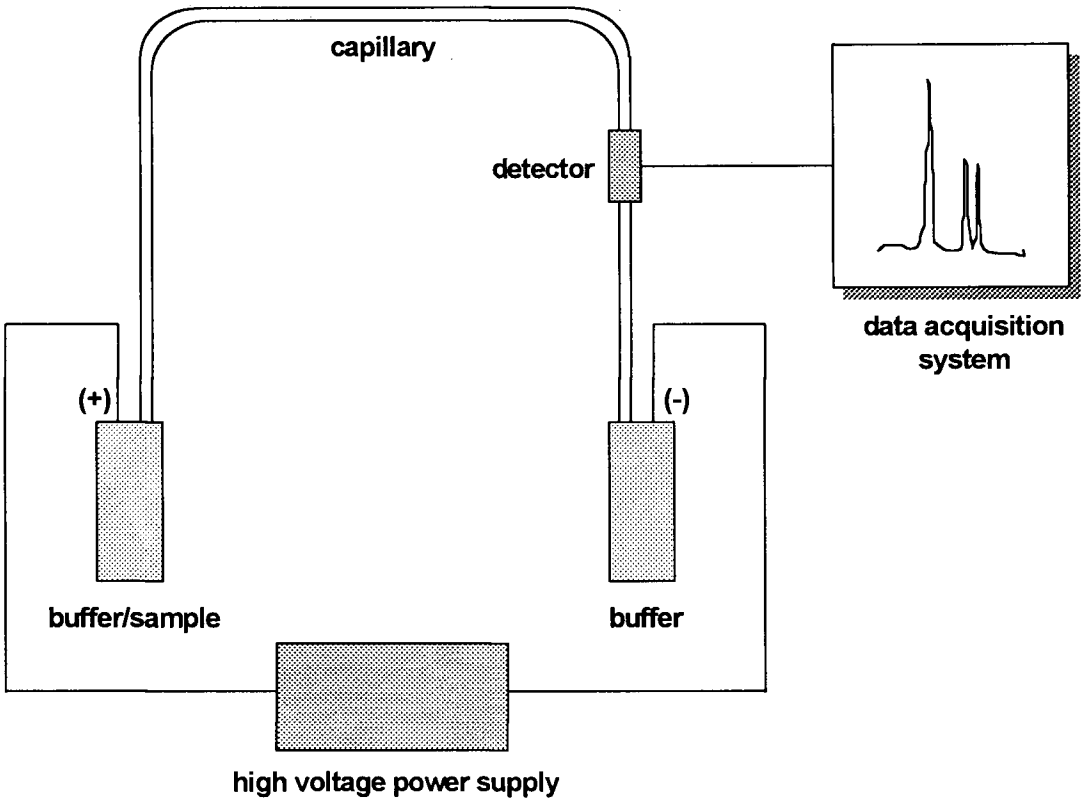

Figure 3. Schematic of a capillary electrophoresis instrument.

Detection in CE is usually on-column. A small section of the polyimide coating on the capillary is removed and will serve as a detector window. The on-column detection contributes to the high efficiencies observed in CE in that it eliminates band spreading due to connections. However, the small capillary diameter results in a very small optical path length which can compromise sensitivity (Goodall *et al.*, 1990). The currently available detectors for CE are UV (single wavelength and diode array detection) (Ong *et al.*, 1991, Ma *et al.*, 1992, Heiger *et al.*, 1994), laser-induced fluorescence (LIF) (Cheng and Dovichi, 1988, Albin *et al.*, 1991, Liu *et al.*, 1991), electrochemical (Olefirowicz and Ewing, 1990), and mass spectrometric detectors

(Thibault *et al.*, 1991a, Thibault *et al.*, 1991b, Pleasance *et al.*, 1992a, Pleasance *et al.*, 1992b, Tsuji *et al.*, 1992, Naylor *et al.*, 1994).

The simplest mode of CE, called capillary zone electrophoresis (CZE) or free solution capillary electrophoresis (FSCE) was described above. Many adaptations of the technique were developed in order to overcome its limitations and increase its applicability. Micellar electrokinetic chromatography (MEKC) was developed by Terabe *et al.* (1984) and allows the separation of very closely related compounds and neutral molecules. This mode of CE employs surfactants added to the background electrolyte to form micelles. The analytes will partition into the micelles, which serve as a pseudo-stationary phase, to different extents, and separation will occur (Nishi and Terabe, 1990, Evenson and Wiktorowicz, 1992, Lurie, 1992, Terabe, 1992, Donato *et al.*, 1993, Gaus *et al.*, 1993, Lukkari *et al.*, 1993, Thormann *et al.*, 1993, Wätzig and Lloyd, 1995). Other modes include capillary gel electrophoresis (CGE) for the separation of protein and DNA fragments (Sudor *et al.*, 1991, Paulus, 1992, Brunner *et al.*, 1995), capillary isoelectric focusing (CIEF) for the separation of proteins based on differences on the isoelectric point of proteins (Frenz *et al.*, 1989, Rabel and Stobaugh, 1993) and capillary isotachophoresis (CITP) for the separation of proteins based on the formation of a potential gradient inside the capillary (Mikkers *et al.*, 1979, Stegehuis *et al.*, 1991).

Several reviews have been published to date describing this valuable technique and listing its numerous applications in the field of pharmaceutical sciences, clinical sciences, forensic analysis, molecular biology, and chemistry (Chen *et al.*, 1991, Goodall *et al.*, 1991, Jellum *et al.*, 1991, Krull and Mazzeo, 1992, Shihabi, 1992, Landers, 1993, Landers *et al.*, 1993, Rabel and Stobaugh, 1993, Tagliaro *et al.*, 1993, Brunner *et al.*, 1995).

1.3.2 Electroosmotic Flow

Electroosmotic flow or electroendosmotic flow (EOF) is the bulk flow of liquid within the capillary (Brunner *et al.*, 1995). Figure 4 shows the origin of the EOF inside a fused silica capillary. Depending on the pH, surface silanol groups on the capillary wall will be ionized, resulting in a negative charge at the wall of the capillary. A mono-layer of cations from the buffer adsorb to this negatively charged surface. The positive charge density decreases away from the surface forming a more diffuse layer. When voltage is applied, the cations forming the diffuse layer are attracted toward the cathode. Because they are solvated with water molecules, their movement drags the bulk solution in the capillary toward the cathode (McLaughlin *et al.*, 1992, Knox, 1994, Brunner *et al.*, 1995).

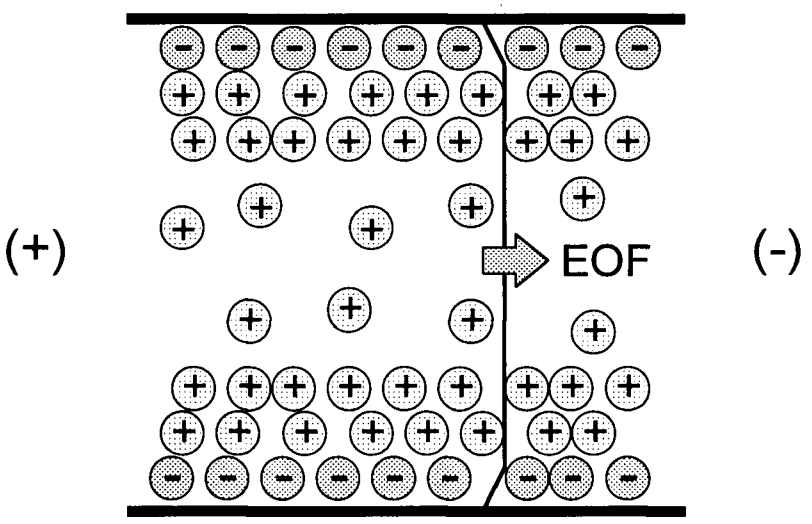

Figure 4. Diagrammatic representation of the electroosmotic flow inside a fused silica capillary at high pH. Negative charges on the walls represent ionized silanol groups. Positive charges represent the double layer of cations on the charged silanol surface and the diffuse layer of hydrated cations.

A unique characteristic of the EOF is the flat profile of the flow generated inside the capillary (Jorgenson and Lukacs, 1981). This is because the driving force of the flow is uniformly distributed along the capillary resulting in uniform flow throughout the entire length. This is in contrast with the flow produced by an external pump, as in HPLC, which yields a laminar or parabolic flow, due to the shear force at the wall (Brunner *et al.*, 1995). The flat flow profile results in higher efficiency for CE since there is less diffusion of solutes compared to the parabolic flow generated in HPLC (Jorgenson and Lukacs, 1981).

The benefit of the EOF is that it promotes the movement of all species, regardless of charge, towards the cathode under normal conditions. Cations will migrate faster since they are also being electrophoretically attracted towards the negative electrode. Neutral molecules are carried at the velocity of the EOF and are not separated from each other. Anions will move more slowly since they are attracted toward the anode, but are still carried by the EOF which is greater than their electrophoretic mobility (Jorgenson and Lukacs, 1981, Altria, 1996).

The observed mobility or apparent mobility (μ_A) of a solute is therefore dependent on both the mobility of the solute (μ_E) and the EOF (μ_{EOF}), and can be determined from the migration time (Altria, 1996):

$$\mu_A = \mu_E + \mu_{EOF} = lL/tV$$

where:

μ_A: apparent mobility

μ_E: mobility of the solute

μ_{EOF}: mobility of the electroosmotic flow

l: effective capillary length (to the detector)

L: total capillary length

t: migration time

V: applied voltage

1.3.3 Optimization Parameters

Unlike HPLC, where a large number of stationary phases are available and a limitless number of mobile phases possible, CE methods have only a limited number of variables that can be adjusted to affect selectivity (Steuer *et al.*, 1990).

1.3.3.1 pH

The pH is the most important optimization parameter in CE since it directly affects the EOF (Steuer *et al.*, 1990). EOF becomes significant above pH 3, and increases as the pH rises and the silanol groups become predominantly ionized. The EOF levels off around pH 7-8 where all silanol groups are ionized (Altria, 1996).

If the pH selection is incorrect, the success of the analysis is compromised. For example, at high pH, EOF can be too fast and the solutes will be carried too quickly and will elute before separation occurs. On the other hand, at low or moderate pH, the slow EOF may allow coulombic interaction of cationic solutes with the capillary wall, resulting in peak broadening. This is particularly significant for protein separations (McCormick, 1988). The pH also affects ionization of the solutes which will directly

influence electrophoretic mobility and hence selectivity of the assay (McLaughlin *et al.*, 1992).

1.3.3.2 Voltage

In CE, the efficiency of separation is proportional to the voltage. As higher voltages are applied, an increase in the number of theoretical plates is observed. In addition, faster analyses times are obtained (Jorgenson and Lukacs, 1981). The relationship between efficiency and voltage in CE is expressed by the formula:

$$N = \frac{1}{2D} (\mu_{EOF} + \mu_e) V$$

where:

N: number of theoretical plates

μ_{EOF}: electrophoretic mobility of the electroosmotic flow

μ_e: electrophoretic mobility of the analyte

D: diffusion coefficient of the analyte

V: voltage applied

The formula considers molecular diffusion as the only parameter affecting zone broadening (Jorgenson and Lukacs, 1981, Knox, 1994). Higher voltages result in shorter migration times which will allow less time for diffusion leading to band broadening. In practice, the linear relationship between efficiency and applied voltage is valid up to a certain voltage only, after which excessive Joule heating is produced. The increase in the temperature inside the capillary produces a decrease in buffer

viscosity with a subsequent increase in diffusion, which will lead to a decrease in efficiency (Jorgenson and Lukacs, 1981, Fanali *et al.*, 1990). Temperature control of the capillary can be used when application of higher voltages is necessary.

1.3.3.3 Buffer Concentration

An increase in buffer concentration decreases the double-layer thickness on the capillary wall and reduces the EOF. Slower migration times are obtained as well as increased theoretical plates and resolution, and improved peak shape. As current increases with increased buffer concentration more heat will be produced and effective temperature control is required in this situation. Smaller diameter capillaries allow the use of high concentration buffers because they can dissipate heat more efficiently (McLaughlin *et al.*, 1992).

1.3.3.4 Buffer Additives

The addition of organic modifiers such as methanol or acetonitrile to the run buffer can affect efficiency and resolution in CE. The organic modifier increases the viscosity of the buffer thus resulting in longer migration times. In addition, they also reduce the current inside the capillary allowing the application of higher voltages (McLaughlin *et al.*, 1992).

Ion pairing agents, e.g. hexanesulfonic acid, can be used to alter migration characteristics of the analytes or their net charge affecting analysis time and improving resolution (McLaughlin *et al.*, 1992).

Surfactants at concentrations below their CMC (critical micelle concentration) can adsorb to the capillary wall and modify EOF. A cationic surfactant, for instance

cetyltrimethylammonium bromide (CTAB), added to the buffer can reduce EOF, as the monomers adhere to the wall through ionic interactions masking the ionized silanol groups. The coating of the capillary wall by the surfactant also limits solute adsorption (Guzman *et al.*, 1992). If the CTAB concentration is high enough, another layer will bind through hydrophobic interactions, producing a positively charged surface which will generate EOF towards the anode thus reversing the direction of the EOF. Furthermore, surfactants can interact with the solute (ionic or hydrophobic interactions) altering its mobility (McLaughlin *et al.*, 1992).

The use of hydrophilic polymers such as hydroxymethyl cellulose (HMC) to mask or deactivate the silanol groups on the capillary wall has also been described as a means of decreasing EOF, reducing solute/wall interactions, and also increasing buffer viscosity (McLaughlin *et al.*, 1992).

1.3.3.5 Capillary Length and Diameter

An increase in capillary length produces longer migration times, with increased number of theoretical plates and resolution. Less heat is generated because resistance of the capillary is greater and current is diminished (McLaughlin *et al.*, 1992).

An increase in diameter results in increased heat due to higher charge density and consequently higher current (Sepaniak *et al.*, 1992). On the other hand a wider capillary allows larger sample loading (McLaughlin *et al.*, 1992).

1.3.3.6 Capillary Wall Coating

Wall-coated capillaries can be used with the purpose of eliminating or modifying EOF, as well as to eliminate interaction of the solutes with the capillary wall (especially proteins) (McLaughlin *et al.*, 1992, Yao *et al.*, 1993). The coating can be covalently bonded via a Si-O-Si-R bond or via a more stable Si-C bond. Agents such as polyacrylamide, polyethylene glycol, polyvinylpyrrolidinone, aryl pentafluoro, maltose, as well as HPLC phases such as C_2, C_8, and C_{18} are used as coating materials chemically bonded to the capillary wall. Physically adhered or adsorbed agents including cellulose, polyethylene glycol, and polyvinyl alcohol are also available, but exhibit poor long-term stability (McCormick, 1988, Yao *et al.*, 1993).

Extremes of pH are also effective in reducing ionic interactions with the capillary wall but can alter the protein structure at non-biological pH values (McCormick, 1988). High concentration can also limit ionic interactions but its use is limited due to the production of excessive Joule heating (McLaughlin *et al.*, 1992).

1.3.4 Chiral Separations by Capillary Electrophoresis

The majority of the chiral separations by CE are achieved with the use of chiral selectors added to the background electrolyte, using free solution capillary electrophoresis (FSCE) (Altria, 1996). Transient diastereomeric complexes are formed, and enantioseparation will arise from differences in effective electrophoretic mobilities, resulting from different stability constants of the complexes formed between the enantiomers and the chiral selector (Vespalec & Bocek, 1994). A variety of chiral selectors are available, but the most widely used are the cyclodextrins. Other chiral selectors used in CE include crown ethers, transition metal/amino acid complexes,

carbohydrates (maltodextrins), proteins (bovine serum albumin, ovomucoid), and glycopeptides (vancomycin) (D'Hulst and Verbeke, 1992, Armstrong *et al.*, 1994, D'Hulst and Verbeke, 1994, Altria, 1996).

Capillary gel electrophoresis has also been employed, with the capillary filled with a gel containing cyclodextrins (Guttman *et al.*, 1988, Cruzado & Vigh, 1992) or a protein (Birnbaum & Nillson, 1992). Capillary electrochromatography (CEC) involves the use of capillaries filled or coated with packing materials used in HPLC. Capillaries coated with β-cyclodextrin (Mayer and Schurig, 1993), and capillaries filled with HPLC packing material coated with α_1-acid glycoprotein have been used for chiral separations by CE (Li and Lloyd, 1993).

Derivatization of the analytes, prior to the electrophoretic process, in order to form diastereomers, has also been employed to obtain chiral separation by CE. Assays involving the use of Marfey's reagent (Tran *et al.*, 1990), and 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) have been reported (Vespalec & Bocek, 1994).

1.3.4.1 Chiral CE Using Cyclodextrins

There are a large number of publications that describe the use of cyclodextrins to achieve chiral separation of drugs by CE (Fanali, 1991, Snopek *et al.*, 1991, Schutzner and Fanali, 1992, Chankvetadze *et al.*, 1994, Sänger-van de Griend *et al.*, 1996). Other applications include monitoring enantiospecific enzymatic biotransformation (Rogan *et al.*, 1993), analysis of pharmaceutical formulations (Fanali and Bocek, 1990, Ong *et al.*, 1991, Peterson and Trowbridge, 1992, Baeyens *et al.*,

1993), and determination of drug enantiomers in plasma or serum (Pruñonosa *et al.*, 1992b, Soini *et al.*, 1992).

Cyclodextrins (CDs) are nonionic, cyclic oligosaccharides consisting of several glucose units connected by glycosidic linkages (Figure 5). They have the shape of a hollow truncated cone. The internal diameter is determined by the number of glucose units (usually 6, 7, or 8, corresponding to α, β, and γ CD). The cavity is hydrophobic while the entrance to the cavity is lined with secondary hydroxyl groups and is thus hydrophilic (Guttman, 1995) (Figure 6). Interaction with a solute will occur by inclusion of a hydrophobic portion of the solute in the cavity and from hydrogen bonding to the chiral hydroxyl groups of the rim (Fanali, 1989, Fanali & Bocek, 1990, Fujimura, *et al.*, 1990).

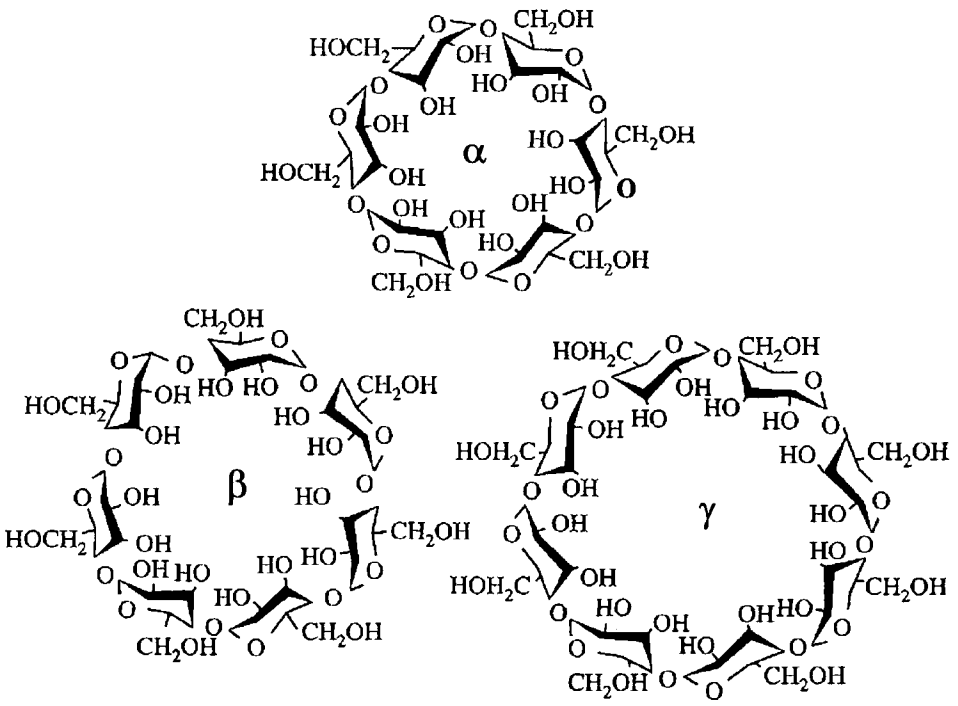

Figure 5. Structure of the native cyclodextrins. (Adapted from Rogan and Altria, 1993)

Derivatized CDs are available where the hydroxyl groups in positions 2, 3, and 6 have been chemically modified with functional groups such as methyl or hydroxypropyl. These modifications will result in changes in enantioselectivity, as well as in the solubility of the cyclodextrin (Fujimura *et al.*, 1990, Guttman, 1995).

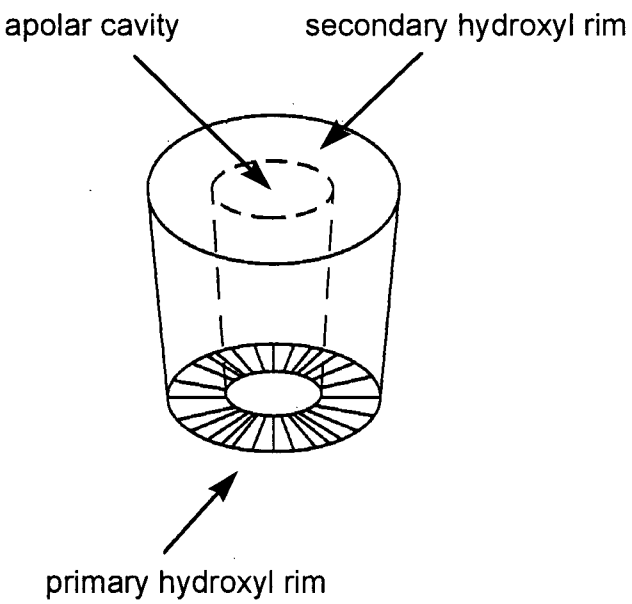

Figure 6. Representation of the cone-like structure of the cyclodextrins indicating the primary and secondary hydroxyl rims. (Adapted from Rogan and Altria, 1993)

1.3.4.1.1 Mechanism of Chiral Recognition

Chiral separation using cyclodextrins, in CE, will occur due to the formation of an inclusion complex between the analyte and the cyclodextrin. The analyte should have at least one aromatic ring or condensed aromatic rings, which will be situated inside the hydrophobic cavity of the cyclodextrin (Vespalec & Bocek, 1994). The chiral centre, or a substituent on the chiral centre, should be near the hydroxyl groups on the rim of the

cavity, and produce hydrophilic interactions (hydrogen bonding) (Sänger-van de Griend *et al.*, 1996).

In the case of propranolol, it is believed that the naphthyl group of propranolol fits inside the cyclodextrin cavity forming hydrophobic interactions with the hydrophobic cavity of the cyclodextrin, and the hydroxyl and amino groups of propranolol form hydrogen bonds with the secondary hydroxyl groups at the rim of the cyclodextrin (Armstrong *et al.*, 1986, Guttman and Cooke, 1994).

Enantiomers have the same electrophoretic mobility. However, when cyclodextrins are added to the buffer, the enantiomers will form transient complexes with the cyclodextrins. The extent of the complexation depends on the affinity of each enantiomer to the cyclodextrins. Therefore they will retain the two enantiomers to a different degree and separation will result. Enantiomeric resolution is thus a result of the amount of time that the enantiomers spend in the free form, or in the complex form with the cyclodextrin (Wren, 1993, Wren *et al.*, 1994). If the difference between the resulting apparent electrophoretic mobility is large enough, enantiomeric resolution will occur (Schutzner and Fanali, 1992).

Chiral resolution will depend on the type of CD and concentration used, as well as the pH of the buffer (St. Pierre and Sentell, 1994). The higher the affinity of the analyte for the CD, the lower the CD concentration required to obtain chiral separation (Vespalec and Bocek, 1994). Recently a negatively charged cyclodextrin was developed; the polyanion of the β-cyclodextrin sulfobutyl ether (Chankvetadze *et al.*, 1994). This agent provided very high efficiency, and resolution was obtained using concentrations of the chiral selector of the order of 40-100 μM. The high resolution power of charged cyclodextrins is a result of strong electrostatic interactions with the

charged analytes, as well as a migration in the opposite direction of a positively charged solute or a neutral molecule migrating with the EOF, enhancing differences in electrophoretic mobilities (Chankvetadze *et al.*, 1994, Dette *et al.*, 1994).

Operating pH can have a great effect on chiral separations by CE using cyclodextrins. It has been shown by Vigh and collaborators that three types of interactions can occur, depending on the ionization state of the analyte (Rawjee and Vigh, 1994, Rawjee *et al.*, 1994). In desionoselective separation (Type I), only the nondissociated form of the analyte interacts stereoselectively with the chiral selector. In the ionoselective type (Type II), only the dissociated form will produce enantioselective interaction. Duoselective separation (Type III) occurs when both dissociated and nondissociated forms of the enantiomers can combine in a stereoselective manner with the cyclodextrin (Rawjee and Vigh, 1994, Rawjee *et al.*, 1994, Guttman, 1995, Guttman *et al.*, 1995). Another effect of the buffer pH is that it determines the velocity of the electroosmotic flow. For cationic analytes, where electrophoretic mobilities will have the same direction as the EOF, the presence of the EOF will diminish the electrophoretic mobility differences between the two enantiomers. An increased number of interactions between the analytes and the cyclodextrins will be possible if they have more contact time with the chiral selectors as they move along the capillary (St. Pierre and Sentell, 1994). Therefore, it is important for optimum resolution that the EOF be minimized, by operating at low pH, or alternatively, by the use of a wall-coated capillary (Wren, 1993).

1.3.5 Quantitation Aspects

1.3.5.1 Internal Standardization

Injection volume variability is a major cause of reproducibility problems in CE. Commercial instruments are capable of performing electrokinetic and hydrodynamic injection. The former is done by application of voltage for a certain period of time. The quantity of sample introduced into the capillary upon application of voltage will depend on the mobility of the analytes, their ionization state, and the presence of EOF, and it is generally considered less reproducible (Goodall *et al.*, 1991, McLaughlin *et al.*, 1992). With hydrodynamic injection, the amount of sample injected will be determined by the amount of time pressure is applied, during the injection step. The volume, of the order of nanolitres, is not normally known, although it can be calculated (McLaughlin *et al.*, 1992). Although more reproducible than electrokinetic injection (Goodall *et al.*, 1991, McLaughlin *et al.*, 1992), there is the possibility of pressure variations between injections (Altria *et al.*, 1994). Therefore the use of an internal standard is necessary to ensure assay reproducibility (McLaughlin *et al.*, 1992, Peterson and Trowbridge, 1992, Altria, 1996).

1.3.5.2 Peak Area Normalization

In CE, compounds are migrating through the on-column detector window at different velocities. Analytes with a lower electrophoretic mobility will spend more time at the detector window than the faster-moving electrolytes, generating longer response times and consequently, larger peak areas. In contrast, in HPLC, all analytes pass the detector at the same speed, that is, the mobile phase flow rate. Therefore, to correct

for the differences in residence-time at the detector window on the capillary, the peak area is divided by the corresponding migration time. The final value is denoted "normalized peak area" or "corrected peak area" (McLaughlin *et al.*, 1992, Altria, 1993, Altria *et al.*, 1994).

1.4 Protein Binding of Drugs

1.4.1 Binding Proteins in Serum

Although many proteins are present in human plasma, three major proteins are responsible for the binding of drugs: albumin, α_1-acid glycoprotein and lipoproteins. The normal levels in serum for albumin and α_1-acid glycoprotein range from 3.5-5.5% and 55-140 mg%, respectively (MacKichan, 1992). Albumin accounts for the binding of most neutral and anionic (acidic) drugs, while cationic (basic) drugs bind not only to albumin but also to α_1-acid glycoprotein and lipoproteins (Piafsky, 1980, Kwong, 1985, MacKichan, 1992). The binding of a drug in plasma is a result of a combination of factors related to the binding protein(s), such as association constant, number of binding sites, access to these sites, and factors related to the drug, such as chemical nature, concentration and the presence of competitive ligands (Hervé *et al.*, 1994).

Changes in concentration of the plasma binding proteins are often associated with various disease states and can dramatically affect the unbound levels of circulating drug. A decrease in the plasma concentration of albumin is associated with cancer, cirrhosis, age, burn injury, surgery, and trauma injury (MacKichan, 1992). Lipoprotein levels vary to a large extent within the normal population and hyperlipoproteinaemia is associated with hypothyroidism, obstructive liver disease and

alcoholism (Piafsky, 1980). The levels of α_1-acid glycoprotein are increased in conditions such as acute myocardial infarction, inflammatory disease, surgery, and trauma injury (Piafsky, 1980, MacKichan, 1992). An increased plasma protein binding associated with myocardial infarction has been reported for propranolol (Routledge *et al.*, 1980). Increased binding was observed for propranolol and chlorpromazine in patients with active inflammatory diseases (Crohn's disease, rheumatoid arthritis) in which the levels of α_1-acid glycoprotein were very elevated (165 mg% in patients with Crohn's disease and 149 mg% in rheumatoid arthritis patients) compared to the control group (69 mg%) (Piafsky *et al.*, 1978).

1.4.2 Methods for the Determination of Protein Binding

1.4.2.1 Equilibrium Dialysis

Equilibrium dialysis is still considered the reference method for protein binding determinations. A semipermeable membrane is placed between two chambers, one containing the plasma, and the other containing a buffer. The membrane allows diffusion of drug molecules from the plasma compartment to the buffer, while retaining the proteins and protein-drug complexes. At equilibrium the concentration of free drug in the plasma compartment (retentate) and in the buffer compartment (dialysate) will be the same (Kwong, 1985, Kurz, 1986, MacKichan, 1992). The unbound fraction of drug is determined by the ratio of drug concentration in the buffer compartment (free) to drug concentration in the plasma compartment (bound and free) and is related to the post-dialysis plasma concentration, which is smaller than the initial drug concentration in plasma (Behm and Wagner, 1979, MacKichan, 1992).

The temperature of the chambers should be carefully controlled in order to reproduce physiological conditions, since the extent of binding is temperature-dependent (Pacifici and Viani, 1992). The time necessary to reach equilibrium must be determined for each drug. An error of 59% was reported for the determination of the unbound fraction of suramine due to failure in achieving equilibrium of the dialysis process (Kurz *et al.*, 1977).

Nonspecific adsorption of drug to the membrane can occur. However if the levels of drug in both the dialysate side and the retentate side are being measured no correction for the loss due to adsorption to the membrane is necessary (Kurz *et al.*, 1977, Kinget *et al.*, 1979, Bowers *et al.*, 1984). The Donnan effect originates from an unequal distribution of diffusible charged ions (such as drug molecules) due to the retention of charged protein molecules in one compartment. This can be a source of error in equilibrium dialysis of drugs which are both highly ionized and exhibit low protein binding (Kurz, 1986).

Significant volume shifts from the buffer side to the protein side can occur during long dialysis processes due to differences in osmotic pressure of both sides. These changes in volumes must be measured and corrected for when calculating the extent of drug binding (Lima *et al.*, 1983, Lockwood and Wagner *et al.*, 1983, Tozer *et al.*, 1983, Bowers *et al.*, 1984). Also protein leakage through the membrane into the dialysate can occur leading to an overestimation of the free fraction. Therefore the dialysate should be tested for presence of proteins after the dialysis process is completed (Bowers *et al.*, 1984).

1.4.2.2 Ultrafiltration

Determination of protein binding of drugs by ultrafiltration is based on the passage of plasma water containing free drug through a membrane by applying positive pressure or by centrifugation. The unbound fraction is obtained by the ratio of the concentration of drug in the ultrafiltrate to the drug concentration before ultrafiltration (MacKichan, 1992). The advantage of ultrafiltration is the rapidity and simplicity of the technique (Pacifici and Viani, 1992).

A major limitation of ultrafiltration is the nonspecific binding of drugs to the ultrafiltration devices and membranes (Hinderling *et al.*, 1974, Whitlam and Brown, 1981, Parsons and Fan, 1986, Barre *et al.*, 1988, Ohshima *et al.*, 1988, Pacifici and Viani, 1992). This drug loss leads to an underestimation of the unbound drug level (MacKichan, 1992). The adsorption can be so high as to completely preclude the use of such technique for the measurement of free drug (Zhirkov and Piotrovskii, 1984, Pacifici and Viani, 1992).

Another source of error in ultrafiltration is the sieve effect, which consists of a preferential passage of the smaller water molecules through the membrane, over the passage of drug molecules. The sieve effect is larger at higher molecular weights and filtration pressure (Kurz *et al.*, 1977, MacKichan, 1992).

Ultrafiltration should be performed in temperature-controlled centrifuges to minimize the effect of heat generated by the centrifugation on the binding equilibrium (Koike *et al.*, 1985, Kwong, 1985).

1.4.2.3 Ultracentrifugation

Ultracentrifugation is less widely used and is based on different sedimentation rates of solutes according to their molecular weights. The problem of nonspecific binding is eliminated in this technique since it does not require the use of plastic devices or membranes (MacKichan, 1992). On the other hand, it is a relatively time-consuming method (12 to 15 h) and sedimentation of part of free drug can cause error in the determination (Barre *et al.*, 1988, Kurz, 1986, Kurz *et al.*, 1977).

1.4.3 Stereoselectivity in Binding

Differences in binding for enantiomers can arise from differences in the affinity constants for binding and also from displacement of one enantiomer from the binding sites by the other enantiomer. The result is a higher unbound fraction for each enantiomer when testing the racemate compared to those which are obtained when each enantiomer is examined separately (MacKichan, 1992).

Stereoselective binding in serum (Walle *et al.*, 1983, Albani *et al.*, 1984, Colangelo *et al.*, 1989), in isolated α_1-acid glycoprotein (Walle *et al.*, 1983, Albani *et al.*, 1984, Oravcová *et al.*, 1989), and in isolated human serum albumin (Walle *et al.*, 1983) has been reported for propranolol. Slight differences were observed in the binding of pirprofen enantiomers to human serum albumin (Oravcová *et al.*, 1991b). Stereoselective binding was also reported for disopyramide (Lima *et al.*, 1984), chloroquine (Ofori-Adjei *et al.*, 1986), verapamil (Gross *et al.*, 1988), ibuprofen (Evans *et al.*, 1989) and methadone (Eap *et al.*, 1990).

Oravcová *et al.* (1991a) observed stereoselective binding of propafenone enantiomers to α_1-acid glycoprotein which was enhanced with larger total binding. The

authors attributed the difference in binding to a competitive inhibition of binding of the enantiomers at a single binding site on α_1-acid glycoprotein (Oravcová *et al.*, 1991a).

1.5 Hypothesis

It is hypothesized that derivatization of carvedilol with a fluorogenic reagent will increase the sensitivity of the determination of the drug in serum.

It is also hypothesized that, as for other basic drugs, α_1-acid glycoprotein is the major binding protein for carvedilol enantiomers and that the binding is stereoselective.

1.6 Thesis Objectives

Carvedilol is a high clearance drug and the concentrations achieved in plasma after therapeutic doses are very low. Maximum concentrations in plasma after an oral dose of 50 mg are of the order of 20 ng/ml and 80 ng/ml for (-)-(S)- and (+)-(R)-carvedilol, respectively. The analytical methods reported in the literature so far can detect quantities as low as 0.5 ng/ml of each carvedilol enantiomer in plasma. The assays consist of derivatization of the drug with an optically pure derivatization reagent to form diastereomers and subsequent analysis by conventional HPLC. There are two major studies on the stereoselective pharmacokinetics of carvedilol in humans. One is the study done by Neugebauer *et al.* (1990) which used derivatization of carvedilol with GITC. The other study was performed by Spahn *et al.* (1990) and involved the derivatization of carvedilol with the fluorogenic reagent (+)-(S)-naproxen chloride. Neither of these methods was sufficiently sensitive to adequately describe the terminal elimination phases of the enantiomers. Discrepancies in some of the pharmacokinetic parameters reported are observed. The terminal elimination half-lives reported by

Neugebauer *et al.* (1990) were 9.6 and 22.1 hours for (+)-(R)- and (-)-(S)-carvedilol, respectively, while those reported by Spahn *et al.* (1990) were 3.3 and 5.6 hours. Both studies used a 50 mg oral dose of racemic carvedilol. By examination of the elimination curves it is evident that the elimination rate constants were determined using only 3-4 data points.

It is possible that the disparity in reported values for some of the pharmacokinetic parameters of carvedilol enantiomers, namely half-life and clearance, are due, in part, to inadequate sensitivity of the current analytical methods. It is believed that a sensitive and stereoselective assay can be developed that will enable a more accurate determination of the stereoselective pharmacokinetics of carvedilol enantiomers.

Furthermore, the stereoselective aspects of protein binding of carvedilol to serum proteins have not been completely investigated. Pharmacological activity is correlated to unbound drug present in plasma. Considering the fact that carvedilol enantiomers exhibit distinct pharmacological actions, information on the extent and characteristics of the protein binding will provide a more thorough understanding of the stereoselective drug-protein interactions which could influence the drug's ultimate therapeutic actions.

Therefore the objectives of this study were:

- To develop a highly sensitive high-performance liquid chromatographic assay for the determination of carvedilol enantiomers in serum.
- To demonstrate the applicability of capillary electrophoresis for the determination of carvedilol enantiomers in serum.
- To compare the capillary electrophoresis and the liquid chromatographic assays.
- To determine the stereoselective protein binding of carvedilol enantiomers in serum.
- To identify the proteins involved in binding of carvedilol enantiomers in serum.
- To determine the existence of concentration-dependent binding of carvedilol in serum and in isolated serum protein solutions.
- To compare the binding of carvedilol enantiomers to normal and altered levels of serum proteins.

1.7 Rationale

1. To develop a highly sensitive high-performance liquid chromatographic assay for the determination of carvedilol enantiomers in serum.

The disparity in the pharmacokinetic parameters for carvedilol reported in the literature point to a potential analytical problem: limited sensitivity of the assay to quantify very low concentrations of carvedilol enantiomers after therapeutic doses. The most sensitive assays involve detection of the native fluorescence of carvedilol or the fluorogenic product formed after derivatization with (+)-(S)-naproxen chloride. None of these methods has been sufficiently sensitive to adequately describe the terminal

elimination phases. In fact Neugebauer *et al.* (1990) admitted that in 4 of 10 subjects studied the concentration of (-)-(S)-carvedilol reached the quantitation limits too early and not enough data points were available for a reliable calculation of the half-life. Thus, the need for a more sensitive stereoselective assay for the determination of carvedilol pharmacokinetics is evident. In previous investigations in our laboratory the sensitivity for the analysis of free and total levels of mexiletine enantiomers (a primary amine) was increased several fold with the use of fluorogenic derivatizing reagents such as 2-naphthoyl chloride and 2-anthroyl chloride (McErlane *et al.*, 1987, Kwok *et al.*, 1994). The derivatization of carvedilol with fluorogenic reagents will therefore be investigated to determine if the secondary amine group in carvedilol can be reacted with the same reagents in a quantitative and reproducible manner.

2. To demonstrate the applicability of capillary electrophoresis for the determination of carvedilol enantiomers in serum.

The resolution power of capillary electrophoresis (CE) has now been widely recognized as a valuable alternative to conventional analytical methods for the analysis of drug enantiomers. Compared to HPLC, it offers the advantages of faster method development, higher efficiencies, and lower consumption of solvents and reagents. In addition, chiral separations by CE using cyclodextrins are relatively less expensive than HPLC using chiral stationary phases which are costly and have limited lifetimes. However, the potential of CE for the determination of drugs in biological material has not been completely explored. There are only a few reports in the literature on the use of CE for analysis of drugs in biological fluids. Thus, the use of CE will be assessed as

a potential alternative to HPLC for the stereoselective analysis of carvedilol enantiomers in serum and its application for pharmacokinetics studies with this drug.

3. To compare the capillary electrophoresis and the liquid chromatographic assays.

Capillary electrophoresis will be compared to the HPLC method developed for the determination of carvedilol enantiomers in serum. Since HPLC is a standard analytical technique agreement between the results obtained with the two techniques will indicate that CE is a practical method for the stereoselective analysis of carvedilol.

4. To determine the stereoselective protein binding of carvedilol enantiomers in serum.

Carvedilol was reported to be highly protein bound (> 95%) in human plasma (Ruffolo Jr. *et al.*, 1992). There is only one report on the stereoselective protein binding of carvedilol enantiomers in humans. Using a single concentration (1 μg/ml of each enantiomer) Fujimaki *et al.* (1990) dialyzed human plasma containing carvedilol against phosphate buffer for only 2 hours at room temperature. The free fraction of (-)-(S)-carvedilol was reported to be 1.4 times greater than (+)-(R)-carvedilol. The coefficients of variation obtained by these authors were extremely high (111% for (-)-(S)- and 44% for (+)-(R)-carvedilol). This could be due to the short dialysis time employed by these authors. Alternatively, the high variability encountered could be again a result of limited sensitivity of the assay which explored the native fluorescence of carvedilol after derivatization with GITC. It is believed that the stereoselective protein binding of carvedilol in humans has not been accurately determined and will

therefore be reexamined. The use of alternative methods for free fraction determination such as ultrafiltration will be investigated. The extent of binding of carvedilol enantiomers to serum proteins will also be measured by equilibrium dialysis after determination of the time to reach equilibrium.

5. To identify the proteins involved in binding of carvedilol enantiomers in serum.

In a study of the protein binding of carvedilol enantiomers in rats it was determined that the S/R ratio of unbound drug to albumin was 0.79 which was lower than the unbound fraction of 1.57 determined in control plasma (Stahl *et al.*, 1993a, Stahl *et al.*, 1993c). The results suggest that albumin does not account for all the binding in plasma and it was suggested by these authors that it was likely that other proteins were involved in the stereoselective binding of carvedilol. The binding of carvedilol to isolated solutions of human serum albumin, α_1-acid glycoprotein, and lipoprotein-deficient serum will be evaluated in order to determine the identities of the proteins responsible for the stereoselective binding of carvedilol enantiomers.

6. To determine the existence of concentration-dependent binding of carvedilol in serum and in isolated serum protein solutions.

It has been reported that the binding of racemic carvedilol was independent of drug concentration over the 50 to 1000 ng/ml concentration range (Ruffolo Jr. *et al.*, 1992). However the authors do not describe how this information was obtained. The free fractions of carvedilol enantiomers in serum and in isolated protein solutions will be evaluated over a range of drug concentrations. The results will indicate whether the

unbound fraction of (-)-(S)- and (+)-(R)-carvedilol varies with an increase in drug concentration due to saturation of the binding sites.

7. To compare the binding of carvedilol enantiomers to normal and altered levels of serum proteins.

Disease states such as myocardial infarction, severe burns, trauma or surgery result in altered plasma concentration of α_1-acid glycoprotein. The increased levels of the reactant protein α_1-acid glycoprotein in these states can result in altered protein binding of drugs. Since the enantiomers of carvedilol exhibit different pharmacological activity, changes in the S/R ratio of unbound drug may elicit an unexpected pharmacological response. Therefore information is needed on the effect of altered levels of α_1-acid glycoprotein on the binding of carvedilol enantiomers. The binding of carvedilol to buffered protein solutions simulating the normal levels of the two major binding proteins, albumin and α_1-acid glycoprotein will be examined as well as the binding to a protein solution with increased concentration of the reactant protein α_1-acid glycoprotein.

2. MATERIAL AND METHODS

2.1 Materials and Supplies

2.1.1 Chemicals and Reagents

(±)-Carvedilol, (+)-(R)-carvedilol, and (-)-(S)-carvedilol were kindly donated by Boehringer Mannheim GmbH (Mannheim, Germany). (-)-Propranolol was obtained from Ayerst Laboratories (Montreal, PQ). All chemicals and reagents were of analytical grade. Trifluoroacetic acid, trichloroacetic acid, triethylamine, and sodium phosphate (mono and dibasic) were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium hydroxide and toluene were from Fisher Scientific (Nepean, ON). Dimethyldichlorosilane, tris(hydroxymethyl)aminomethane (TRIZMA base), and hexadecyltrimethylammonium bromide (HTAB) were from Sigma (St. Louis, MO). 2-Naphthoyl chloride, (+)-(S)-1-(1-naphthyl)ethyl isocyanate, oxalyl chloride, thionyl chloride, anthraquinone-2-carboxylic acid, 4-dimethylamino pyridine, N,N-dimethyloctylamine, and tetrahydrofuran were purchased from Aldrich Chem. Co. (Milwaukee, WI). Zinc powder was obtained from BDH Chemicals Ltd. (Poole, England). Triton X-100 was purchased from BIO-RAD Laboratories (Hercules, CA) and sodium dodecyl sulphate was from BDH Chemicals (Toronto, ON).

2.1.2 Solvents

Ethyl alcohol, reagent, denatured (5% v/v isopropyl alcohol), HPLC grade, was from Sigma (St. Louis, MO). Acetonitrile, hexane, methanol, and dichloromethane, HPLC grade, ethyl ether and toluene were obtained from Fisher Scientific (Nepean,

ON). 2-Propanol, HPLC grade, was purchased from Fisher Scientific (Fair Lawn, NJ). Deionized water was obtained with a Milli-Q water purification system (Millipore, Montreal, PQ).

2.1.3 Glassware

For protein binding studies all glassware that was in contact with carvedilol was silanized with a 10% solution of dimethyldichlorosilane in toluene and rinsed with methanol before use.

2.1.4 Chromatographic Columns

An Ultrasphere C_{18} column, ion pair, 250 mm x 4.6 mm I.D., 5 μm, was obtained from Beckman (San Ramon, CA). An Econosphere silica column, 150 mm x 4.6 mm I.D., 5 μm, was from Alltech, (Deerfield, Il). The chiral HPLC columns, (S)-indoline-2-carboxylic acid and (R)-1-(α-naphthyl)ethylamine (3022), analytical (250 mm x 3.2 mm I.D.) and starter (50 mm x 4.6 mm I.D.), were purchased from Phenomenex (Torrance, CA). A Pirkle-type column 1-A, HI-CHROM, 250 mm x 4.6 mm I.D., 5 μm, was obtained from Regis (Morton Grove, Il). The Chiralcel OD column, 250 x 4.6 mm I.D., was purchased from Daicel Chemical Industries (New York, NY).

2.1.5 Supplies for Capillary Electrophoresis

Fused silica capillaries, uncoated, 57 cm length (50 cm effective length), 75 μm I.D. were purchased from Beckman (Palo Alto, CA). Hydroxypropyl-β-cyclodextrin, dimethyl-β-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin were also obtained from

Beckman (Palo Alto, CA). Heptakis ((2,3,6-tri-O-methyl)-β-cyclodextrin) was purchased from Sigma (St. Louis, MO). The capillary cartridge coolant consisted of perfluoro compounds, C_{5-18}, and was obtained from Beckman (Fullerton, CA). Samples were filtered through 0.2 μm Acrodisc LC13 PVDF filters (Gelman, Ann Arbor, MI) prior to injection into the capillary electrophoresis apparatus.

2.1.6 Supplies for Serum Protein Binding Studies

Blood was collected in additive-free Vacutainer tubes, silicone-coated interior, with silicone-lubricated stoppers from Becton Dickinson (Franklin Lakes, NJ). Ultrafiltration was performed in a Centrifree Micropartition System from Amicon (Danvers, MA) using a YMT membrane with 14 mm diameter and molecular weight cut-off of 30,000 Daltons. Millipore units Ultrafree-CL (polysulfone membranes and low-binding regenerated cellulose membranes) with 10,000 and 30,000 Daltons cut-off were obtained from Millipore Corporation (Bedford, MA). Equilibrium dialysis units, with 1 ml cells, were manufactured from Plexiglas by 3-Dimension Plastic (Vancouver, BC). Cellulose tubing from Sigma (St. Louis, MO) with a molecular weight cut off of 12,000 Daltons was used. Human serum albumin, fraction V, human α_1-acid glycoprotein (orosomucoid), and lipoprotein-deficient human serum, lyophilized, were purchased from Sigma (St. Louis, MO). The dialysis buffer consisted of 0.067 M isotonic phosphate buffer, pH 7.4 (1.7868 g potassium phosphate, monobasic; 14.35 g sodium phosphate dibasic heptahydrate; 3.9 g sodium chloride in 1000 ml deionized water).

2.2 Equipment

2.2.1 HPLC

Chromatographic analyses were performed using a Tosohaas TSK-6010 HPLC precision pump, a Shimadzu SIL-9A autoinjector (Kyoto, Japan), a Shimadzu RF-551 fluorescence detector (Kyoto, Japan), and a CR501 Chromatopac Shimadzu integrator (Kyoto, Japan).

2.2.2 LC/MS

LC/MS and MS/MS with flow injection was performed on a HP Series II 1090 Liquid Chromatograph (Hewlett Packard, Avondale, PA), coupled to a VG QUATTRO triple quadrupole Mass Spectrometer equipped with an electrospray interface (Fisons Instruments, Altrincham, Cheshire, England).

2.2.3 Capillary Electrophoresis

Capillary electrophoresis was performed on a P/ACE System 5000 instrument (Beckman Instruments, Fullerton, CA) equipped with a UV detector with 4 filters. Data was collected and analyzed on a 486/66 Hz computer (Dell Computer Corporation, Austin, TX) equipped with System Gold Chromatography Software, version 8.10 (Beckman Instruments, Fullerton, CA).

2.2.4 Spectrofluorometer

Fluorescence scans of carvedilol were obtained on a Shimadzu Spectrofluorophotometer RF-540 (Kyoto, Japan).

2.2.5 Centrifuges

Ultrafiltration experiments were performed using a Beckman J2-21 centrifuge (Beckman Instruments, Irvine, CA) with a 35 degree angle rotor head JA-17, and a microcentrifuge Eppendorf 5415C (Brinkmann Instruments, Westbury, NY). A GP Centrifuge (Beckman Instruments, Palo Alto, CA) was used for blood centrifugation and extraction procedures.

2.3 Stereoselective HPLC Assay for Carvedilol Enantiomers

2.3.1 Derivatization of Carvedilol with Fluorescent Reagents

2.3.1.1 Derivatization with Naphthoyl Chloride

Derivatization with naphthoyl chloride was used by McErlane *et al.* (1987) for the determination of mexiletine enantiomers in serum using a chiral stationary phase. The naphthoyl derivatives of carvedilol were prepared by vortex-mixing 0.1 ml (1.0 μg) of a 10 μg/ml acidic aqueous solution of racemic carvedilol with 0.2 ml 2 M NaOH and 15 μl of naphthoyl chloride solution (1 mg/ml in dichloromethane) for 2 min at room temperature. The derivatives were extracted with 0.6 ml of 5.5% v/v 2-propanol in hexane and 20 μl of the organic phase were injected into the HPLC. The column used was a HI-CHROM dinitrobenzoylphenyl glycine, Pirkle type, ionic (Regis, Morton Grove, IL) and the mobile phase consisted of 5.5% 2-propanol in hexane delivered at a flow rate of 1.4 ml/min. The reaction was also tested using temperature (60 °C for 0.5, 1 and 2 hours). Derivatization was also performed with carvedilol dissolved in dichloromethane using triethylamine as a base catalyst. The detection was by

fluorescence at 230 nm (EX) and 370 nm (EM). The derivatization reaction was tested with racemic mexiletine as a means of validating the conditions.

The reactions were monitored by thin layer chromatography (TLC) using plates coated with silica gel G-25 UV_{254} (Macherey-Nagel, Düren, Germany). The eluent used was toluene:dichloromethane:tetrahydrofuran (5:3:2, v/v/v).

The presence of unreacted carvedilol after derivatization was assessed using a Chiralcel OD column (Daicel, New York, NY). The mobile phase was hexane:ethanol:dimethyloctylamine (50:50:0.1, v/v/v), at a flow rate of 0.55 ml/min, and fluorescence detection at 285 nm (EX) and 355 nm (EM). Carvedilol enantiomers eluted as a pair of peaks, partially resolved at 23.3 and 24.8 min. Dilutions of the reaction mixture and the corresponding starting carvedilol amount were prepared and injected into the chiral column. Remaining carvedilol was determined by comparing peak areas obtained from the reaction mixture with those obtained after injection of carvedilol not submitted to derivatization.

2.3.1.1.1 Mass Spectrometric Analysis of the Naphthoyl Derivatives of Carvedilol

In order to elucidate the structure of the derivatives formed after derivatization of racemic carvedilol with naphthoyl chloride, liquid chromatographic/mass spectrometric analysis (LC/MS) of the products was performed. The LC system consisted of a HP Series II 1090 Liquid Chromatograph (Hewlett Packard, Avondale, PA) equipped with a Beckman ODS column, 250 x 4.6 mm I.D.. The mobile phase was acetonitrile:water (90:10, v/v), delivered at a flow rate of 0.5 ml/min. The MS system consisted of a VG QUATTRO triple quadrupole Mass Spectrometer equipped with an electrospray

interface (Fisons Instruments, Altrincham, Cheshire, England). The reaction mixture was injected into the column and the molecular weight of the products determined.

MS/MS analysis with flow injection was performed with samples extracted from TLC plates of the reaction mixture which were believed to be derivatization products. Samples were injected into the mass spectrometer via the HPLC system described and daughter ions of the derivatives were obtained.

2.3.1.2 Derivatization with Anthroyl Chloride

Anthroyl chloride was synthesized according to Kwok *et al.* (1994). The first step involved the reduction of anthraquinone-2-carboxylic acid with zinc in aqueous ammonia solution. The resulting 2-anthracene carboxylic acid was isolated and dissolved in dry dichloromethane. Oxalyl chloride was added and the mixture refluxed for 1.5 hours. The acid chloride was purified by recrystalization from a dichloromethane solution by addition of hexane.

Derivatization of carvedilol (5 μg) with anthroyl chloride (3.3 μg, 10x excess) was performed in dichloromethane at room temperature or at 70 °C for 2 hours. Different base catalysts (such as triethylamine and 4-dimethylaminopyridine) were tested. Derivatives were analyzed using a Phenomenex 3022 column, 50 mm x 4.6 mm I.D. (Torrance, CA), containing (S)-indoline-2-carboxylic acid and (R)-1-(α-naphthyl)ethylamine as the stationary phase. The mobile phase was hexane: dichloromethane: HPLC grade ethanol (60:30:10, v/v/v), and 0.5 % trifluoroacetic acid, delivered at a flow rate of 0.3 ml/min. The detection was by fluorescence at 285 nm (EX) and 355 nm (EM).

2.3.1.3 Derivatization with (+)-(S)-Naproxen Chloride

(+)-(S)-Naproxen was obtained by extracting tablets (Apo-naproxen, 375 mg) with methanol. The solvent was evaporated and the residue was recrystallized from acetone by the addition of hexane. The crystals were filtered under vacuum and dried in air. The melting point of the material obtained was 151-152 °C (Merck Index: 152-154 °C). The derivatizing reagent (+)-(S)-naproxen chloride was obtained according to Spahn (1988) by reacting (+)-(S)-naproxen dissolved in toluene with thionyl chloride with reflux for 1 hour. The solvent was evaporated under vacuum. Infrared spectra of the starting material and of the product were obtained.

Carvedilol (28 μg) was allowed to react with 0.06 mg of (+)-(S)-naproxen chloride in 0.2 ml dichloromethane for 1 hour at 50 °C, following the procedure used by Spahn *et al.* (1990) for the derivatization of carvedilol. The solvent was evaporated and the residue was reconstituted in mobile phase for injection into the HPLC. A silica column, Econosphere silica, 5 μm (Alltech, Deerfield, Il) was used to analyze the diastereomers formed, with a mobile phase consisting of hexane:dichloromethane: ethanol (120:28:1.7, v/v/v), at a flow rate of 0.9 ml/min. The detection was by fluorescence at 285 nm (EX) and 355 nm (EM). The reaction was also monitored by TLC using the system described in section 2.3.1.1.

2.3.1.4 Derivatization with (+)-(S)-1-(1-Naphthyl)ethyl Isocyanate

The derivatization with (+)-(S)-1-(1-naphthyl)ethyl isocyanate (NEIC) was performed in dry acetonitrile, dichloromethane and methanol, at room temperature or at 75 °C for 1 hour. Five micrograms of racemic carvedilol were allow to react with 12 μg (5x excess) of NEIC. The diastereomers formed were analyzed by HPLC using an

Econosphere silica column, 150 mm x 4.6 mm I.D., 5 μm (Alltech, Deerfield, Il). The mobile phase consisted of hexane:dichloromethane:ethanol (120:28:2, v/v/v), with a flow rate of 1 ml/min. The detection was by fluorescence at 285 nm (EX) and 355 nm (EM).

In this system, unreacted carvedilol cannot be detected since it is retained by the silica column. Therefore the completeness of the derivatization reaction was examined using an Ultrasphere C_{18} column, ion pair, 250 mm x 4.6 mm I.D., 5 μm (Beckman, San Ramon, CA). The mobile phase used was methanol:0.2% phosphoric acid (70:30, v/v), at a flow rate of 0.5 ml/min. The peak areas of carvedilol enantiomers after reaction were compared to those obtained by injection of equivalent amounts of drug without undergoing derivatization.

2.3.1.4.1 Mass Spectrometric Analysis of the Reaction of Carvedilol with (+)-(S)-1-(1-Naphthyl)ethyl Isocyanate

The derivatization reaction of carvedilol with (+)-(S)-1-(1-naphthyl)ethyl isocyanate was analyzed by mass spectrometry with flow injection. The MS system consisted of a VG QUATTRO triple quadrupole Mass Spectrometer equipped with an electrospray interface (Fisons Instruments, Altrincham, Cheshire, England). The reaction mixture was injected into the MS and the molecular weight of the products determined.

2.3.2 Direct Resolution of Carvedilol Enantiomers by HPLC Using a Chiral Stationary Phase

2.3.2.1 Extraction Procedure

Serum samples or retentate samples (0.2 ml) were treated with 0.2 ml 10% trichloroacetic acid to precipitate the proteins, and subsequently basified with 0.2 ml 1 M sodium hydroxide. The internal standard (100 µl of a 4 µg/ml solution of (-)-propranolol in HPLC grade ethanol) was added. The samples were extracted twice with 3 ml ethyl ether and vortex-mixed for 2 min. After evaporation of the extracts under nitrogen, the samples were reconstituted in 0.5 ml HPLC grade ethanol for injection into the HPLC.

Buffer samples and dialysate samples (0.85 ml) were basified with 0.2 ml 1 M sodium hydroxide. Fifty microlitres of a 0.4 µg/ml solution of (-)-propranolol in HPLC grade ethanol was used as internal standard. Samples were then submitted to extraction with ethyl ether (2 x 3 ml, 2 min vortex-mixing) and the organic phase was evaporated to dryness under nitrogen. The residue was reconstituted in 0.1 ml HPLC grade ethanol for injection into the HPLC.

2.3.2.1.1 Determination of Extraction Recovery of Carvedilol from Serum and from Dialysis Buffer

The extraction recoveries of carvedilol enantiomers in serum were determined at 100 and 1000 ng/ml per enantiomer. Three replicate samples at each concentration were submitted to the extraction procedure described in section 2.3.2.1. The internal standard solution was then added, and samples were analyzed by the HPLC method

described in section 2.3.2.3. Peak area ratios enantiomer/internal standard were compared to those obtained by the analysis of identical amounts of carvedilol and internal standard not submitted to the extraction procedure.

Recovery of drug from dialysis buffer was determined in triplicate at 10 and 100 ng/ml per enantiomer in the same fashion.

2.3.2.2 HPLC Analysis of Carvedilol Enantiomers Using a Chiralcel OD Chiral Stationary Phase

The Chiralcel OD column (Daicel, New York, NY) was used for the analysis of carvedilol enantiomers. The mobile phase consisted of hexane:ethanol: dimethyloctylamine (50:50:0.1, v/v/v) delivered with a flow rate of 0.55 ml/min. The detection was by fluorescence at 285 nm (EX) and 355 nm (EM).

2.3.2.3 HPLC Analysis of Carvedilol Enantiomers Extracted from Serum Using a Phenomenex 3022 Chiral Stationary Phase

Carvedilol enantiomers were extracted from serum, serum retentate, or dialysate buffer according to section 2.3.2.1. The chiral chromatographic assay used (S)-indoline-2-carboxylic acid and (R)-1-(α-naphthyl)ethylamine (Phenomenex 3022, Torrance, CA) as the stationary phase. The mobile phase consisted of hexane:dichloromethane:HPLC grade ethanol (50:35:15, v/v/v), and 0.25% (v/v) trifluoroacetic acid at a flow rate of 0.55 ml/min. The injection volume was 10 μl. The internal standard used was (-)-propranolol and peak area ratios were used for quantitations. The detection was by fluorescence. The optimum excitation and emission wavelengths were determined by scanning the excitation and emission

spectra of a carvedilol solution in mobile phase. Maximum fluorescence signal was obtained at 284 nm for excitation and 343 nm for emission. The elution order of the enantiomers was determined by injecting a solution of the racemate enriched with each enantiomer separately. The first peak to elute from the column was determined to be (+)-(R)-carvedilol, followed by (-)-(S)-carvedilol.

2.3.2.3.1 Determination of Detector Linearity for Carvedilol Enantiomers in Serum

Calibration samples of carvedilol in serum were prepared in duplicate using stock solutions of racemic carvedilol in water (pH 2) at 104 ng/ml and 1040 ng/ml. The solutions were added to serum to obtain, at the low concentration range, final concentrations of 1, 2.6, 7.3, 15.6, 31.2, and 62.4 ng/ml of each enantiomer. Fifty microlitres of internal standard solution (0.4 μg/ml (-)-propranolol in HPLC grade ethanol) were added to the samples (0.5 ml) which were then submitted to protein precipitation, basification and extraction with ethyl ether. The extracts were evaporated to dryness under nitrogen and reconstituted in 0.1 ml HPLC grade ethanol for injection.

At the high concentration range, racemic carvedilol (100 μg/ml in water, pH 2) was added to serum to obtain final concentrations of 1, 2, 3, 4, and 5 μg/ml of each enantiomer. An aliquot (100 μl) of internal standard solution (4 μg/ml (-)-propranolol in HPLC grade ethanol) was added to the samples, which were then submitted to protein precipitation and extraction as described in section 2.3.2.1.

Samples were analyzed by the chiral HPLC method described in section 2.3.2.3. Results were expressed as peak area ratios of each enantiomer to the internal standard *versus* enantiomer concentrations in serum.

2.3.2.3.2 Determination of Detector Linearity for Carvedilol Enantiomers in Dialysis Buffer

Calibration samples of carvedilol were prepared in duplicate, by dilutions of a stock solution of racemic carvedilol (100 μg/ml in water, pH 2) to obtain final concentrations of 10, 25, 50, 100, and 200 ng/ml of each enantiomer in dialysis buffer. An aliquot (50 μg/ml) of internal standard solution (0.4 μg/ml (-)-propranolol in HPLC grade ethanol) was added to the samples, which were then submitted to extraction as described in section 2.3.2.1. Samples were analyzed by the chiral HPLC method described in section 2.3.2.3. Results were expressed as peak area ratios of each enantiomer to the internal standard *versus* enantiomer concentrations in dialysis buffer.

2.3.2.3.3 HPLC Intra- and Inter-Assay Variability

The reproducibility of the HPLC assay for the determination of carvedilol enantiomers in serum and dialysis buffer using a chiral column (Phenomenex 3022) was determined. Serum was adjusted to pH 7.4 with sodium phosphate salts (5.2 mg sodium phosphate monobasic monohydrate and 43.4 mg sodium phosphate dibasic heptahydrate in 1 ml of serum). Racemic carvedilol (50 μg/ml) was added to serum adjusted to pH 7.4 to obtain final concentrations of 2 and 4 μg/ml per enantiomer. Three replicates of 0.2 ml of serum were extracted as described in section 2.3.2.1 and analyzed by the HPLC method described in 2.3.2.3. For the analysis in dialysis buffer, racemic carvedilol (5 μg/ml) was added to isotonic phosphate buffer, pH 7.4, to obtain final concentrations of 10 and 100 ng/ml of each enantiomer. Three replicates of 0.85 ml of buffer containing carvedilol were extracted as described in section 2.3.2.1 and analyzed by the HPLC method described in 2.3.2.3. Peak area ratios of carvedilol

enantiomer/internal standard were calculated. Intra-assay variability was determined in terms of coefficient of variation (C.V.) in percent of the 3 determinations. Inter-assay variability was obtained by performing the same determinations on three different days.

2.4 Development of a CE Assay for Carvedilol Enantiomers in Serum

The electrophoretic analyses were performed using a 75 μm I.D. fused silica capillary, 57 cm (50 cm to the detector) in length. The background electrolyte consisted of phosphate buffer, pH 2.5, and cyclodextrins as the chiral selectors. Samples were injected into the capillary by pressure, for 4 s. The electrophoresis was carried out by applying high voltage to the capillary, with the cathode being at the detector end. The cartridge temperature was maintained constant by a liquid coolant containing a fluoroorganic fluid (perfluoro compounds, $C_{5\text{-}18}$, Beckman, Fullerton, CA). The capillary was washed between runs with a sequence of rinses: 0.1 M sodium hydroxide (1 min), water (0.5 min), 0.1 M hydrochloric acid (0.5 min), and water (0.5 min), to ensure reproducibility of the assay. Before sample injection, the capillary was rinsed with the run buffer for 1 min. Detection was by UV at 200 nm. Buffer concentration, pH, type, and concentration of cyclodextrin, as well as applied voltage were optimized. The appropriate UV filter was selected and the optimum cartridge temperature determined. The internal standard used was (-)-propranolol. The migration order of the enantiomers was determined by injecting a solution of the racemate enriched with each enantiomer separately. The first peak to pass the detector window was determined to be (+)-(R)-carvedilol, followed by (-)-(S)-carvedilol. Corrected peak area ratios (Introduction, section 1.3.5.2) were used for quantitations.

2.4.1 Choice of Chiral Selector

Several types of cyclodextrins (native and derivatized forms) in different concentrations were tested in order to obtain separation of carvedilol enantiomers. A stock solution of racemic carvedilol (10 μg/ml) in ethanol was injected into the capillary. The cyclodextrins and concentrations examined were: γ-cyclodextrin, at 10 and 50 mM, β-cyclodextrin, at 3 and 15 mM, dimethyl-β-cyclodextrin, at 5 and 20 mM, trimethyl-β-cyclodextrin, at 10 mM, and hydroxypropyl-β-cyclodextrin, at 10 and 50 mM.

2.4.2 Effect of Cyclodextrin Concentration on Resolution

Resolution of the enantiomers was compared using various concentrations of cyclodextrin in the run buffer. Phosphate buffers containing 5, 10, 20, and 50 mM of hydroxypropyl-β-cyclodextrin were prepared, and capillary electrophoresis of racemic carvedilol in ethanol was performed. Resolution factors (R_s) for the enantiomers were calculated and the optimal cyclodextrin concentration for the analysis determined.

2.4.3 Effect of Voltage on Efficiency

The effect of the applied voltage on the efficiency of separation of carvedilol enantiomers by capillary electrophoresis was examined over the 10-22 kV range. A solution of racemic carvedilol in ethanol was used for the injections. Efficiency of the assay, expressed as the number of theoretical plates (N) for the peak corresponding to (+)-(R)-carvedilol (first enantiomer detected), was calculated using the formula:

$$N = 16 (l / w)^2$$

where:

N : number of theoretical plates

l : migration distance

w : peak width

A plot of N *versus* voltage was constructed and the optimum voltage identified.

2.4.4 Effect of Buffer Concentration on Efficiency

The effect of buffer concentration on the separation efficiency of carvedilol enantiomers was assessed by performing the electrophoretic separation using phosphate buffer, pH 2.5, at 10, 15, 20, 25, and 50 mM, with 10 mM hydroxypropyl-β-cyclodextrin as the chiral selector.

2.4.5 Effect of Cartridge Temperature on Resolution

The temperature inside the capillary cartridge can affect separation in capillary electrophoresis because it affects solute mobility and the electroosmotic flow (EOF) (Altria, 1996). At the operating pH of 2.5, EOF is minimal. Therefore temperature will solely affect electrophoretic mobility. In order to determine the optimum temperature for the resolution of carvedilol enantiomers, electrophoretic runs at different cartridge temperatures (15, 18, 20, and 25 °C) were performed, and the separation of the enantiomers examined.

2.4.6 Optimized CE Assay for Carvedilol Enantiomers in Serum

Serum containing carvedilol was adjusted to pH 7.4 with sodium phosphate salts (5.2 mg sodium phosphate monobasic monohydrate and 43.4 mg sodium phosphate dibasic heptahydrate in 1 ml of serum). An aliquot (200 μl) of internal standard solution (10 μg/ml of (-)-propranolol in HPLC grade ethanol) was added. The samples (0.7 ml) were submitted to precipitation of proteins with 0.4 ml 10% trichloroacetic acid, basified with 0.4 ml 1 M sodium hydroxide and subsequently extracted with 2 x 3 ml ethyl ether and vortex-mixing for 2 min. The extracts were evaporated to dryness under nitrogen. The residues were reconstituted in 200 μl HPLC grade ethanol and filtered through 0.2 μm Acrodisc LC13 PVDF filters (Gelman, Ann Arbor, MI) for injection into the capillary. Studies have shown no difference between the corrected peak area ratios carvedilol enantiomer/internal standard for filtered and non-filtered solutions. The optimized electrophoretic conditions were: background electrolyte: 25 mM phosphate buffer, pH 2.5, 10 mM hydroxypropyl-β-cyclodextrin; voltage: 18 kV; capillary: fused silica, uncoated, 57 cm length (50 cm to detector), 75 μm I.D.; cartridge temperature: 20 °C; injection: pressure, 4 s; detection: UV at 200 nm.

2.4.6.1 Determination of Detector Linearity for Carvedilol Enantiomers in Serum

Detector linearity for the CE assay was assessed in the serum concentration range of 50-4000 ng/ml per enantiomer. Samples were prepared in duplicate. Racemic carvedilol was added to serum (pH 7.4) to obtain final concentrations of 50, 100, 250, 500, 1000, 2000, and 4000 ng/ml of each enantiomer. The internal standard was added and samples were extracted and analyzed as described in section 2.4.6.

A calibration curve was constructed by plotting corrected peak area ratios of carvedilol enantiomers/internal standard *versus* the corresponding enantiomer concentration.

A higher concentration range of 1, 2, 3, 4, and 5 μg/ml of each carvedilol enantiomer (used for protein binding studies) was also tested.

2.4.6.2 Determination of the Reproducibility of the CE Assay

Calibration curves consisting of carvedilol enantiomers concentrations of 50, 100, 500, 1000, and 2000 ng/ml in serum were prepared in duplicate. Five replicate samples of carvedilol in serum at concentrations of 100 and 1000 ng/ml were also prepared. Samples (0.7 ml) were extracted and analyzed by the optimized CE method as described in section 2.4.6. Graphs of corrected peak area ratios of enantiomer/internal standard *versus* enantiomer concentration in serum were constructed and used to calculate carvedilol concentration in the samples. Intra-assay variability was expressed in terms of coefficient of variation (C.V.) of the determination, in percent, for the 5 replicates at each drug concentration. Inter-assay reproducibility was assessed by performing the same analysis described above on 3 different days.

2.5 Comparison Between the HPLC and the CE Assays

Repeated calibration curves of carvedilol enantiomers in serum in concentrations of 1, 2, 3, 4, and 5 μg/ml per enantiomer were prepared. Samples for HPLC analysis (0.2 ml) were extracted as described in section 2.3.2.1. Extracts were then analyzed using the HPLC method described in section 2.3.2.3. Samples for CE analysis (0.7 ml) were extracted and analyzed as described in section 2.4.6. The slopes and intercepts obtained for each calibration curve were used to calculate the

concentration of carvedilol enantiomers in each sample. A graph of the concentrations obtained by CE *versus* those obtained by the HPLC method was constructed and the correlation coefficient determined.

2.6 *In vitro* Serum Protein Binding of Carvedilol Enantiomers

2.6.1 Determination of Nonspecific Binding of Carvedilol to Centrifree Ultrafiltration Units

Serum ultrafiltrate was obtained by ultrafiltration of blank serum adjusted to pH 7.4 using the Centrifree Micropartition filtration units (Amicon, Danvers, MA) with YMT membranes, at 1600 x g for 20 min at room temperature. Racemic carvedilol (2 μg/ml in isotonic phosphate buffer, pH 7.4) was added to the ultrafiltrate obtained to achieve a final concentration of 100 ng/ml of each enantiomer. Triplicate 0.5 ml samples were added to the ultrafiltration units, left in contact for 60 min and subsequently centrifuged as described above. Aliquots of 0.45 ml of the filtrate were collected for analysis. The internal standard (60 μl of a 0.35 μg/ml solution of (-)-propranolol in ethanol) was then added. The solution was basified with 200 μl 1 M NaOH and extracted with 2 x 3 ml ethyl ether and vortex-mixing for 2 min. The extracts were evaporated to dryness under nitrogen and the residues were reconstituted in HPLC grade ethanol. Samples were analyzed using the HPLC method described in section 2.3.2.3. The amount of carvedilol in the samples after ultrafiltration was compared to ultrafiltrate samples containing carvedilol that were not submitted to ultrafiltration. The results were expressed in terms of percentage recovery of drug after ultrafiltration.

2.6.1.1 Treatment of the Centrifree Ultrafiltration Units to Avoid Nonspecific Binding

Ultrafiltration units were submitted to various treatments prior to ultrafiltration in order to obtain improved recovery of carvedilol. The units (all parts, except membranes) were soaked overnight with one of the following: **(1)** 2 μg/ml racemic carvedilol solution in isotonic phosphate buffer, pH 7.4, **(2)** 2% triethylamine (TEA) in water, **(3)** 10 μg/ml propafenone in isotonic phosphate buffer, pH 7.4, **(4)** 5% sodium dodecyl sulphate (SDS) in water, and **(5)** 5% Triton X-100 in water. Membranes were soaked for three hours in the same solutions prior to the test, and then blotted in tissue paper. The two last treatments (called "passivation") are recommended by the supplier to reduce binding of proteins to the units. Samples (0.5 ml) consisting of 0.5 μg/ml of each carvedilol enantiomer in isotonic phosphate buffer, pH 7.4 were loaded to the treated devices as well as to untreated units, and left in contact for 60 minutes. Samples were centrifuged at room temperature for 20 min at 1600 x g. The filtrate (0.45 ml) was collected for analysis. The internal standard (100 μl of a 3.5 μg/ml solution of (-)-propranolol in ethanol) was then added and samples were basified and extracted as described in the previous section. The amount of drug after ultrafiltration was determined by HPLC according to section 2.3.2.3 and compared to those samples that were not submitted to ultrafiltration.

2.6.2 Determination of Nonspecific Binding of Carvedilol to Millipore Ultrafiltration Units

Ultrafree-CL centrifugal filters available from Millipore (Bedford, MA) were tested for nonspecific binding of carvedilol enantiomers. These units were of 2 ml capacity and contained either polysulfone membranes or low binding regenerated cellulose membranes, both with a molecular weight cut-off of 10,000 or 30,000 Daltons. Four replicates of 1.5 ml of a carvedilol solution containing 60 ng/ml of each enantiomer in isotonic phosphate buffer, pH 7.4, were loaded to the ultrafiltration units and left in contact for 1 hour. Units were then centrifuged in a Beckman centrifuge J2-21, with a 35 degree angle rotor JA-17, at 1600 x g for 30 min. Aliquots of 1 ml of the filtrates were collected and internal standard solution was then added. Samples were basified, extracted with ethyl ether, and analyzed by HPLC as described in section 2.3.2.3. Four aliquots of 1 ml of carvedilol solution were submitted to the same extraction and analysis procedure without being submitted to ultrafiltration. The amount of carvedilol in the samples after ultrafiltration was determined and compared to samples that were not submitted to ultrafiltration. The results were expressed in terms of percentage recovery of drug after ultrafiltration.

2.6.3 Determination of Nonspecific Binding of Carvedilol to Equilibrium Dialysis Units

2.6.3.1 Determination of Nonspecific Binding Using Serum

Triplicate 1 ml samples of carvedilol in serum (adjusted to pH 7.4 with 5.2 mg sodium phosphate monobasic monohydrate and 43.4 mg sodium phosphate dibasic

heptahydrate in 1 ml of serum) at concentrations of 24 and 240 ng/ml of each enantiomer were loaded into the dialysis cells, and dialyzed against an equal volume of 0.067 M isotonic phosphate buffer, pH 7.4, at 37 °C for 6 hours. After dialysis, the serum retentate and buffer dialysate volumes were collected and combined. Duplicate aliquots of 0.9 ml of the combined dialysate/retentate solutions were prepared and internal standard solution was added. Protein precipitation and extraction were undertaken as described in section 2.3.2.1. Reconstituted samples were analyzed by HPLC using the assay described in 2.3.2.3. Triplicate 1 ml samples of carvedilol in serum at 24 and 240 ng/ml of each enantiomer were combined with an equal volume of isotonic phosphate buffer. Aliquots of 0.9 ml of the combined serum/buffer solutions were analyzed for carvedilol enantiomers without being submitted to the dialysis process. Peak area ratios were determined for dialyzed and non-dialyzed samples. The results were expressed as percent recovery of carvedilol from the samples after dialysis compared to non-dialyzed samples.

2.6.3.2 Determination of Nonspecific Binding Using Dialysis Buffer

Triplicate 1 ml samples of carvedilol in 0.067 M isotonic phosphate buffer, pH 7.4, at concentrations of 24 and 240 ng/ml of each enantiomer were loaded into the dialysis cells, and dialyzed against an equal volume of buffer at 37 °C for 6 hours. After dialysis, the volumes of liquid from the two dialysis cells for each sample were collected and combined. Duplicate aliquots of 0.9 ml of the combined solutions were prepared. The internal standard solution was then added and samples were submitted to extraction as described in section 2.3.2.1. Triplicate 1 ml samples of carvedilol solution in isotonic phosphate buffer at 24 and 240 ng/ml were directly combined with

an equal volume of phosphate buffer and duplicate aliquots of 0.9 ml of the diluted solutions were prepared. Samples were submitted to extraction according to section 2.3.2.1. All samples were analyzed for carvedilol enantiomers by HPLC as described in section 2.3.2.3. Peak area ratios carvedilol enantiomer/internal standard were determined for dialyzed and non-dialyzed samples. The results were expressed as percent recovery of carvedilol from buffer after dialysis compared to non-dialyzed buffer.

2.6.4 Determination of Dialysis Equilibrium Time for Carvedilol

Serum was adjusted to pH 7.4 with sodium phosphate salts (5.2 mg sodium phosphate monobasic monohydrate and 43.4 mg sodium phosphate dibasic heptahydrate in 1 ml of serum). Racemic carvedilol was added to serum to achieve a concentration of 2 μg/ml of each enantiomer. Samples were dialyzed against an equal volume of 0.067 M isotonic sodium phosphate buffer, pH 7.4, in a water-bath at 37 °C for up to 12 hours. The dialysate of 4 cells was collected at 2, 4, 6, 8, 10, and 12 hours and individually analyzed for carvedilol enantiomers after the addition of internal standard and following the extraction procedure outlined in 2.3.2.1. Volume shift from the buffer compartment to the serum compartment was monitored by weighing the serum retentate and the buffer dialysate volumes after collection from the dialysis cells. The buffer dialysate was also tested for the presence of protein due to leakage through the dialysis membrane by addition of 10% TCA by observing any turbidity development.

2.6.5 Free Fraction of Carvedilol Enantiomers in Serum Determined by Equilibrium Dialysis

Protein binding of the enantiomers of carvedilol to serum proteins was determined *in vitro* by equilibrium dialysis. Blood was obtained from healthy volunteers in additive-free Vacutainers, allowed to clot for 2 hours at room temperature, and then submitted to centrifugation at 3000 rpm for 20 min. Serum was stored at -20 °C until required for analysis. Prior to the experiments, serum was adjusted to pH 7.4 with sodium phosphate salts (5.2 mg sodium phosphate monobasic monohydrate and 43.4 mg sodium phosphate dibasic heptahydrate in 1 ml of serum). Racemic carvedilol (100 μg/ml in water, pH 2) was added to serum to obtain final concentrations of 2 and 4 μg/ml of each enantiomer. Serum containing carvedilol was incubated at 37 °C for 30 min, and 1 ml was dialyzed against an equal volume of 0.067 M isotonic phosphate buffer, pH 7.4, at 37 °C for 6 hours. After dialysis, samples were removed from the dialysis cells and weighed to determine occurrence of volume shift during the dialysis process. The dialysate samples were tested for the presence of proteins by the addition of 10% TCA and by observing if any turbidity developed thereafter. Samples were then submitted to extraction with ethyl ether as described in section 2.3.2.1. Due to the sensitivity limitations of the capillary electrophoresis assay with UV detection, and considering the fact that carvedilol binding to serum proteins is higher than 98%, dialysate samples (buffer compartment) were analyzed by HPLC according to section 2.3.2.3. Retentate samples (serum compartment) were analyzed by the CE method described in section 2.4.6. The post-dialysis free fraction (f_u) was calculated by dividing the concentration of free drug in the dialysate by the total concentration (bound and free) in the retentate.

2.6.6 Free Fraction of (+)-(R)-Carvedilol and (-)-(S)-Carvedilol in Serum

The free fraction of each enantiomer in serum was independently determined by equilibrium dialysis. Each enantiomer (50 μg/ml in water, pH 2) was added to serum adjusted to pH 7.4 to obtain final concentrations of 2 and 4 μg/ml. Samples were submitted to dialysis and analyzed as described in section 2.6.5.

2.6.7 Free Fraction of Carvedilol Enantiomers in Isolated Serum Protein Solutions

2.6.7.1 Free Fraction in 4% Human Serum Albumin

Human serum albumin (HSA) was dissolved in 0.067 M isotonic phosphate buffer, pH 7.4, at a concentration of 4%, which represents the normal level of this protein in serum (MacKichan, 1992). Racemic carvedilol (100 μg/ml in water, pH 2) was added to the HSA solution to obtain final concentrations of 2 and 4 μg/ml of each enantiomer. Drug-protein solutions were incubated at 37 °C for 30 min. One millilitre of drug-protein solution was dialyzed against an equal volume of isotonic phosphate buffer, pH 7.4, at 37 °C for 6 h. Retentate samples were analyzed by CE as described in section 2.4.6, and dialysate samples were analyzed by the HPLC method presented in section 2.3.2.3. The post-dialysis free fraction (f_u) was calculated by dividing the concentration of free drug in the dialysate by the total concentration (bound and free) in the retentate.

2.6.7.2 Free Fraction in 100 mg% and 400 mg% α_1-Acid Glycoprotein

α_1-Acid glycoprotein was dissolved in 0.067 M isotonic phosphate buffer, pH 7.4 at a concentration of 100 mg%, which represents the normal levels of this protein in serum (MacKichan, 1992). Binding to 400 mg% α_1-acid glycoprotein, simulating an acute phase increase on the serum levels of this reactant protein, as could occur in myocardial infarction and trauma, was also tested. Racemic carvedilol (100 μg/ml in water, pH 2) was added to the AAG solutions to obtain final concentrations of 2 and 4 μg/ml per enantiomer. Samples were incubated at 37 °C for 30 min. Drug-protein solutions (1 ml) were dialyzed against an equal volume of isotonic phosphate buffer at 37 °C, for 6 h. The retentate was analyzed by the CE method presented in section 2.4.6, whereas the dialysate was analyzed by HPLC as described in section 2.3.2.3. The post-dialysis free fraction (f_u) was calculated by dividing the concentration of free drug in the dialysate by the total concentration (bound and free) in the retentate.

2.6.7.3 Free Fraction in 4% Human Serum Albumin Combined with 100 mg% and 400 mg% α_1-Acid Glycoprotein

Human serum albumin and α_1-acid glycoprotein were dissolved in 0.067 M isotonic phosphate buffer, pH 7.4, to obtain concentrations of 4% HSA + 100 mg% AAG, and 4% HSA + 400 mg% AAG. Racemic carvedilol (100 μg/ml in water, pH 2) was added to the protein solutions to obtain final concentrations of 2 and 4 μg/ml per enantiomer. Drug-protein solutions were incubated at 37 °C for 30 min. Samples (1 ml) were dialyzed against an equal volume of isotonic phosphate buffer, pH 7.4, at 37 °C, for 6 h. The retentate was analyzed by the CE method presented in section

2.4.6. The dialysate was analyzed by HPLC as described in section 2.3.2.3. The post-dialysis free fraction (f_u) was calculated by dividing the concentration of free drug in the dialysate by the total concentration (bound and free) in the retentate.

2.6.7.4 Free Fraction of Carvedilol Enantiomers in Lipoprotein-Deficient Serum

Lyophilized lipoprotein-deficient serum was reconstituted with 0.067 M isotonic phosphate buffer, pH 7.4. Racemic carvedilol (100 μg/ml in water, pH 2) was added to the serum to obtain concentrations of 2 and 4 μg/ml of each enantiomer, and solutions were left at 37 °C for 30 min. Sample volumes of 1 ml were dialyzed for 6 h, at 37 °C, against an equal volume of 0.067 M isotonic phosphate buffer, pH 7.4. Retentate samples were subsequently analyzed by CE as described in section 2.4.6 and the dialysate was analyzed for carvedilol by HPLC as outlined in section 2.3.2.3. Post-dialysis free fractions (f_u) were calculated by dividing the concentration of free drug in the dialysate by the total concentration (bound and free) in the retentate.

2.6.8 Concentration-Dependent Binding of Carvedilol Enantiomers in Serum and in Isolated Serum Protein Solutions

2.6.8.1 Concentration-Dependent Binding of Carvedilol in Serum

Serum was obtained from a healthy male volunteer and adjusted to pH 7.4 with sodium phosphate salts (5.2 mg sodium phosphate monobasic monohydrate and 43.4 mg sodium phosphate dibasic heptahydrate in 1 ml of serum). Racemic carvedilol (100 μg/ml in water, pH 2) was added to the serum to obtain final concentrations of 0.5, 1, 2, 3, and 4 μg/ml of each enantiomer. Serum containing carvedilol was incubated at

37 °C for 30 min. Samples (1 ml) were dialyzed against 1 ml 0.067 M isotonic phosphate buffer, pH 7.4, at 37 °C for 6 h. Serum retentate samples were analyzed by CE according to section 2.4.6. Buffer dialysate was analyzed by HPLC as described in section 2.3.2.3. Post-dialysis free fractions (f_u) were calculated and plotted against the corresponding drug serum concentrations.

2.6.8.2 Concentration-Dependent Binding to 4% Human Serum Albumin

Racemic carvedilol (100 μg/ml in water, pH 2) was added to 4% HSA in 0.067 M isotonic phosphate buffer, pH 7.4, to obtain final concentrations of 0.5, 1, 2, 3, and 4 μg/ml of each enantiomer. Drug-protein solutions were incubated at 37 °C for 30 min. Samples were dialyzed against isotonic phosphate buffer at 37 °C for 6 h. Retentate samples were analyzed by CE as outlined in section 2.4.6 and buffer dialysate was analyzed by HPLC as described in section 2.3.2.3. Post-dialysis free fractions (f_u) were calculated and plotted against the corresponding drug concentrations in the protein solutions.

2.6.8.3 Concentration-Dependent Binding to 100 mg% α_1-Acid Glycoprotein

Racemic carvedilol (100 μg/ml in water, pH 2) was added to a 100 mg% AAG solution in 0.067 M isotonic phosphate buffer, pH 7.4, to obtain final concentrations of 0.5, 1, 2, 3, and 4 μg/ml per enantiomer. Drug-protein solutions were incubated at 37 °C for 30 min. Samples were then dialyzed against isotonic phosphate buffer at 37 °C for 6 h. Retentate samples were analyzed by CE as described in section 2.4.6 and buffer dialysate samples were analyzed by HPLC as outlined in section 2.3.2.3.

Post-dialysis free fractions (f_u) were calculated and plotted against the corresponding drug concentrations in the protein solutions.

3. RESULTS AND DISCUSSION

3.1 Stereoselective HPLC Assay for Carvedilol Enantiomers

Previous investigations in our laboratory have established the use of fluorescent derivatizing reagents such as 2-naphthoyl chloride and 2-anthroyl chloride for the analysis of mexiletine enantiomers using a Pirkle type 1-A chiral stationary phase (McErlane *et al.*, 1987, Kwok *et al.*, 1994). These reagents provided an increase in the detection limits for mexiletine and a reliable assay for the study of the stereoselective pharmacokinetics of this drug. Derivatization with 2-anthroyl chloride provided a 10 fold increase on the limit of detection for that drug in plasma compared to the assay using 2-naphthoyl chloride (Kwok *et al.*, 1994). The reaction of the acid chlorides was determined to be complete and reproducible with the primary amine group of mexiletine.

Although carvedilol contains a secondary amine group, it was believed that 2-anthroyl chloride would be adequately reactive towards carvedilol and provide a very sensitive chiral HPLC assay to study the stereoselective pharmacokinetics of carvedilol. The rationale for the use of 2-anthroyl chloride was the possibility that the anthracene group of the derivatizing reagent would be more fluorescent than the carbazole ring of carvedilol. Preliminary experiments supported that idea: the fluorescence signal of a solution of anthracene carboxylic acid in the HPLC mobile phase was about 3 fold higher than the signal produced by a carvedilol solution of equal molar concentration. That indicated that 2-anthroyl chloride would be a suitable fluorescent derivatizing reagent which could produce an improvement on the limit of quantitation for carvedilol in serum.

2-Anthroyl chloride is not commercially available and had to be synthesized. Therefore, optimization of the reaction conditions and characterization of the derivatization products were initially performed using 2-naphthoyl chloride, which could be purchased. However, the ultimate goal was the derivatization with 2-anthroyl chloride.

3.1.1 Derivatization of Carvedilol with Fluorescent Reagents

3.1.1.1 Derivatization with 2-Naphthoyl Chloride

In order to establish the reactivity of the derivatization reagent, mexiletine enantiomers were used as a "test compound" since this drug has been well established (McErlane *et al.*, 1987) to react readily with 2-naphthoyl chloride.

The derivatization reactions of carvedilol and mexiletine with 2-naphthoyl chloride were analyzed by HPLC using a dinitrobenzoylphenylglycine (Pirkle type 1-A) chiral column. The fact that 2 well resolved peaks were detected for mexiletine indicated that the reaction was occurring in the conditions studied. The analysis of the reaction mixture with carvedilol revealed the presence of a single peak eluting at 19 min that was not carvedilol nor the reagent. Therefore this peak could correspond to the naphthamide derivatives of carvedilol but was not resolved with that chiral stationary phase. While such interactions can be predicted based on the relative positions of the π-bonding groups, the hydrogen-bonding groups and the steric interaction of the analyte and the stationary phase, it is not possible to predict the steric geometry interactions with certainty.

In order to determine if a derivative had, in fact, formed, the reaction was monitored by thin layer chromatography (TLC) using silica gel as the stationary phase. Two extra spots were detected on the TLC plate with R_f 0.50 and 0.67, which could correspond to derivatization products. When testing the reaction mixture, a band was also detected at R_f 0.04 which was identified as carvedilol by comparison of the R_f value with carvedilol standard. The results suggested that the reaction was not complete as detected by the presence of a spot corresponding to carvedilol near the origin and also that more than one derivative may have been formed.

It had been established in preliminary studies in our laboratory that carvedilol enantiomers could be partially resolved by HPLC using the Chiralcel OD chiral column, which contains a cellulose carbamate derivative coated on silica gel as the stationary phase. Therefore, this system was also used to detect the presence of unreacted drug after derivatization with 2-naphthoyl chloride. The reaction did not produce complete derivatization at room temperature or at 75 °C for 60 min, as revealed by the presence of partially resolved peaks for unreacted carvedilol enantiomers in the reaction mixture. The amount of unreacted drug varied from about 5 to 15% of total starting material. The HPLC system using the Chiralcel OD column revealed the presence of unreacted carvedilol and excess reagent but there were no additional peaks that would have been indicative of a derivative of carvedilol.

3.1.1.1.1 Mass Spectrometric Analysis of the Naphthoyl Derivatives of Carvedilol

In order to elucidate the structure of the reaction products formed after derivatization of carvedilol enantiomers with 2-naphthoyl chloride, the reaction mixture was examined by LC/MS. An ODS column with a mobile phase consisting of

acetonitrile:water (90:10, v/v) was used for sample introduction into the mass spectrometer. Carvedilol enantiomers will only elute from such a column stationary phase in the ionized form, using a low pH buffer. Thus the unreacted carvedilol enantiomers were strongly retained by the column. The naphthoyl derivatives, however, could be detected by MS using positive electrospray. Two pseudo-molecular ions were monitored at m/z 561 and 715, corresponding to the mono- and the di-derivatives of carvedilol. The peaks eluted from the column at 6.3 and 9.9 min, respectively. The di-derivative was detected at much lower intensity, about 10% of the area of the mono-derivative. The occurrence of multiple derivatization was also detected by LC/MS when the reaction was performed at room temperature and the percentage of di-derivative increased to about 20% when the reaction was performed at 75 °C for 1 hour.

MS/MS with flow injection was performed in order to elucidate the structures of the reaction products. As reported in the previous section two fractions with R_f values of 0.50 and 0.67 were detected by TLC of the reaction mixture. These fractions were extracted from the TLC plates and introduced into the mass spectrometer by flow injection. The fractions were determined to contain ions m/z 561 and 715, which correspond to the mono-derivative and di-derivatives of carvedilol with 2-naphthoyl chloride, respectively. Figure 7 shows the spectrum of the daughters of ion m/z 561 and the structure of the mono-derivative formed in the reaction with the fragmentation pattern indicated. The most intense ions obtained were the molecular ion (m/z 561); m/z 437, corresponding to the loss of the methoxyphenoxy group; m/z 378, which corresponds to the loss of the carbazol group; m/z 222, resulting from the loss of the naphthoyl and carbazol groups; m/z 155 corresponding to the naphthoyl group. The

second fraction collected from the TLC plate contained an ion with m/z 715, which corresponds to the di-derivative of carvedilol with 2-naphthoyl chloride. Daughter ions of ion m/z 715 were obtained. The mass spectrum and the chemical structure of the di-derivative with the fragmentation pattern are shown in Figure 8. The presence of a fragment with m/z 532, corresponding to the loss of the carbazol moiety indicated that multiple derivatization of carvedilol occurred at the secondary amine group and at the hydroxyl group on the side chain. Analysis of the fragments obtained by negative electrospray are shown in Figure 9 and Figure 10 and indicated that the mono-derivative results from the attack of 2-naphthoyl chloride at the secondary amine group on the side chain of carvedilol. The presence of an abundant ion with m/z 171 corresponding to naphthoyl carboxylic acid was only present in the spectrum of the di-derivative (Figure 10) and originated from the fragmentation at the ester bond. The absence of this fragment in the spectrum of the mono-derivative (Figure 9) suggests that the derivatization at the hydroxyl group did not occur.

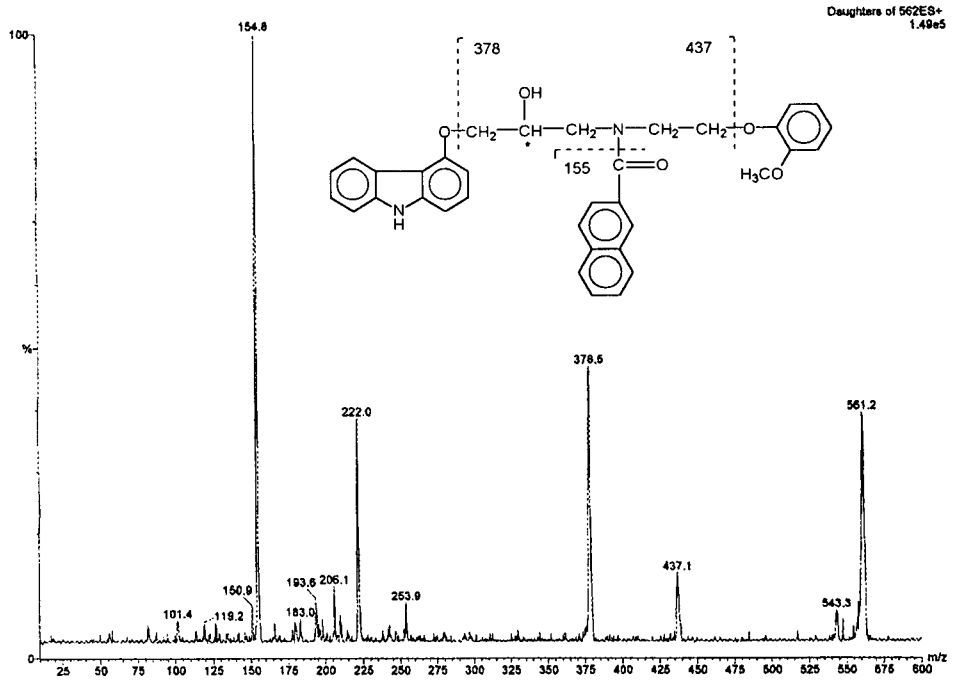

Figure 7. MS/MS (Positive Electrospray) of ion m/z 561, resulting from the derivatization of carvedilol with 2-naphthoyl chloride.

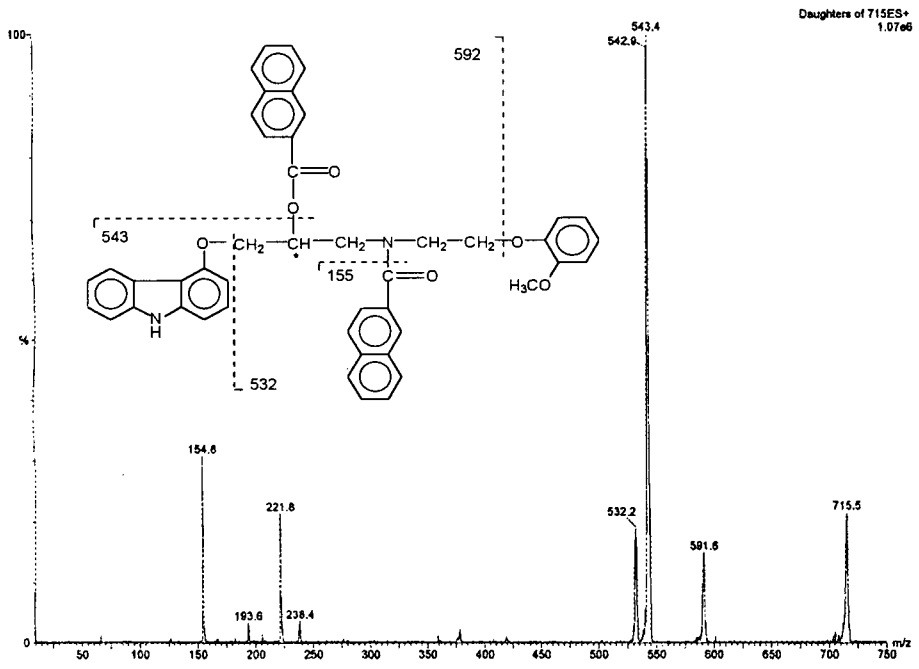

Figure 8. MS/MS (Positive Electrospray) of ion m/z 715, resulting from the derivatization of carvedilol with 2-naphthoyl chloride.

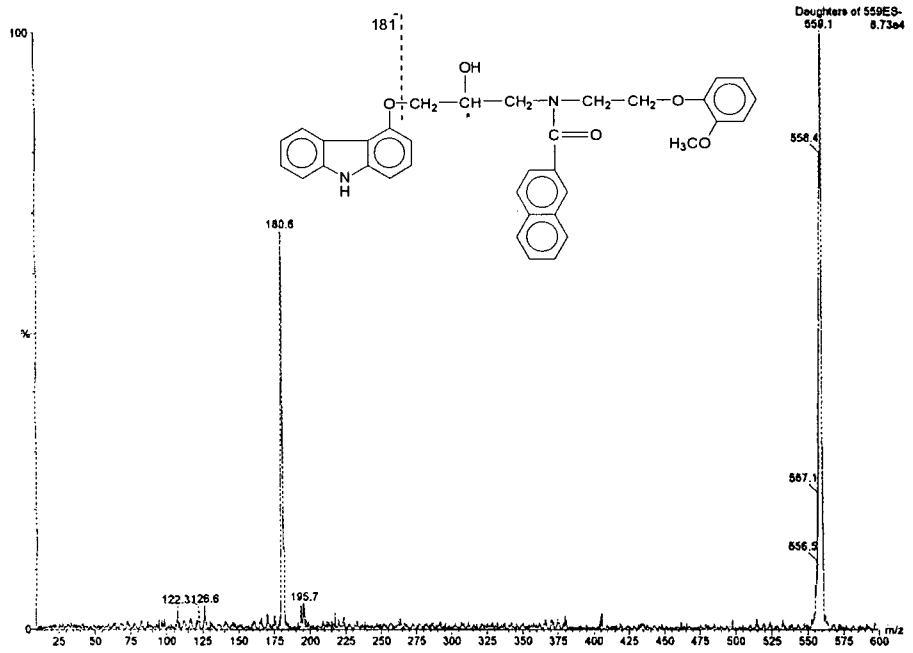

Figure 9. MS/MS (Negative Electrospray) of ion m/z 559, resulting from the derivatization of carvedilol with 2-naphthoyl chloride.

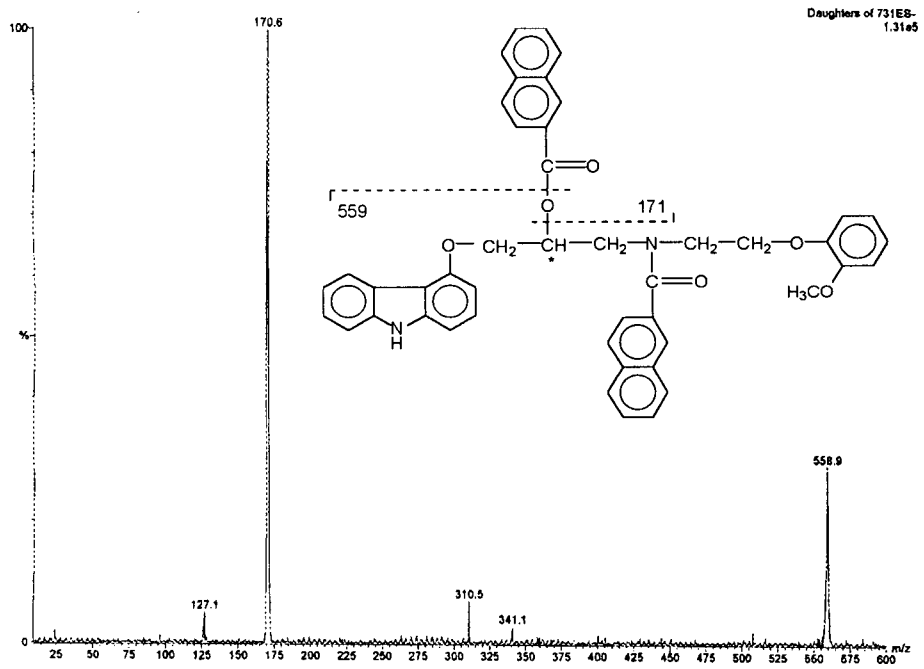

Figure 10. MS/MS (Negative Electrospray) of ion m/z 731 (714 plus ammonia), resulting from the derivatization of carvedilol with 2-naphthoyl chloride.

3.1.1.2 Derivatization with 2-Anthroyl Chloride

Although incomplete reaction and multiple derivatives resulted when carvedilol was reacted with 2-naphthoyl chloride, the strongly fluorescing reagent 2-anthroyl chloride was also tested in order to further investigate the reactivity of the acid chlorides for the derivatization of carvedilol. The resulting derivatives of carvedilol enantiomers with 2-anthroyl chloride were analyzed using the chiral stationary phase Phenomenex 3022. Figure 11 shows a chromatogram obtained after the injection of the reaction mixture. It can be observed that the reaction was not quantitative, as detected by the presence of unreacted carvedilol enantiomers eluting at about 18.2 and 20.4 min. The percentage of carvedilol remaining after reaction was of about 13% for the reaction at room temperature and 8% for the reaction at 70 °C for 2 hours. A pair of partially resolved peaks was observed with retention times of about 13.2 and 14.4 min and were believed to correspond to the enantiomers of derivatized carvedilol. Due to the fact that carvedilol is not soluble in aqueous solvents the choice of the base catalyst was limited to organic bases (triethylamine, 4-dimethylaminopyridine). Neither triethylamine nor 4-dimethylaminopyridine used as base catalysts produced complete derivatization of carvedilol with 2-anthroyl chloride.

Due to the fact that the derivatization reaction with 2-anthroyl chloride was not complete and reproducible, it was deemed inappropriate to pursue this avenue of research.

3.1.1.3 Derivatization with (+)-(S)-Naproxen Chloride

(+)-(S)-Naproxen chloride was developed to be used as a chiral fluorescent derivatizing reagent for optically active primary and secondary amines and alcohols

(Spahn, 1988). The reaction was used for the derivatization of carvedilol enantiomers in one of the two major studies on the stereoselective pharmacokinetics of carvedilol in humans (Spahn *et al.*, 1990). The reaction was performed in dichloromethane at 50 °C for 60 min.

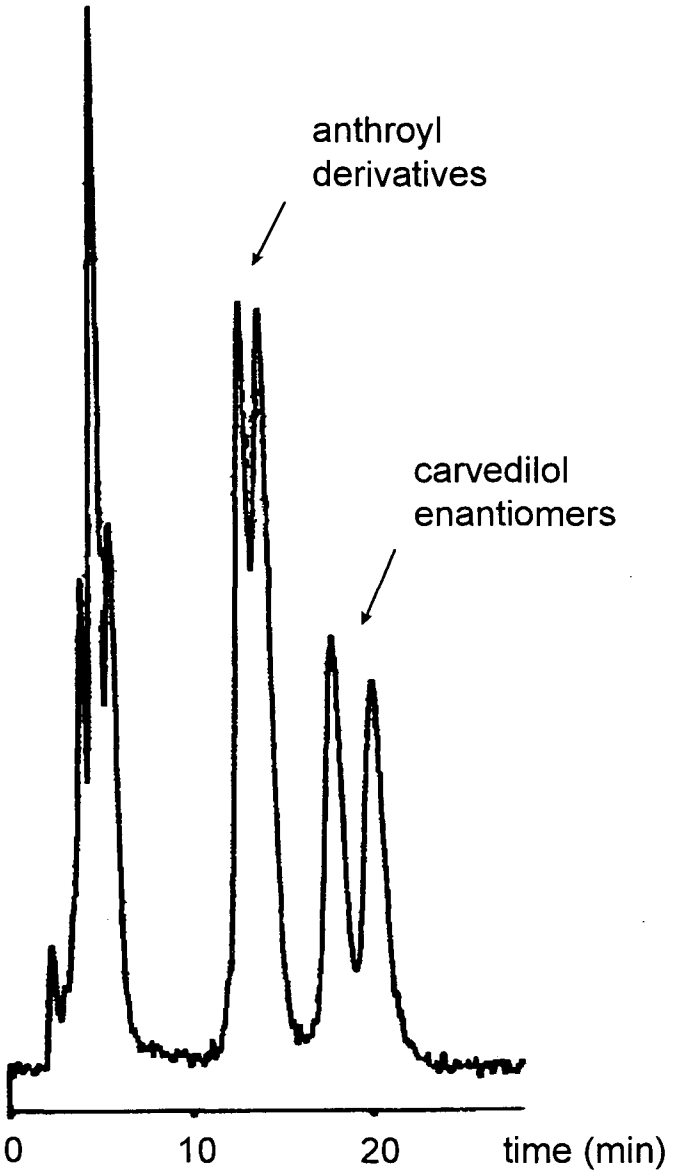

Figure 11. HPLC analysis of the derivatization reaction of carvedilol enantiomers with 2-anthroyl chloride at 70 °C for 2 hours. **Conditions:** column: Phenomenex 3022, 50 mm x 4.6 mm I.D.; mobile phase: hexane:dichloromethane:ethanol:trifluoroacetic acid (60:30:10:0.5, v/v/v/v); flow: 0.3 ml/min; detection: fluorescence at 285 nm (EX) and 355 nm (EM).

The authors reported that at temperatures higher than 80 °C the yield of derivatives decreased, possibly due to either decomposition or bis-derivatization both at the amino and hydroxyl groups of carvedilol (Spahn *et al.*, 1990). The maximum reaction yield was reported to be after 60 minutes of reaction. The derivatives were analyzed by HPLC using a silica column. Unfortunately it was not reported if the derivatization was quantitative although a calibration curve with an r^2 of 0.994 was obtained. Since carvedilol is retained by the silica column, the presence of unreacted drug was apparently not even considered. This omission in developing the method gave cause for concern in our work, especially in view of the fact that Spahn *et al.* (1990) reported a yield of only 45% when preparing the derivatives for characterization purposes.

The fluorescent derivatizing reagent (+)-(S)-naproxen chloride had to be first synthesized from (+)-(S)-naproxen. The infrared spectrum of the product was characterized by the disappearance of the broad band in the 2784-3358 cm^{-1} region, caused by O-H stretching, which was observed in the spectrum of (+)-(S)-naproxen. A shift was also observed for the C=O stretching from 1771 cm^{-1} for (+)-(S)-naproxen, to 1788 cm^{-1} for (+)-(S)-naproxen chloride.

Following the procedure outlined by Spahn *et al.* (1990) for derivatization of carvedilol with (+)-(S)-naproxen chloride, the resulting diastereomers were analyzed on an HPLC system using a silica column. The naproxen derivatives appeared as a pair of peaks eluting at 22.6 and 25.4 min (Figure 12). Unreacted carvedilol could not be detected in this system since the drug was strongly retained by the silica column. However, remaining carvedilol in the reaction mixture could be detected by TLC, by the presence of a band near the origin (about 20% of the total amount).

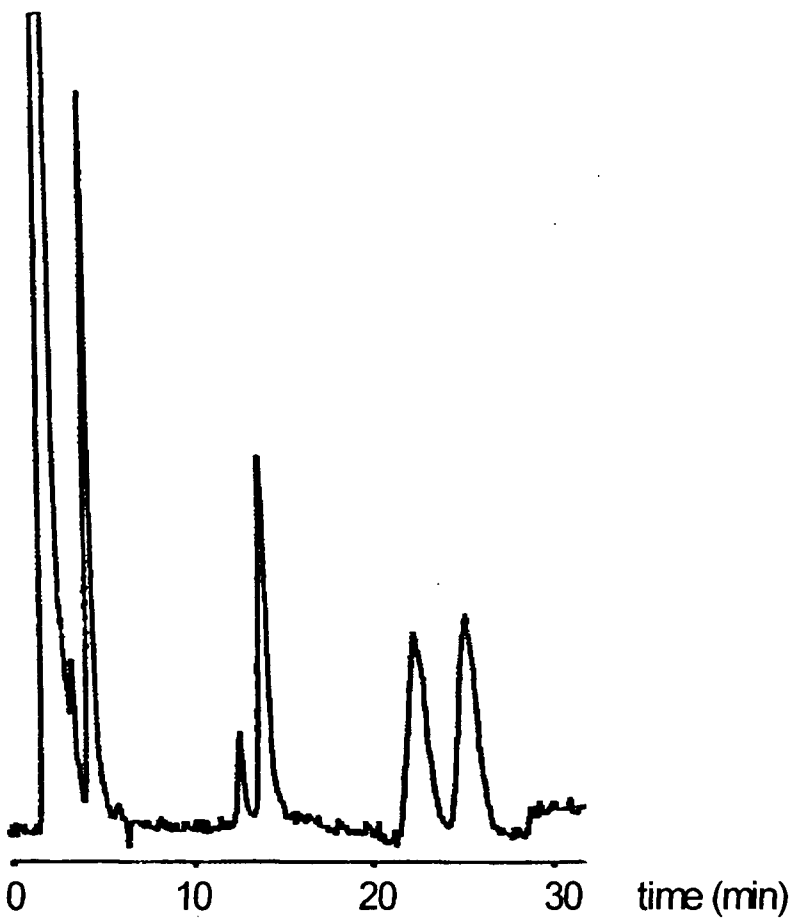

Figure 12. HPLC analysis of the derivatization reaction of carvedilol enantiomers with (+)-(S)-naproxen chloride. **Conditions:** column: silica, 150 x 4.6 mm I.D., 5 μm; mobile phase: hexane:dichloromethane:ethanol (120:28:1.7, v/v/v); flow rate: 0.9 ml/min; detection: fluorescence at 285 nm (EX) and 355 nm (EM). The pair of peaks eluting at 22.6 and 25.4 min correspond to the diastereomers formed in the reaction.

3.1.1.4 Derivatization with (+)-(S)-1-(1-Naphthyl)ethyl Isocyanate

Since the acid chlorides used previously did not produce complete derivatization of carvedilol, and in addition, lead to multiple derivatives at the hydroxyl and secondary amine groups, an alternative reagent, (+)-(S)-1-(1-naphthyl)ethyl isocyanate (NEIC),

was examined. The rationale behind this choice was based on the fact that NEIC has been reported to react readily with secondary amines. The reaction with optically pure NEIC has been used to resolve the enantiomers of several β-blockers, such as betaxolol (Darmon and Thenot, 1986, Stagni *et al.*, 1991), acebutolol (Piquette-Miller *et al.*, 1990), diacetolol (Piquette-Miller and Foster, 1990), and metoprolol (Bhatti and Foster, 1992).

The diastereomers formed after derivatization of carvedilol enantiomers with NEIC were analyzed by adsorption mode HPLC, using a silica column. A pair of peaks was detected eluting at 18.0 and 18.7 min which were believed to correspond to the diastereomers. A second pair of peaks, with smaller areas, appeared late in the chromatogram (30 min), and was considered to be due to the diastereomers formed after derivatization of carvedilol at both the amino and the hydroxyl groups.

The presence of unreacted carvedilol in the reaction mixture was monitored by HPLC using a C_{18} column. In order to examine solvent effects on the derivatization reaction different solvents with different dielectric constants and polarities such as acetonitrile, methanol, and dichloromethane were evaluated. Unfortunately, in none of the solvents tested was derivatization complete. When using acetonitrile as the reaction solvent about 5% of unreacted carvedilol was detected compared to 11% of remaining carvedilol after reaction using dichloromethane, and 13% using methanol.

It is well established that temperature increase in endothermic reactions leads to more rapid formation of product. Additionally, increasing the time of such reactions can also lead to larger yields. Therefore the reaction with NEIC was tested at room temperature or at 75 °C for 1 hour. Somewhat surprisingly, increasing temperature from room temperature to 75 °C, resulted in even more unreacted carvedilol: 13% with

acetonitrile, 14% with methanol, and 17% with dichloromethane. The effect of the temperature on reaction yield was not further investigated but the presence of more unreacted carvedilol at higher temperature could indicate that the reaction was reversible.

Darmon and Thenot (1986) reported a lower yield when the reaction was performed at 60 °C as compared to room temperature. These authors speculated that it could be due to derivatization at both the amino and hydroxyl group of betaxolol. In fact, two extra peaks eluting at about 40 min were observed in the assay using an ODS HPLC stationary phase (Darmon and Thenot, 1986). The yield for the derivatization of tertatolol with NEIC at room temperature for 12 h was reported to be about 70% for each enantiomer (Lave *et al.*, 1991).

3.1.1.4.1 Mass Spectrometric Analysis of the Derivatization Reaction with (+)-(S)-1-(1-Naphthyl)ethyl Isocyanate

Mass spectra of the derivatization reactions of carvedilol enantiomers with NEIC were obtained. Figure 13 shows the mass spectrum of the reaction in acetonitrile at room temperature, for 60 min. The presence of peak at m/z 407 corresponding to the molecular ion of carvedilol indicated that the derivatization was incomplete. The peak with m/z 604 was attributed to the mono-derivative of carvedilol at the aliphatic amino group. Other authors confirmed by mass spectrometry the formation of the urea derivative rather then the carbamate derivative after reaction of β-blockers with NEIC (Darmon and Thenot, 1986, Piquette-Miller *et al.*, 1990, Lave *et al.*, 1991). Hsyu and Giacomini (1986) have also confirmed the formation of the urea derivative in the reaction of pindolol enantiomers with (+)-(R)-α-methylbenzyl isocyanate. The results

obtained from the MS/MS experiment with the naphthoyl derivatives of carvedilol (section 3.1.1.1.1) also support the formation of the urea derivative of carvedilol.

A small peak with m/z 801 was also detected and probably corresponds to the di-derivative of carvedilol at the amino and at the hydroxyl group, which was also identified in the derivatization reaction with naphthoyl chloride. The peak corresponding to the di-derivative was also present when the reaction was performed in methanol or dichloromethane.

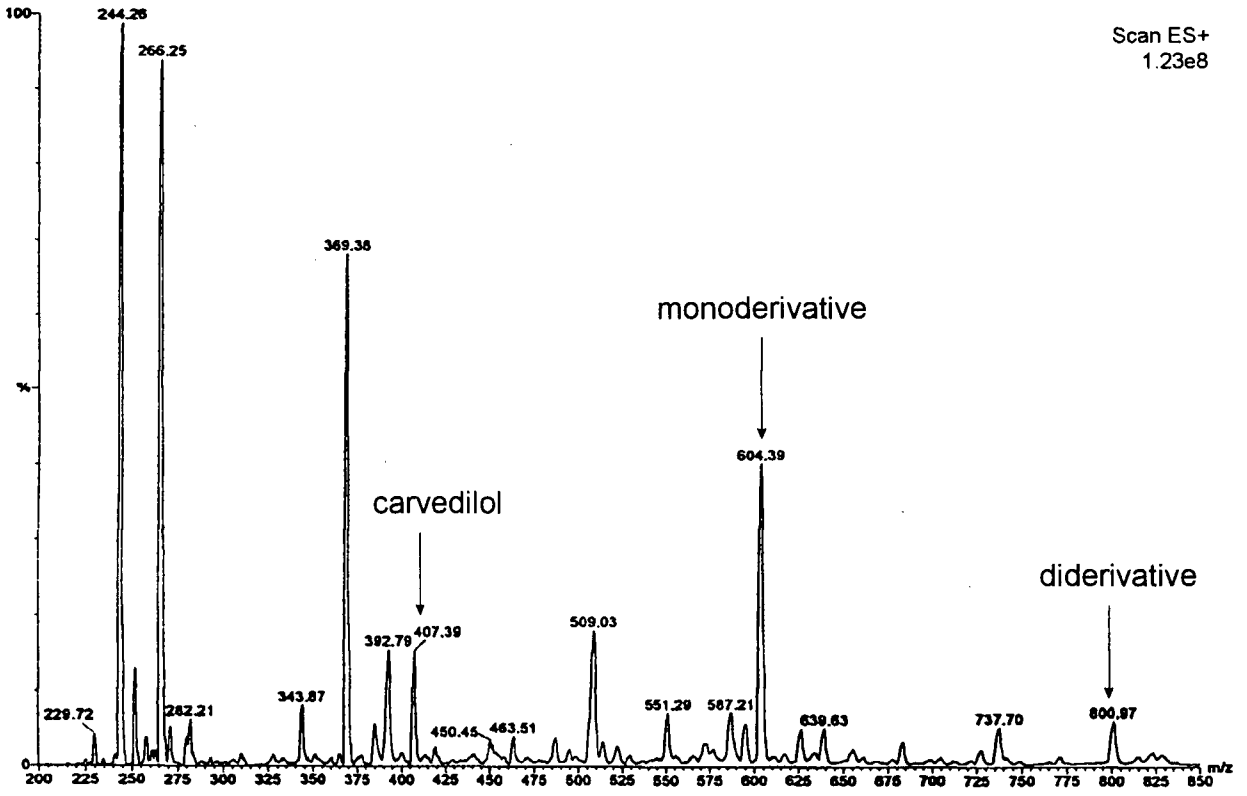

Figure 13. Mass spectrum of the derivatization reaction of carvedilol with (+)-(S)-1-(1-naphthyl)ethyl isocyanate (NEIC). Ion m/z 604 corresponds to the mono-derivative of carvedilol with NEIC at the secondary amine nitrogen on the side chain and ion m/z 801 is believed to be the di-derivative at both the amine group and the hydroxyl group of carvedilol.

3.1.2 Direct Resolution of Carvedilol Enantiomers by HPLC Using Chiral Stationary Phases

As it appeared from the results obtained that reaction with acid chlorides or reaction with isocyanate did not lead to complete derivatization of the enantiomers of carvedilol, or, more importantly, lead to multiple derivatives, it was considered essential to develop an analytical method that would not rely on derivatization methods. Therefore, a study to identify a chiral stationary phase that would be capable of resolving the enantiomers of carvedilol without the need for derivatization was undertaken.

There is only one report in the literature (Fujimaki *et al.*, 1991) describing the direct analysis of carvedilol enantiomers using chiral stationary phases. As reviewed in section 1.1.4.2 (Introduction, Stereoselective Analysis of Carvedilol Enantiomers), the analytical methods currently in use for carvedilol involve the formation of diastereomers and separation in either adsorption or partition mode using HPLC. Fujimaki *et al.* (1991) described the use of a chiral HPLC column (Chiralcel OF) for preparative purposes, to obtain sufficient quantities of ^{14}C-labeled (+)-(R)- and (-)-(S)-carvedilol enantiomers, to be used in a metabolism study in the rat. Thus, a similar stationary phase, Chiralcel OD, which was available in our laboratory was evaluated in this study.

3.1.2.1 HPLC Analysis of Carvedilol Enantiomers Using a Chiralcel OD Column

The Chiralcel OD column contains a cellulose carbamate derivative coated on silica gel as the stationary phase. Although Fujimaki *et al.* (1991) obtained baseline resolution of carvedilol enantiomers using the Chiralcel OF column, the Chiralcel OD column produced only partial resolution of carvedilol enantiomers with a pair of peaks

eluting at about 25 min (Figure 14). Attempts to optimize the mobile phase composition did not improve the resolution; therefore, it was concluded that this chiral stationary phase was unsuitable for the analysis of carvedilol enantiomers.

Figure 14. Separation of carvedilol enantiomers by HPLC using a Chiralcel OD column. **Conditions:** mobile phase: hexane:ethanol:dimethyloctylamine (50:50:0.1, v/v/v); flow rate: 0.55 ml/min; detection: fluorescence at 285 nm (EX) and 355 nm (EM). The peaks eluting at about 25 min correspond to carvedilol enantiomers.

3.1.2.2 HPLC Analysis of Carvedilol Enantiomers Using a Phenomenex 3022 Column

As the Chiracel OD column did not provide adequate resolution an alternative chiral stationary phase was evaluated. An examination of the chiral interactions between several chiral stationary phases marketed by Phenomenex and compounds with structures similar to carvedilol indicated that the most appropriate stationary phase was the (S)-indoline-2-carboxylic acid and (R)-1-(α-naphthyl)ethylamine (3022) chiral phase.

The HPLC method developed using the Phenomenex column 3022 provided partial resolution of carvedilol enantiomers, with an R_s of 1.17. Figure 15 shows a chromatogram of blank serum and serum spiked with racemic carvedilol. There was no interference due to the sample matrix. The retention times obtained for the enantiomers were 20.7 min for (+)-(R)-carvedilol, and 22.8 min for (-)-(S)-carvedilol. The internal standard eluted at 10.1 min. The total analysis time was 27 min for each run.

Figure 16 presents the structures of carvedilol and the chiral stationary phase (S)-indoline-2-carboxylic acid and (R)-1-(α-naphthyl)ethylamine (Phenomenex 3022). The chiral stationary phase consists of an optically pure carboxylic acid, (S)-indoline-2-carboxylic acid, covalently bound to γ-amino propyl silica and derivatized via an urea linkage with a π-electron group, (R)-1-(α-naphthyl)ethylamine. The dotted lines represent the potential interaction points between the drug enantiomers and the stationary phase. It is considered reasonable that the carbazol group of carvedilol is involved in π-π interactions with the naphthyl group of the stationary phase. Other interactions include hydrogen bonding between the ether oxygen of carvedilol with the

amide nitrogen of the stationary phase, the hydrogen bonding of the secondary amine of carvedilol with the carbonyl group of the indoline portion of the stationary phase and, most importantly, the chiral hydrogen bonding of the hydroxyl group with the opposite carbonyl group of the indoline portion of the stationary phase.

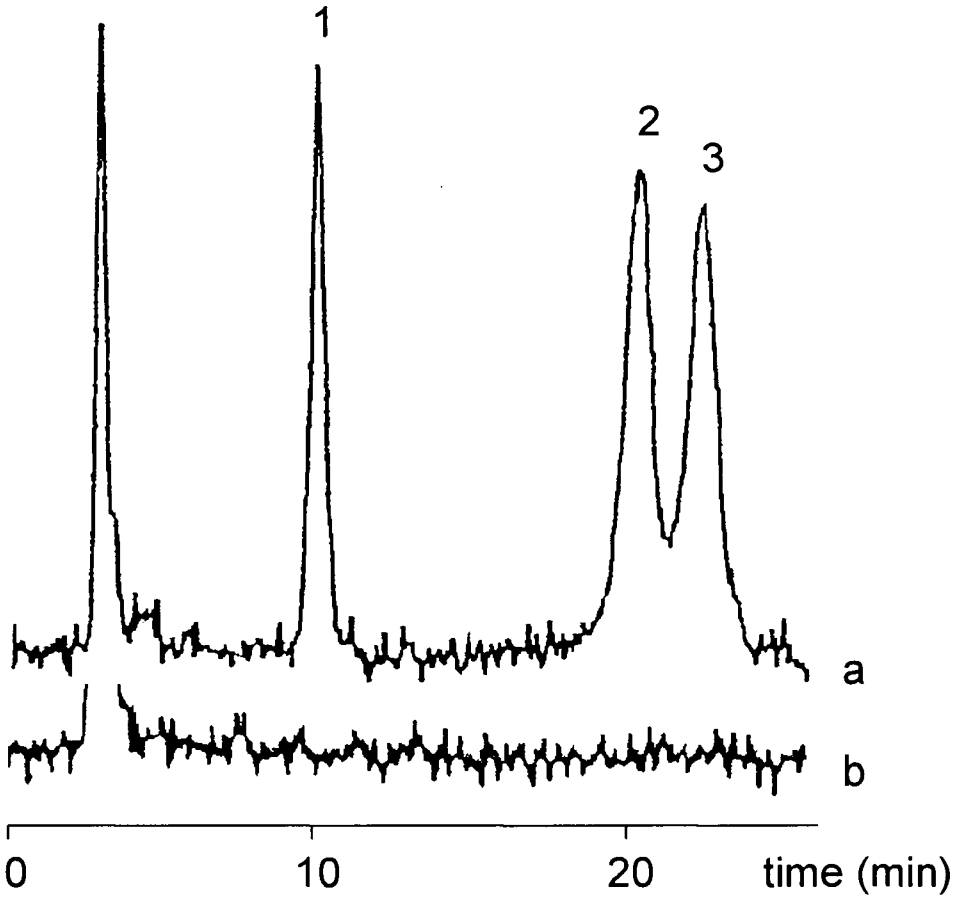

Figure 15. Representative chromatogram of an extraction from serum. **(a)**: serum containing (+)-(R)-carvedilol **(2)**, (-)-(S)-carvedilol **(3)**, and (-)-propranolol **(1)**; **(b)**: blank serum. **Chromatographic conditions:** HPLC column: (S)-indoline-2-carboxylic acid and (R)-1-(α-naphthyl)ethylamine, 250 mm length x 3.2 mm I.D.; mobile phase: hexane:dichloromethane:ethanol:trifluoroacetic acid (50:35:15:0.25, v/v/v/v); flow rate: 0.55 ml/min; detection: fluorescence; 284 nm (EX) / 343 nm (EM).

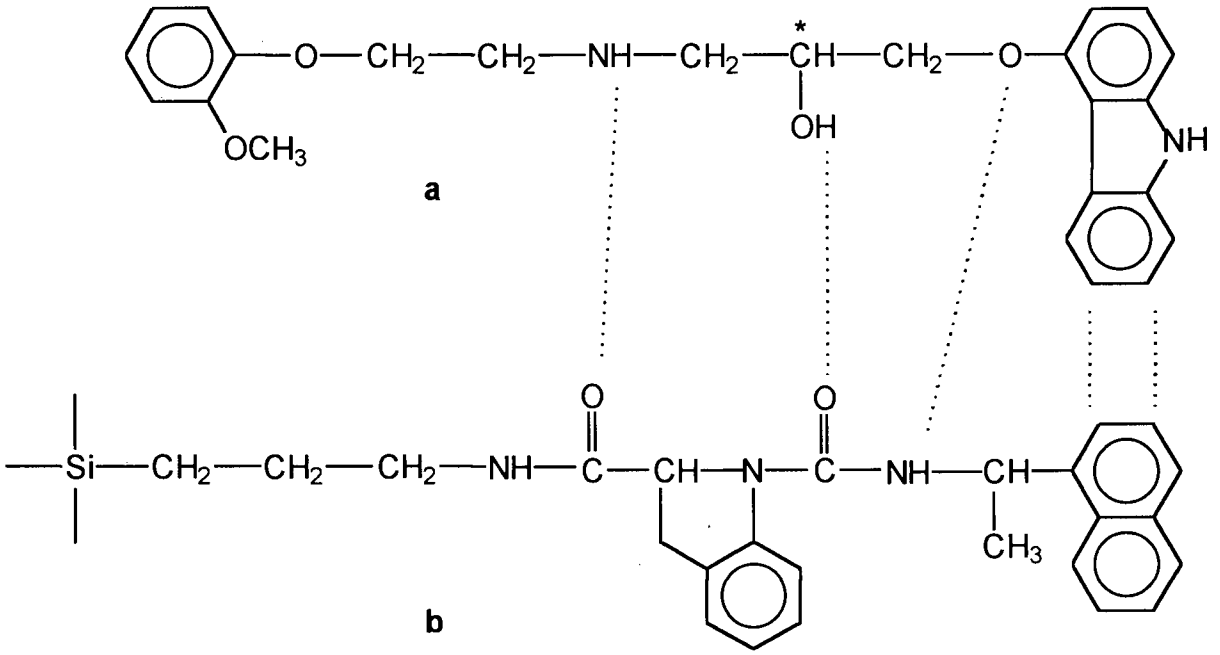

Figure 16. Proposed chiral interaction for carvedilol **(a)** and the chiral stationary phase (S)-indoline-2-carboxylic acid and (R)-1-(α-naphthyl)ethylamine (Phenomenex 3022) **(b)**. The dotted lines represent the proposed interactions between the drug and the stationary phase.

Dreiding stereomodels were constructed which support the proposed interactions between the two molecules. It could also be determined from the models that the hydroxyl group on the chiral centre of carvedilol is critical for the chiral recognition mechanism. The S-enantiomer of carvedilol has a much better fit of the hydroxyl group for hydrogen bonding with the carbonyl group of the stationary phase than the R-enantiomer. This is reflected by the retention times of the two enantiomers: (+)-(R)-carvedilol is the first enantiomer to elute from the column.

As the chiral HPLC method developed offered the advantage of direct resolution of the enantiomers without the need of derivatization and its apparent problems, the validity of the method was established using human serum samples.

3.1.2.2.1 Determination of Detector Linearity for Carvedilol Enantiomers in Serum

Linearity of detector response was assessed over two concentration ranges. A serum concentration range of 1-62 ng/ml per enantiomer, which reflects the normal serum levels after carvedilol administration, showed excellent detector linearity, with coefficients of determination (r^2) of 0.996 and 0.999 for (+)-(R)-carvedilol and (-)-(S)-carvedilol, respectively (Figure 17).

The limit of quantitation (LOQ) of the direct chiral HPLC assay developed for carvedilol enantiomers in serum was 1 ng/ml for each enantiomer. Thus the native fluorescence of carvedilol provided a highly sensitive assay for the determination of the drug in serum. The LOQ obtained is about the same as the values reported in the literature, with assays involving diastereomer formation (Eisenberg *et al.*, 1989, Fujimaki *et al.*, 1990, Neugebauer *et al.*, 1990, Spahn *et al.*, 1990, Stahl *et al.*, 1993a, Zhou and Wood, 1995).

A higher serum concentration range of 1-5 μg/ml of each enantiomer, used for the *in vitro* protein binding studies, was also tested. Coefficients of determination (r^2) higher than 0.999 were obtained for both carvedilol enantiomers.

3.1.2.2.2 Determination of Detector Linearity for Carvedilol Enantiomers in Dialysis Buffer

In order to confirm that the stereoselective HPLC method would be applicable to further studies in stereoselective protein binding, linearity of detection for carvedilol in dialysis buffer was determined at the concentration range of 10-250 ng/ml of each enantiomer. The calibration curves are presented in Figure 18. Coefficients of

determination of 0.9997 and 0.9995 were obtained for (+)-(R)-carvedilol and (-)-(S)-carvedilol, respectively.

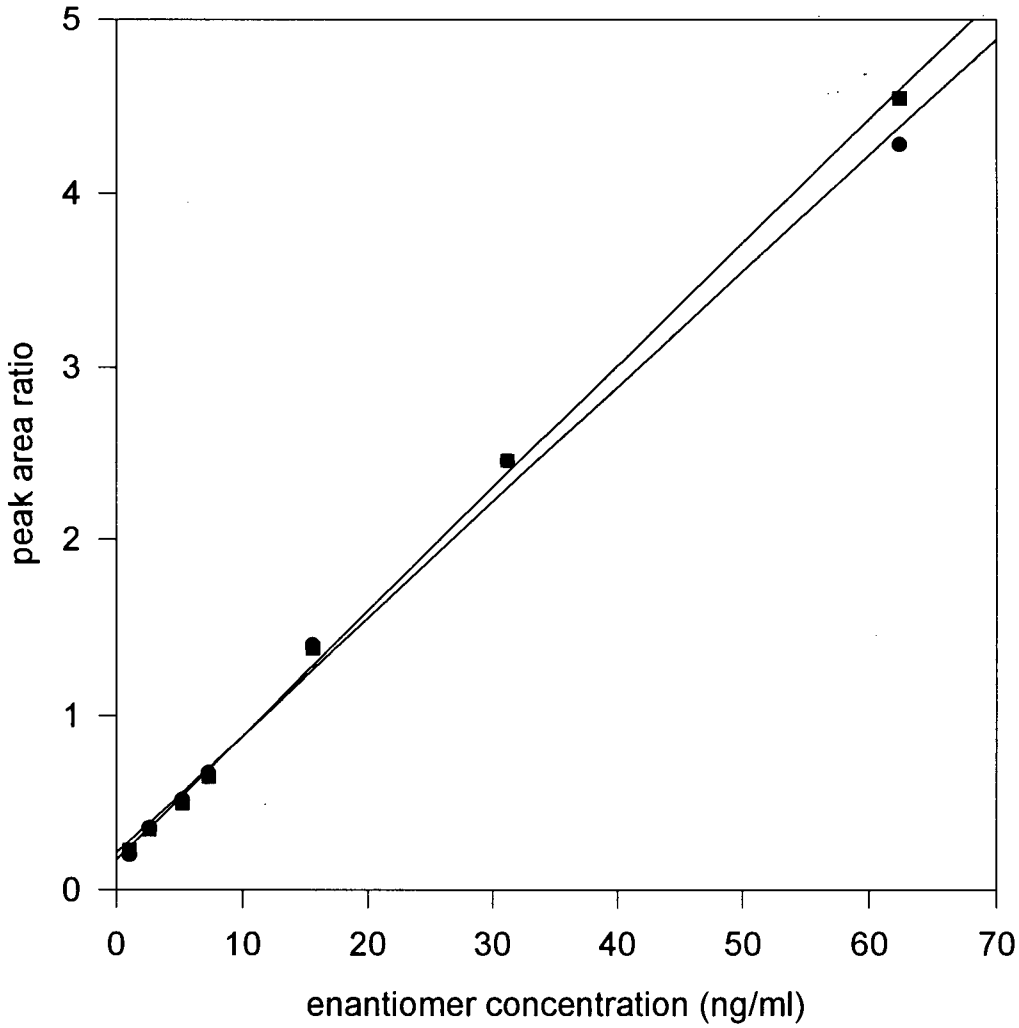

Figure 17. Standard curve for carvedilol enantiomers in serum analyzed by HPLC. (●): (+)-(R)-carvedilol; (■): (-)-(S)-carvedilol. **Conditions:** column: (S)-indoline-2-carboxylic acid and (R)-1-(α-naphthyl)ethylamine, 250 mm length x 3.2 mm I.D.; mobile phase: hexane:dichloromethane:ethanol:trifluoroacetic acid (50:35:15:0.25, v/v/v/v); flow rate: 0.55 ml/min; detection: fluorescence; 284 nm (EX) / 343 nm (EM).

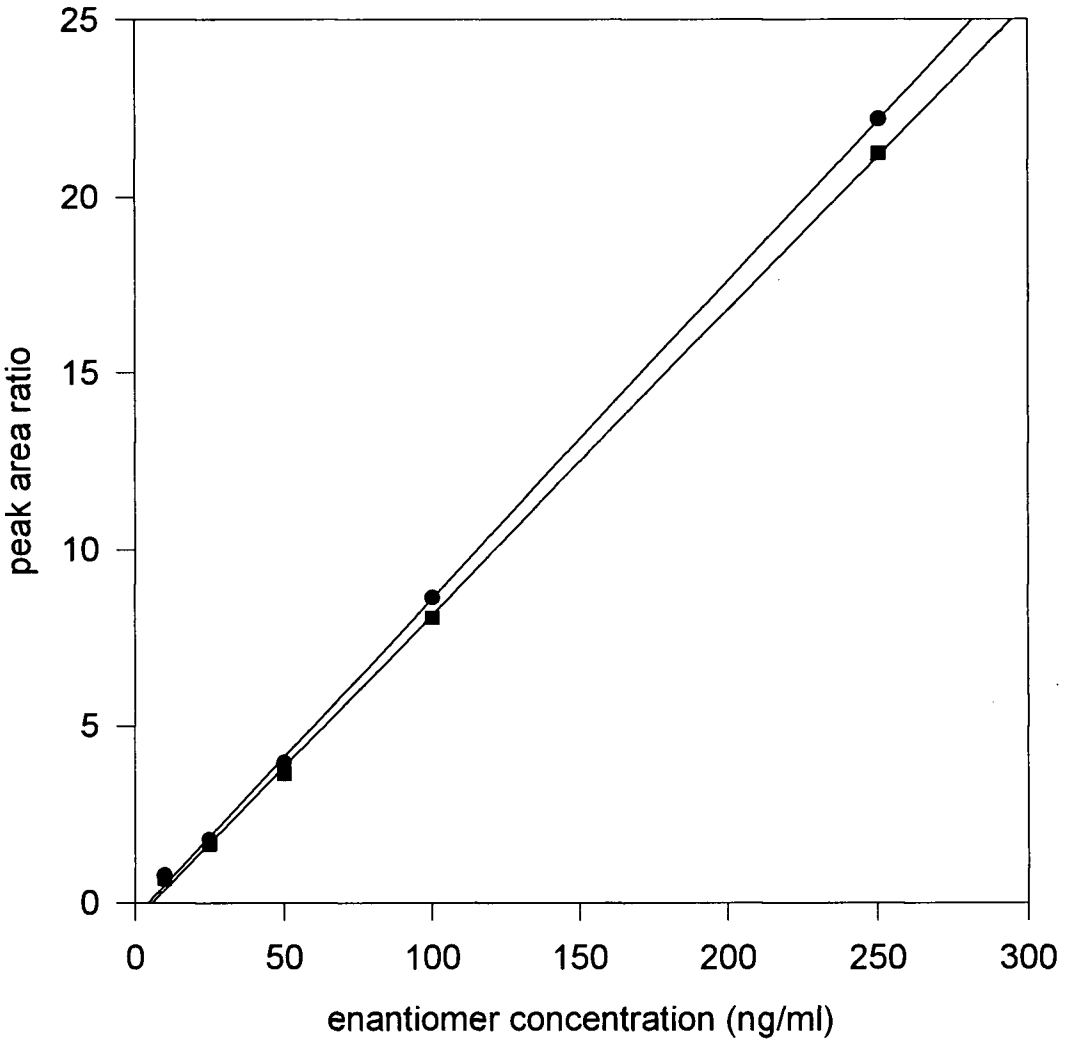

Figure 18. Standard curve for carvedilol enantiomers in dialysis buffer analyzed by HPLC. (●): (+)-(R)-carvedilol; (■): (-)-(S)-carvedilol. **Conditions:** column: (S)-indoline-2-carboxylic acid and (R)-1-(α-naphthyl)ethylamine, 250 mm length x 3.2 mm I.D.; mobile phase: hexane:dichloromethane:ethanol:trifluoroacetic acid (50:35:15:0.25, v/v/v/v); flow rate: 0.55 ml/min; detection: fluorescence; 284 nm (EX) / 343 nm (EM).

3.1.2.2.3 HPLC Intra- and Inter-Assay Variability

The reproducibility of the HPLC assay for the determination of carvedilol enantiomers in serum and in dialysis buffer using the chiral column Phenomenex 3022 was determined. The results are presented in Table I and in Table II. Intra- and inter-assay variability under 7%, expressed as coefficient of variation (C.V.), were obtained for the determination of carvedilol enantiomers in dialysis buffer. For the determination of the enantiomers in serum, C.V.s below 6% were obtained for intra- and inter-assay variability.

Table I - Intra- and inter-assay coefficient of variation (%) for the determination of carvedilol enantiomers in dialysis buffer by HPLC

Concentration (ng/ml)	Intra-assay C.V. (%)[a]		Inter-assay C.V. (%)[b]	
	(+)-(R)	(-)-(S)	(+)-(R)	(-)-(S)
10	5.7	6.8	6.9	5.9
100	5.4	1.5	1.4	1.3

[a] n = 4 determinations
[b] n = 3 days

Table II - Intra- and inter-assay coefficient of variation (%) for the determination of carvedilol enantiomers in serum by HPLC

Concentration (μg/ml)	Intra-assay C.V. (%)[a]		Inter-assay C.V. (%)[b]	
	(+)-(R)	(-)-(S)	(+)-(R)	(-)-(S)
2	2.1	5.7	4.2	5.1
4	3.6	5.6	5.6	5.7

[a] n = 4 determinations
[b] n = 3 days

3.2 Separation of Chiral Drugs by Capillary Electrophoresis

Although the HPLC method using the Phenomenex 3022 column was relatively sensitive and reliable, the resolution of the enantiomers of carvedilol was incomplete. Therefore an alternative method was investigated. Capillary electrophoresis methods offer numerous advantages over HPLC, including faster method development, low consumption of solvents and reagents, and principally much higher separation efficiencies. In terms of chiral separations, CE offers the advantage of a variety of very efficient chiral selectors at a lower cost compared to chiral HPLC columns.

In our early investigations the robustness of stereoselective methods by CE was evident with the use of cyclodextrins as chiral selectors. Figure 19 shows the separation of several chiral cardioactive drugs by capillary electrophoresis. By using only two types of derivatized cyclodextrins, namely dimethyl-β-cyclodextrin (DM-β-CD) and trimethyl-β-cyclodextrin (TM-β-CD), the enantiomers of drugs in ethanolic solutions

or after extraction from plasma could be baseline (or near baseline) resolved, with a minimum of method optimization. The electrophoresis was conducted at 16 kV using a fused silica capillary, uncoated, 75 μm I.D., 57 cm (50 cm to detector), the injection was by pressure for 5 s, the buffer consisted of 18 mM tris(hydroxymethyl)aminomethane (TRIZMA base), 0.03 mM hexadecyltrimethylammonium bromide (HTAB), pH 2.5, the cartridge temperature was set to 20 °C, and the detection was by UV at 214 nm. Methylcellulose (MC) was added to the buffer in order to increase enantiomer resolution.

There are a few reports in the literature on the use of CE for the determination of drugs in biological fluids (Lloyd *et al.*, 1991, Meier and Thormann, 1991, Thormann *et al.*, 1991, Honda *et al.*, 1992, Pruñonosa *et al.*, 1992b, Reinhoud *et al.*, 1992, Soini *et al.*, 1992, Brunner *et al.*, 1993, Gareil *et al.*, 1993, Johansson *et al.*, 1993, Wolfisberg *et al.*, 1993, Brunner *et al.*, 1994, Dethy *et al.*, 1994, Fernandez *et al.*, 1995, Hu *et al.*, 1995, Luksa and Josic, 1995, Hadwiger *et al.*, 1996, Lloyd, 1996). However, these methods have not yet been used in complete pharmacokinetic studies. Pruñonosa *et al.* (1992) developed a non-stereoselective method for the determination of the pharmacokinetics of cicletanine in humans. Up to the present, chiral CE methods have not been used for pharmacokinetics studies, including protein binding studies. The applicability of CE using cyclodextrins for the determination of carvedilol enantiomers was therefore investigated.

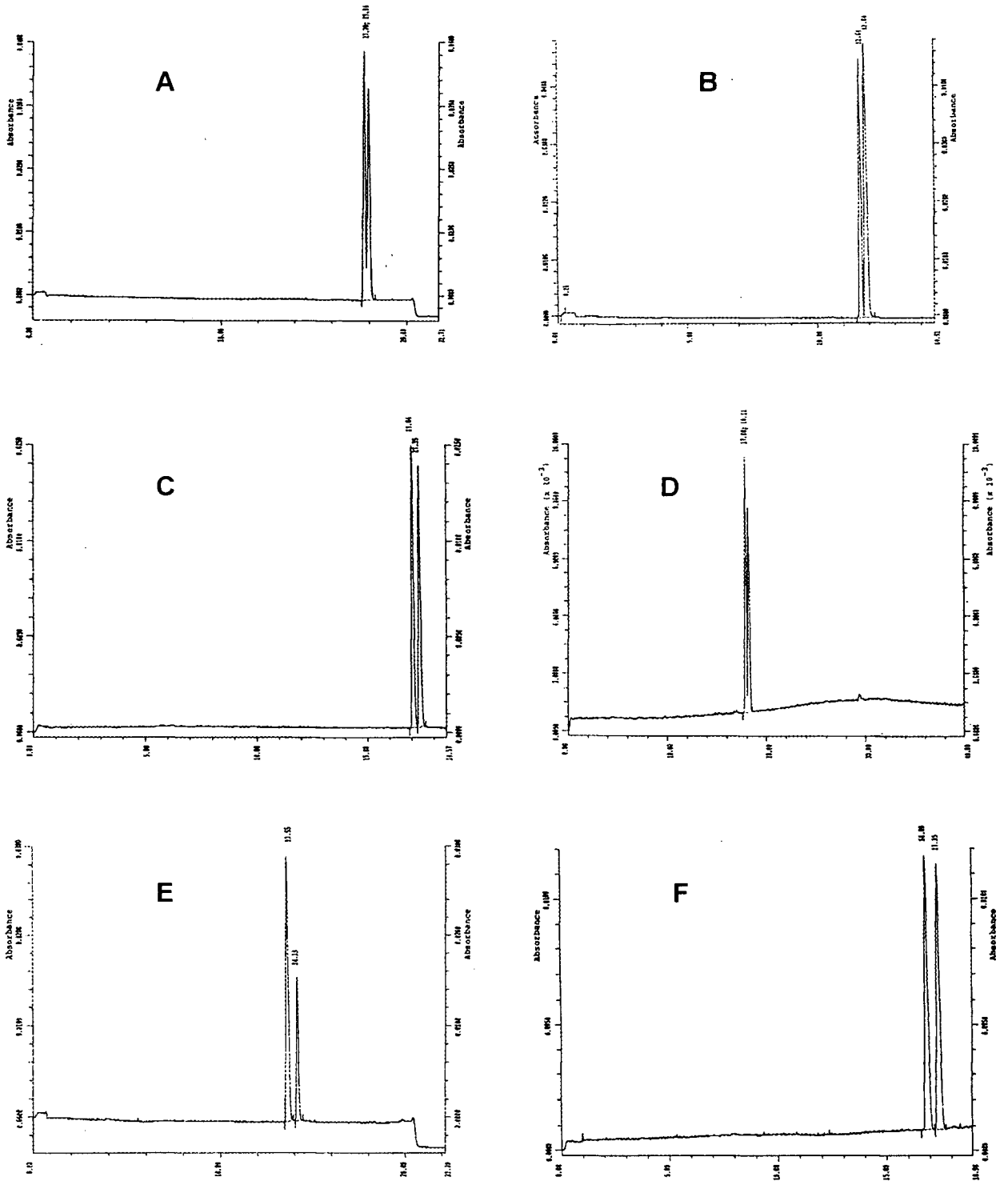

Figure 19. Separation of enantiomeric drugs by capillary electrophoresis. **Conditions:** as described in text. Samples and chiral selector: **A:** racemic propranolol and **B:** tocainide enantiomers, 20 mM TM-β-CD; **C:** racemic pindolol, 10 mM DM-β-CD, 0.02% MC; **D:** racemic sotalol, same as in C, 0.1% MC; **E:** mexiletine enantiomers in plasma, same as in A; **F:** racemic verapamil in plasma, 10 mM TM-β-CD, 0.01% MC.

3.3 Development of a Capillary Electrophoresis Assay for Carvedilol Enantiomers in Serum

3.3.1 Choice of the Chiral Selector

Several types of cyclodextrin were initially tested in high and low concentration in the run buffer, and resolution of carvedilol enantiomers was examined. γ-Cyclodextrin at 10 and 50 mM did not produce resolution of the enantiomers. This could be due to the large size of the cavity of the cyclodextrin (γ-cyclodextrin has 8 glucose units) which did not allow stereoselective interaction with carvedilol. Partial resolution was obtained with 3 and 15 mM β-cyclodextrin, as can be observed in Figure 20. This cyclodextrin has a smaller cavity, as it contains only 7 glucose units in its structure, apparently providing better interaction of the enantiomers of carvedilol.

Substitution at the secondary hydroxyl rim on the surface of the cyclodextrin can dramatically affect selectivity of the separation as it will provide interaction points to form diastereomeric complexes with the analyte with different association constants (Fanali, 1989, Schutzner and Fanali, 1992, Altria, 1996). A substituted β-cyclodextrin, dimethyl-β-cyclodextrin, was tested, and the electropherograms of the separation obtained are shown in Figure 21. When used at a concentration of 5 mM in the buffer, it produced partial resolution of the enantiomers. An increase in the concentration to 20 mM resulted in even less separation of the enantiomers. Trimethyl-β-cyclodextrin, at 10 mM in the buffer, did not favor stereoselective interaction and a single peak was obtained for racemic carvedilol. Optimal results were obtained using a β-cyclodextrin substituted with hydroxypropyl groups. Hydroxypropyl-β-cyclodextrin, at 10 mM in the

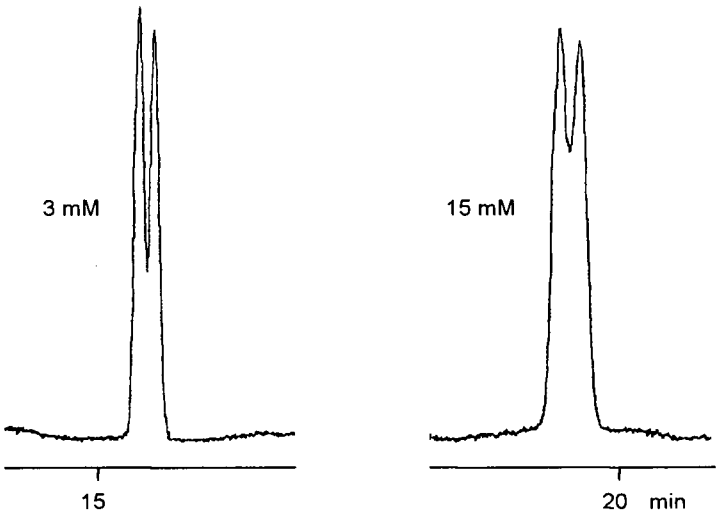

Figure 20. Separation of carvedilol enantiomers using β-cyclodextrin as the chiral selector. **Electrophoretic conditions:** buffer: 25 mM sodium phosphate, pH 2.6; capillary: fused silica, uncoated, 57 (50) cm length, 75 μm I.D.; voltage: 20 kV; temperature: 20 °C; injection: pressure, 5 s; detection: UV at 214 nm.

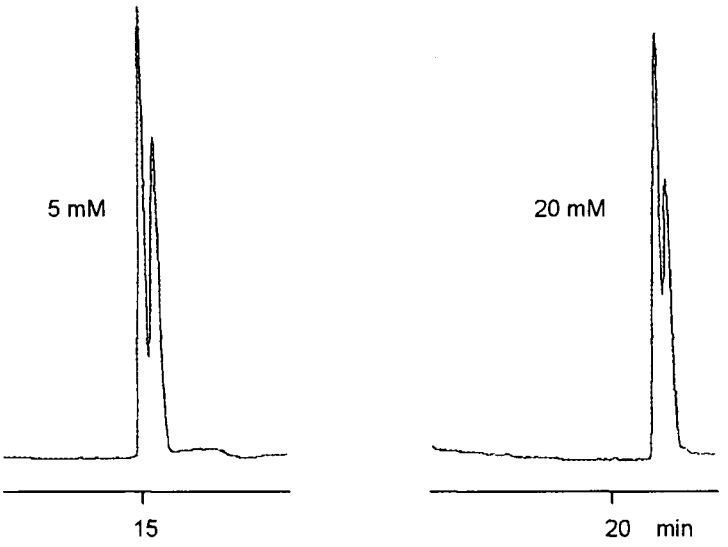

Figure 21. Separation of carvedilol enantiomers using dimethyl-β-cyclodextrin as the chiral selector. **Electrophoretic conditions:** buffer: 25 mM sodium phosphate, pH 2.6; capillary: fused silica, uncoated, 47 (40) cm length, 75 μm I.D.; voltage: 12 kV; temperature: 20 °C; injection: pressure, 4 s; detection: UV at 200 nm.

run buffer, produced very good separation of the enantiomers, although only partial resolution was obtained at 50 mM.

It has been postulated that the substitution of the secondary hydroxyl groups on the cyclodextrin rim with hydroxypropyl groups provides a less restricted hydroxyl group and at an appropriate length for hydrophilic interactions with the hydroxyl group at the chiral centre and the amine group close to the chiral centre for propranolol (St. Pierre and Sentell, 1994). The authors observed improved resolution for propranolol enantiomers when changing the chiral selector from unsubstituted β-cyclodextrin to hydroxypropyl-β-cyclodextrin. The same explanation could be valid for carvedilol where the same effect of the type of cyclodextrin on resolution was obtained and in which the positions of the interacting groups are the same.

The substitution of the secondary hydroxyl groups on the rim with methyl groups as in the dimethyl-β-cyclodextrin also provides less restricted interaction sites as compared to the unsubstituted native β-cyclodextrin. However, these interactions are hydrophobic, weaker interactions as compared to the interactions that can occur with the hydroxypropyl group, and therefore result in less enantioselectivity compared to that provided by hydroxypropyl-β-cyclodextrin (St. Pierre and Sentell, 1994).

3.3.2 Effect of Cyclodextrin Concentration on Resolution

The optimum concentration of hydroxypropyl-β-cyclodextrin (HP-β-CD) for the separation of carvedilol enantiomers was determined. Figure 22 shows the electropherograms corresponding to each HP-β-CD concentration tested, illustrating the effect of the chiral selector concentration on the resolution of carvedilol enantiomers.

As the HP-β-CD concentration increased from 5 to 10 mM, an increase in the separation of carvedilol enantiomers was observed. Maximum separation of the peaks was obtained at 10 mM HP-β-CD in the run buffer. Buffers containing ± 1 mM of the HP-β-CD concentration of maximum resolution (10 mM) were also tested and a slight decrease in resolution resulted. The existence of an optimum concentration of chiral selector, which enhances the difference between the apparent electrophoretic mobilities of the enantiomers, has been demonstrated by Wren and coworkers (Wren, 1993, Wren and Rowe, 1993, Wren *et al.*, 1994). However, at cyclodextrin concentrations higher than the optimum, enantioselective interactions appear to be overwhelmed by nonspecific hydrophobic interactions and enantiomer resolution is precluded (Sepaniak *et al.*, 1992). The migration time increased with an increase in cyclodextrin concentration. This is due to longer residence time of the drug in the complex form as well as to an increase in the viscosity of the buffer with a reduction of the mobility of the analytes. This has also been observed by other investigators for propranolol (Wren and Rowe, 1993, Guttman and Cooke, 1994) and clenbuterol (Altria *et al.*, 1992). Table III presents the resolution factors (R_s) calculated for each electropherogram. Near baseline resolution, corresponding to an R_s = 1.46, was obtained using 10 mM HP-β-CD.

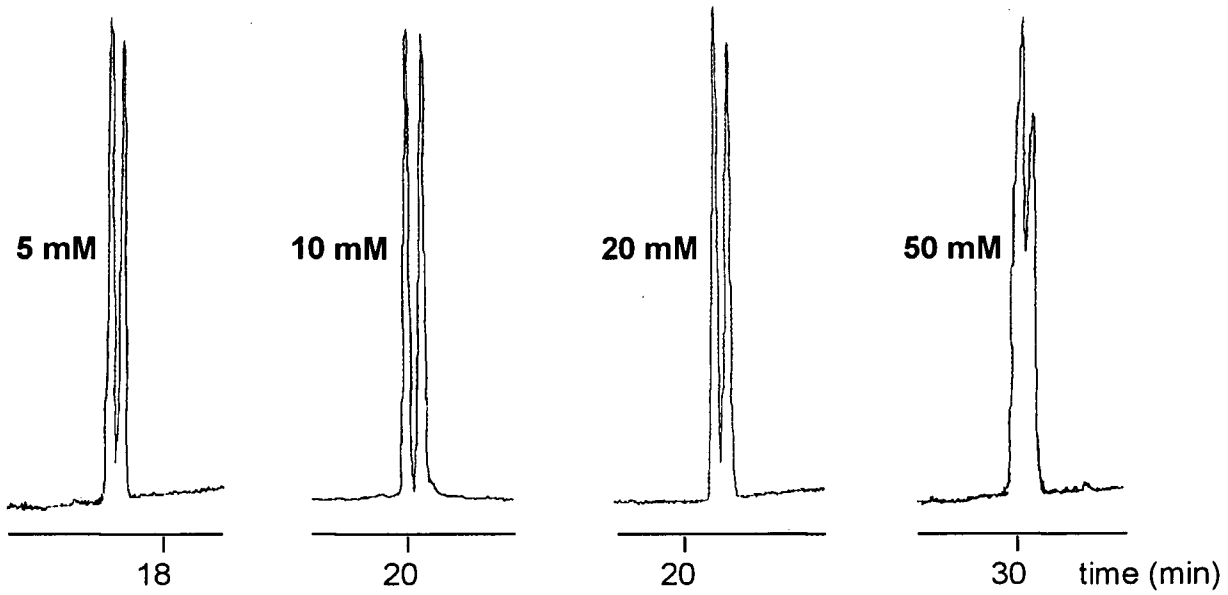

Figure 22. Electropherograms of the separation of carvedilol enantiomers by CE using different concentrations of hydroxypropyl-β-cyclodextrin in the run buffer. **Electrophoretic conditions:** background electrolyte: 25 mM sodium phosphate buffer, pH 2.5; capillary: fused silica, uncoated, 57 (50) cm length, 75 μm I.D.; voltage: 14 kV; temperature: 20 °C; injection: pressure, 2 s; detection: UV at 214 nm.

Table III - Effect of hydroxypropyl-β-cyclodextrin concentration on the resolution of carvedilol enantiomers

Concentration	R_s
5 mM	1.20
10 mM	1.46
20 mM	1.18
50 mM	0.68

3.3.3 Effect of Voltage on Efficiency

In capillary electrophoresis, voltage plays a major role in determining analyses times. It can also affect the efficiency of analysis since efficiency is directly proportional to the voltage (McLaughlin *et al.*, 1992).

In order to determine the optimum separation voltage for the analysis of carvedilol by CE, several runs were performed with gradual increases in the applied voltage. Figure 23 shows a plot of the calculated number of theoretical plates (N) *versus* voltage applied for the (+)-(R)-carvedilol peak. There was an increase in efficiency following an increase in applied voltage from 10 to 18 kV, with a maximum of 102,156 theoretical plates obtained at 18 kV.

Further increases in voltage resulted in decreased efficiencies. The explanation for this is that the capillary becomes less effective in heat dissipation after a certain voltage level, where excessive Joule heat is generated (Fanali *et al.*, 1990). As heat is produced inside the capillary, the viscosity of the buffer decreases, and sample diffusion resulting in peak broadening becomes significant.

An applied voltage of 18 kV was thus selected for further analyses.

3.3.4 Effect of Buffer Concentration on Efficiency

A series of buffer concentrations (10, 15, 20, 25, 50 mM phosphate buffer, pH 2.5) were evaluated for their effect on efficiency of enantiomer resolution and migration times. Buffer concentrations from 10 to 25 mM resulted in similar resolution for carvedilol enantiomers with a gradual decrease in migration times. Efficiency and also the peak areas increased as the buffer concentration increased. This could be explained by a reduction in drug-capillary wall interactions which can cause peak

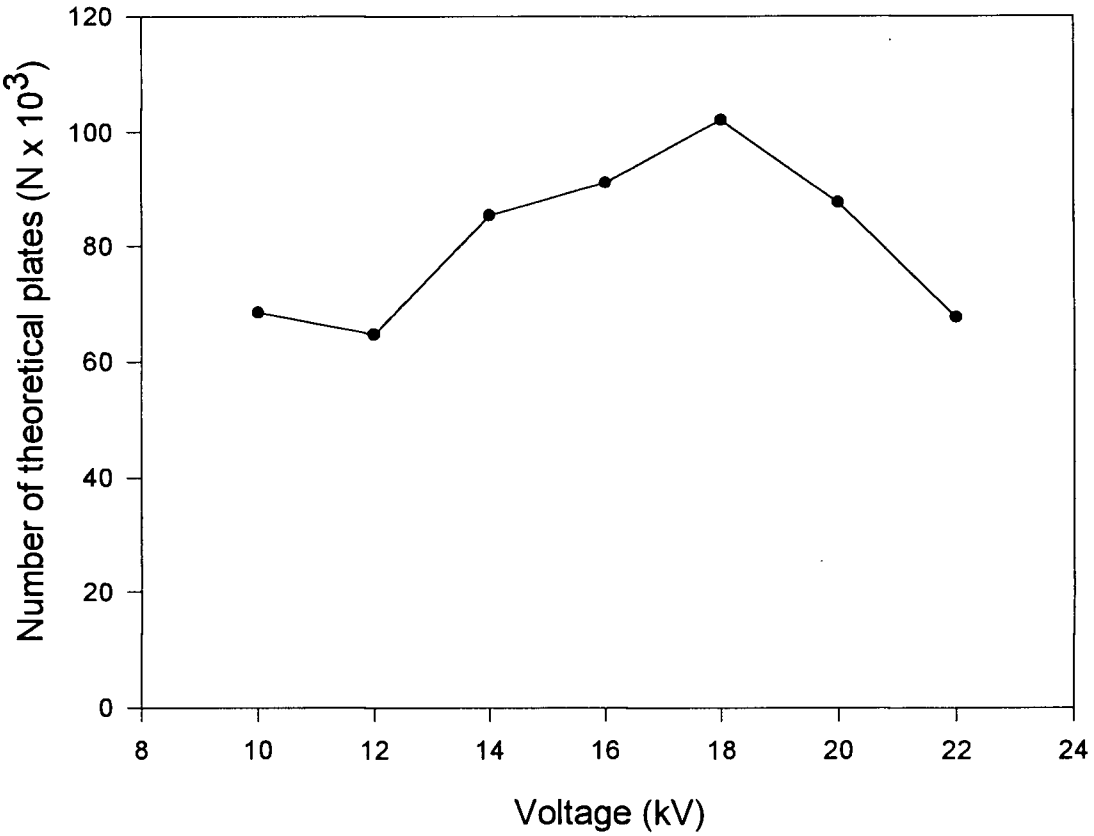

Figure 23. Effect of applied voltage on efficiency of separation in the analysis of carvedilol by capillary electrophoresis.

Electrophoretic conditions: background electrolyte: 25 mM sodium phosphate buffer, pH 2.5, 10 mM hydroxypropyl-β-cyclodextrin; capillary: fused silica, uncoated, 57 (50) cm length, 75 μm I.D.; temperature: 20 °C; injection: pressure, 4 s; detection: UV at 200 nm.

broadening and lower mass recovery. However, the use of buffer concentrations higher than 25 mM decreased greatly enantiomer resolution probably due to high current generated inside the 75 μm I.D. capillary. The use of high buffer concentrations would thus require the use of a capillary with smaller internal diameter. Based on these

observations an optimum buffer concentration of 25 mM was selected for further analyses.

3.3.5 Effect of Cartridge Temperature on Resolution

Capillary temperature control is extremely important for reproducibility of the assay. When current passes along a capillary, part of the electrical energy is converted into Joule heating. Temperature changes viscosity of the buffer and therefore the migration velocity of the analytes affecting the migration times and consequently the resolution of the analytes (Altria *et al.*, 1992). To control or minimize the effects of Joule heating, temperature can be controlled with fan-blown air or by a recirculating liquid, with the capillary mounted in a cartridge. The Beckman P/ACE 5000 equipment used for this study uses a circulating coolant containing perfluoro compounds, $C_{5\text{-}18}$, to maintain the temperature inside the capillary cartridge.

The resolution of carvedilol enantiomers decreased slightly with an increase in temperature from 15 to 25 °C. Temperature can also influence the kinetics of the inclusion complexation with the cyclodextrins. For example, the stability constant of the cyclodextrin inclusion complex would decrease with temperature, thus resulting in a decrease in resolution (Schutzner and Fanali, 1992).

Little change in migration times were observed over the range of temperatures tested (15-25 °C). However, at temperatures lower than 20 °C, it was noticed that the capillary electrophoresis instrument was not as efficient in controlling the temperature, and that equilibration time was rather long. A convenient operational temperature of 20 °C was thus selected for the analyses.

3.3.6 Optimized CE Assay for Carvedilol Enantiomers in Serum

The optimum conditions for the determination of carvedilol enantiomers in serum were determined. The final assay consisted of an extraction from serum and reconstitution in HPLC grade ethanol for injection into the capillary. The optimized electrophoretic conditions selected were: background electrolyte: 25 mM sodium phosphate buffer, pH 2.5, 10 mM hydroxypropyl-β-cyclodextrin; capillary: fused silica, uncoated, 57 (50) cm length, 75 μm I.D.; voltage: 18 kV; temperature: 20 °C; injection: pressure, 4 s; detection: UV at 200 nm; internal standard: (-)-propranolol.

Ideally, in CE, the sample should be dissolved in an aqueous buffer to avoid differences in conductivity along the capillary length. However, the serum extract containing carvedilol was not soluble in aqueous buffer, so it was necessary to reconstitute the extracts in ethanol for injection into the capillary. The sample plug of ethanol formed inside the capillary was poorly conductive, therefore the length of the sample zone (and consequently the injection time) was limited. It was observed that resolution of carvedilol enantiomers in ethanol decreased as the injection time increased. On the other hand, as one of the aims of analytical method development for carvedilol is high sensitivity, larger volumes of sample introduced into the capillary were desirable. Therefore, a balance between sample load, resolution and efficiency had to be achieved. An injection by pressure for 4 s was found to provide a reasonable sample load and maintain resolution.

Low operational pH was found to be essential for the resolution of carvedilol enantiomers. As discussed in section 1.3.4.1.1 (Introduction, Mechanism of Chiral Recognition), the stereoselective interaction with the cyclodextrins is highly dependent on the ionization state of the analyte. In the case of carvedilol, which exhibits an

ionoselective type of interaction, only the ionized form interacts stereoselectively with hydroxypropyl-β-cyclodextrin, therefore it was necessary to use a buffer with low pH to ensure that the drug was ionized.

Figure 24 displays a representative electropherogram of an extract from serum. Carvedilol enantiomers passed the detector window at 14.1 and 14.3 min, corresponding to (+)-(R)- and (-)-(S)-carvedilol, respectively. The internal standard migrated past the detector at 12.5 min. A separation time of 16 min was sufficient for the analysis which, when combined with the rinsing time (2.5 min + 1 min), resulted in a total analysis time of 19.5 min. An injection of an extract from blank serum did not show any interfering peaks. In fact, components from serum were found to elute after 20 min of separation under 18 kV, which is later than the migration times of carvedilol and the internal standard. As opposed to HPLC, where the final analysis time depends on the elution of all sample components from the column, before the subsequent sample can be injected, in CE this is not necessary. As soon as the substance of interest passes the detector window and is recorded as a peak, the separation can be stopped. The capillary can then be rinsed with appropriate solutions to remove all the remaining components of the sample thereby contributing to the faster analysis times observed in CE. In our early investigations on chiral separation of drugs by CE using cyclodextrins, the absence of interference from the sample matrix was also evident as can be observed in the electropherograms of verapamil and mexiletine enantiomers extracted from plasma shown in Figure 19 (E, F). The absence of major interference from the sample matrix is one of the advantages of CE. Direct injection of serum and saliva after an ultrafiltration procedure (Thormann *et al.*, 1993) and injection of urine

samples after a simple filtration (Lukkari *et al.*, 1993) have been reported using micellar electrokinetic capillary chromatography.

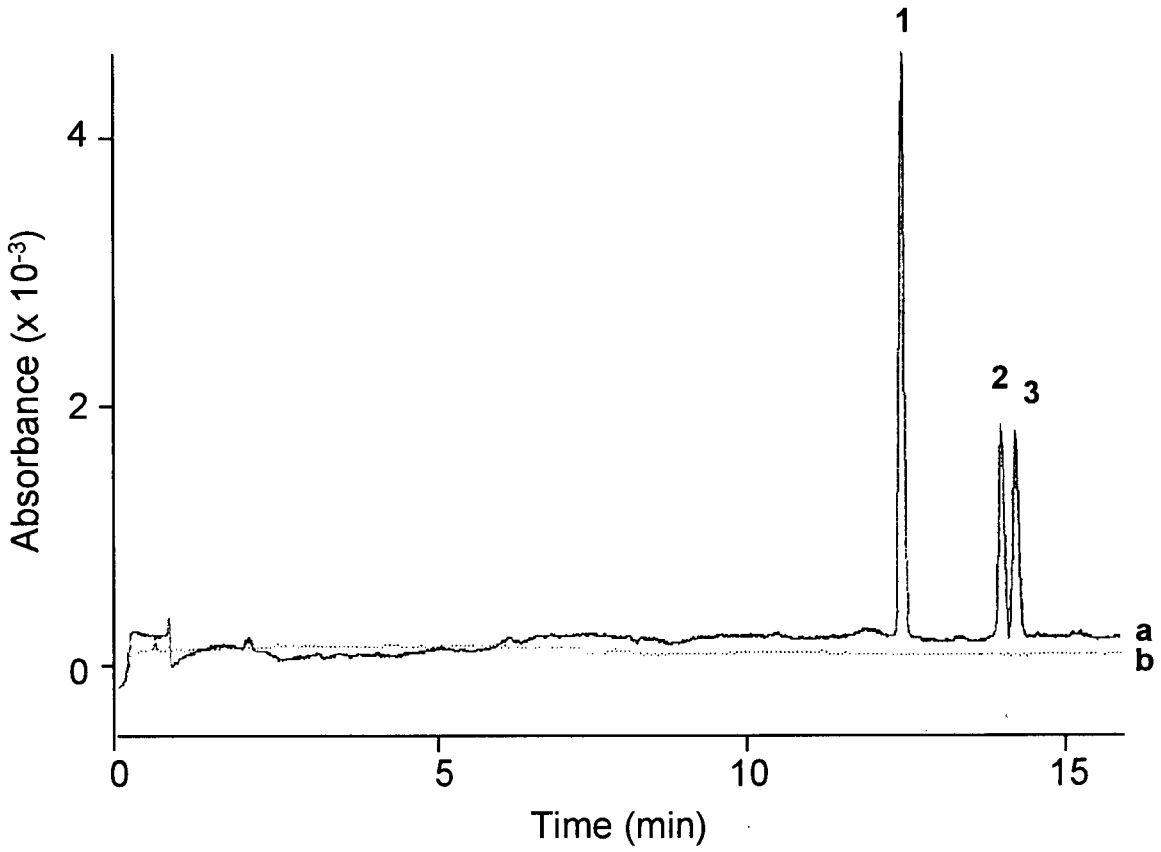

Figure 24. Representative electropherogram of an extraction from serum. **(a)**: serum spiked with (±)-carvedilol and internal standard; **(b)**: blank serum; **(1)**: (-)-propranolol, **(2)**: (+)-(R)-carvedilol; **(3)**: (-)-(S)-carvedilol. **Electrophoretic conditions:** background electrolyte: 25 mM sodium phosphate buffer, pH 2.5, 10 mM hydroxypropyl-β-cyclodextrin; capillary: fused silica, uncoated, 57 (50) cm length, 75 μm I.D.; voltage: 18 kV; temperature: 20 °C; injection: pressure, 4 s; detection: UV at 200 nm.

3.3.6.1 Determination of Detector Linearity for Carvedilol Enantiomers in Serum

Excellent correlation was obtained between corrected peak area ratios and carvedilol concentration in the 50-4000 ng/ml per enantiomer range, with r^2 values of 0.999 for both enantiomers. The limit of quantitation of the assay was 50 ng/ml per enantiomer in serum. Figure 25 shows the calibration curves for carvedilol enantiomers added to serum.

Since carvedilol is highly bound to serum proteins the unbound concentrations of the enantiomers are extremely low. Due to the relatively high LOQ obtained with the CE method (50 ng/ml per enantiomer) it was not possible to use the CE assay for the determination of free drug levels. Therefore, the free drug concentrations had to be determined using the chiral HPLC method developed using the Phenomenex column. However, the CE method was suitable for the determination of total levels in serum. Calibration curves in the serum concentration range of 1-5 μg/ml per enantiomer were used for protein binding studies. Coefficients of determination (r^2) values of 0.999 were also obtained for the enantiomers at this concentration range.

3.3.6.2 Determination of the Reproducibility of the CE Assay

The intra-assay variability of the CE assay for the determination of carvedilol enantiomers in serum was determined by analyzing 5 replicate samples of carvedilol in serum at concentrations of 100 and 1000 ng/ml per enantiomer. Intra-assay variabilities were less than 8% at the lower concentration, and less than 5% at the higher concentration. Inter-assay reproducibility was determined by performing the analysis described above on three different days. Coefficients of variation were less

than 3% at 100 ng/ml, and less than 1% at 1000 ng/ml per enantiomer in serum. The results obtained for intra- and inter-assay reproducibility are listed in Table IV.

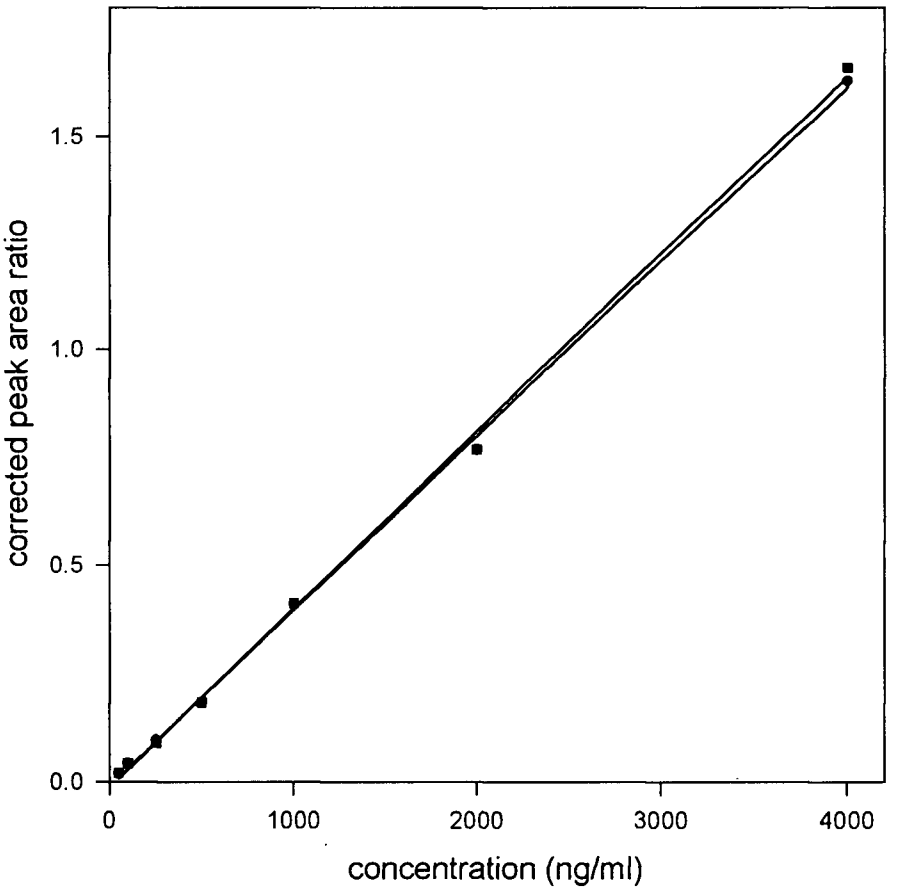

Figure 25. Standard curve for carvedilol enantiomers in serum analyzed by CE. (●): (+)-(R)-carvedilol; (■): (-)-(S)-carvedilol. **Electrophoretic conditions:** background electrolyte: 25 mM phosphate buffer, pH 2.5, 10 mM hydroxypropyl-β-cyclodextrin; capillary: fused silica, uncoated, 57 (50) cm length, 75 μm I.D.; voltage: 18 kV; temperature: 20 °C; injection: pressure, 4 s; detection: UV at 200 nm. **Line parameters (y = a + bx):** (+)-(R)-carvedilol: a = -0.010; b = 4.06 x 10^{-4}; r^2 = 0.999; (-)-(S)-carvedilol: a = -0.014; b = 4.13 x 10^{-4}; r^2 = 0.999.

Table IV - Concentrations obtained and intra- and inter-assay variability data for the determination of carvedilol enantiomers in serum by capillary electrophoresis

	100 ng/ml			1000 ng/ml		
	(+)-(R)	**(-)-(S)**	**R/S**	**(+)-(R)**	**(-)-(S)**	**R/S**
day 1[a]	96.2	98.5	0.98	1016.2	983.7	1.03
s.d.	4.4	7.8		48.0	35.8	
C.V. (%)	4.6	7.9		4.7	3.6	
day 2[a]	101.6	102.9	0.99	1019.7	997.3	1.02
s.d.	7.3	7.6		43.7	44.7	
C.V. (%)	7.2	7.4		4.3	4.5	
day 3[a]	99.3	101.7	0.98	1004.4	978.7	1.03
s.d.	7.9	5.4		35.7	31.6	
C.V. (%)	8.0	5.3		3.6	3.2	
average[b]	99.0	101.0	0.98	1013.4	986.6	1.03
s.d.	2.7	2.3		8.1	9.6	
C.V. (%)	2.7	2.3		0.8	1.0	

[a] average of 5 replicates
[b] average of 3 days

3.4 Comparison Between the HPLC and the CE Assays

HPLC is an established analytical method widely used for chiral separations. The results obtained by the developed chiral CE assay were compared with the HPLC method as a means of validating the CE assay. As pointed out by Altria *et al.* (1994), agreement between two different separation techniques reinforces the validity of the method under investigation.

Serum concentration values obtained by CE correlated well with those obtained by HPLC, with a correlation coefficient (r) of 0.995. A graph of the correlation between the concentrations obtained by the two methods is presented in Figure 26. The parameters of the line obtained (value ± confidence limit) were slope (b) = 0.990 ± 0.044, and intercept (a) = 0.057 ± 0.151. Statistical analysis (Student's *t* test) of the parameters obtained for the correlation line indicate that the slope is not different from the theoretical slope of unity (t = 0.477, p>0.50), and the intercept is not different from zero (t = 0.785, p>0.20) (n = 25).

Good correlation between CE and HPLC were also reported by Pruñonosa *et al.* (1992a) for the determination of cicletanine in plasma, and by Nielen (1993) in a study of enantiomeric purity of phenoxy acid herbicides.

In terms of analysis times, CE offers substantial advantages compared to HPLC. In the present method the analysis time required for enantiomer resolution using the CE method was about 15 min plus a rapid conditioning cycle with 5 consecutive rinses (total rinse time = 3.5 min) to ensure reproducibility of the assay and also to remove serum components which tend to adhere to the capillary wall. Thus, the total analysis time was 18.5 min for CE, whereas for the HPLC assay, an analysis time of 27 min was

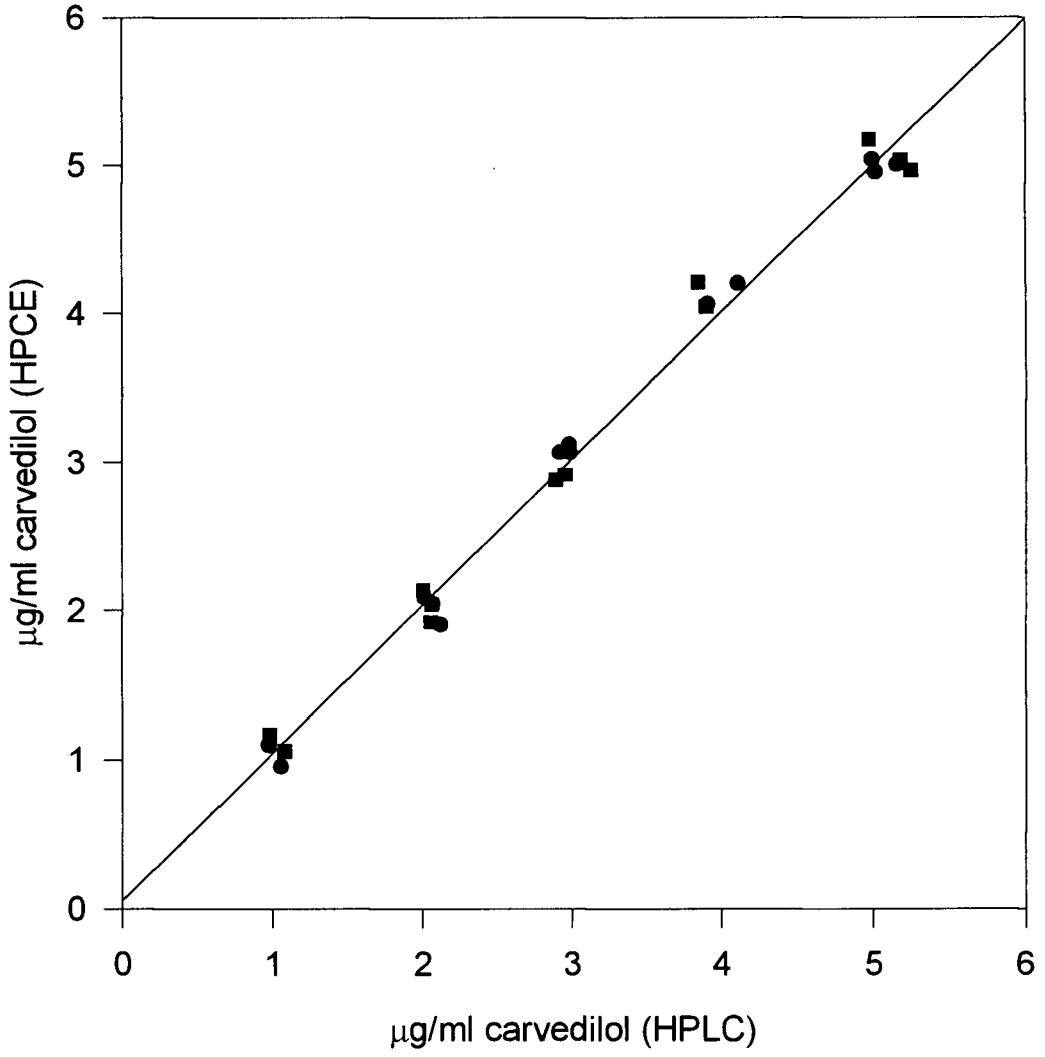

Figure 26. Correlation graph of serum concentration values for (+)-(R)-carvedilol (●), and (-)-(S)-carvedilol (■), obtained by CE and HPLC. **Line parameters:** correlation coefficient (r) = 0.995; slope (b) = 0.990 (not different from the theoretical slope of unity, t = 0.477, p>0.50); intercept (a) = 0.057 (not different from zero, t = 0.785, p>0.20) (n = 25).

necessary. While the HPLC chiral column required an equilibration time of about 1 hour prior to analysis, this is not required with the CE technique.

Overall, method development was much faster with CE since it did not require extensive equilibration times compared with the chiral HPLC columns. Several buffer systems can be tested in CE in less than one hour, while the equilibration time with a new mobile phase required by the chiral HPLC columns is usually greater than 30 minutes. The low consumption of solvents and buffer additives is also a major advantage in CE. Only 10 ml of buffer were sufficient to perform about 20 determinations. The chiral selectors used in CE are also relatively less expensive than the chiral HPLC columns.

One of the principal limitations of CE is the detection sensitivity. Due to the extremely small diameter of the capillary, volumes of up to only 20 nl are typically injected. For the same reason the light path at the on-capillary detector window is very short, reducing the signal produced by the sample. For the chiral CE method developed for carvedilol in serum a limit of quantitation (LOQ) of 50 ng/ml per enantiomer was obtained whereas HPLC provided an LOQ of 1 ng/ml. In terms of injection volumes, in HPLC, injections of 10 µl were used, whereas the injection volumes calculated for CE were of the order of 18 nl. Thus the amounts of drug at the detector were approximately 0.003 ng and 0.04 ng of each enantiomer for CE and HPLC, respectively. It can be seen that the sensitivity at the detector was actually higher for CE compared to HPLC. It should also be noted that the detection systems used were different. While HPLC used fluorescence detection, detection in the CE method was by UV absorption. The fact that mostly aqueous buffers are used in CE allows monitoring at lower wavelengths as compared to HPLC. Hence the sensitivity

limitations of capillary electrophoresis are related to the small quantities injected and consequently dependent on the concentration of the starting material. This is particularly significant in pharmacokinetic studies where drug concentrations are often extremely low, an example being carvedilol. Several methods have been developed to overcome this problem such as on-column concentration techniques (isotachophoresis, pH differences), the use of extended light-path capillaries and laser-induced fluorescence detection but they are able to only partially ameliorate detection limitations.

3.5 *In Vitro* Serum Protein Binding of Carvedilol Enantiomers

3.5.1 Determination of Extraction Recovery of Carvedilol from Serum and from Dialysis Buffer

The recovery of carvedilol enantiomers extracted from serum was studied at 100 and 1000 ng/ml of each enantiomer. The results obtained for each enantiomer are presented in Table V. About 83% recovery was achieved at a concentration of 100 ng/ml, and 86% at 1000 ng/ml per enantiomer.

Table VI presents the results obtained for the extraction of carvedilol enantiomers from dialysis buffer. Recoveries of approximately 87% at 10 ng/ml, and 82% at 100 ng/ml per enantiomer were achieved.

Table V - Peak area ratios carvedilol enantiomer/internal standard of control and extracted samples and extraction recoveries of carvedilol enantiomers from serum at 100 and 1000 ng/ml using ethyl ether

Concentration	Peak Area Ratios[a]				Recovery (%)	
	Control		Extraction			
(ng/ml)	(+)-(R)	(-)-(S)	(+)-(R)	(-)-(S)	(+)-(R)	(-)-(S)
100	1.61 ± 0.06	1.48 ± 0.10	1.34 ± 0.06	1.23 ± 0.02	83.2	83.1
1000	21.11 ± 1.78	20.09 ± 1.78	18.53 ± 2.19	16.97 ± 2.14	87.8	84.5

[a] values are mean ± standard deviation of 3 determinations

Table VI - Peak area ratios carvedilol enantiomer/internal standard of control and extracted samples and extraction recoveries of carvedilol enantiomers from dialysis buffer at 10 and 100 ng/ml using ethyl ether

Concentration	Peak Area Ratios[a]				Recovery (%)	
	Control		Extraction			
(ng/ml)	(+)-(R)	(-)-(S)	(+)-(R)	(-)-(S)	(+)-(R)	(-)-(S)
10	1.16 ± 0.07	1.11 ± 0.05	1.02 ± 0.10	0.95 ± 0.11	87.9	85.6
100	14.27 ± 2.57	12.73 ± 1.91	11.53 ± 1.17	10.45 ± 0.68	80.8	82.1

[a] values are mean ± standard deviation of 3 determinations

3.5.2 Determination of Nonspecific Binding of Carvedilol to Centrifree Ultrafiltration Units

The binding of carvedilol to the Centrifree Micropartition System was determined. The recovery in percentage of carvedilol enantiomers from the units after ultrafiltration was only 37.2 ± 2.0 and 37.9 ± 1.0 (mean ± s.d., n = 3 determinations) for (+)-(R)- and (-)-(S)-carvedilol, respectively. The extremely high nonspecific binding of carvedilol to the ultrafiltration devices makes the use of such a system inadequate for the determination of free levels of the drug in serum.

Nonspecific loss of drug to the ultrafiltration device was also reported by Parsons and Fan (1986) for propranolol. The authors report that there was significant loss of drug to the membrane, but principally to the O-ring. It was also noted that the equilibration with the membrane was fast, while the loss of propranolol to the O-ring was initially rapid, followed by a slow and continuous binding. The time-dependency of the non-specific binding makes a correction factor for recovery unreliable (Parsons and Fan, 1986). Other studies have also reported binding of drugs to the ultrafiltration membranes (Hinderling *et al.*, 1974, Zhirkov and Piotrovskii, 1984, Ohshima *et al.*, 1988, Liu *et al.*, 1992).

3.5.2.1 Treatment of the Centrifree Ultrafiltration Units to Avoid Binding

Table VII lists the recoveries of drug obtained after ultrafiltration following the treatment of the Amicon units. None of the treatments evaluated was able to counteract the non-specific binding of carvedilol to the units. Higher recoveries (about 78%) were obtained by treating the ultrafiltration units with a carvedilol solution. Carvedilol probably acted by saturating the binding sites on the surface of the device.

Although not determined, unpredictable release of drug could occur during ultrafiltration with the use of such treatment. Furthermore, one of the main advantages of ultrafiltration is speed and the need for a preliminary treatment of the units would eliminate this convenience.

Table VII - Effect of different treatments of the Centrifree Micropartition ultrafiltration units on the recovery of carvedilol enantiomers after ultrafiltration

	Recovery (%)[a]	
Treatment	**(+)-(R)-Carvedilol**	**(-)-(S)-Carvedilol**
untreated	30.4 ± 5.0	28.9 ± 3.7
2 μg/ml carvedilol	81.7 ± 1.6	78.7 ± 2.8
2% TEA	24.2 ± 2.7	22.8 ± 2.2
10 μg/ml propafenone	24.5 ± 2.1	24.4 ± 3.4
5% SDS	19.1 ± 1.4	18.3 ± 2.3
5% Triton X-100	36.0 ± 0.4	34.5 ± 1.0

[a] Values are mean ± s.d. of 3 determinations

3.5.3 Determination of Nonspecific Binding of Carvedilol to Millipore Ultrafiltration Units

The nonspecific binding of carvedilol to Millipore ultrafiltration units with different membrane materials was assessed. The recoveries of drug after ultrafiltration compared to samples not submitted to ultrafiltration are presented in Table VIII. Carvedilol could not be detected when the polysulfone membranes were used, indicating complete loss of drug to the ultrafiltration unit. The regenerated cellulose membranes resulted in higher recoveries: about 58% of carvedilol was recovered after ultrafiltration when using the lower molecular weight cut-off membranes and about 81% when using the 30,000 Daltons cut-off membranes. However the relatively high extent of nonspecific binding limits the use of the units for the determination of free fractions of a highly protein bound drug such as carvedilol for which the unbound concentrations are already at the limit of quantitation of the analytical assay. Moreover, high variability in the determination was observed (C.V. ranged from 9 to 20%) making the use of such devices unreliable even with the use of a correction factor.

Both ultrafiltration systems tested (Amicon and Millipore) exhibited extensive nonspecific binding of carvedilol making their use unsuitable for the determination of unbound carvedilol in serum and thus these methods, while convenient, were not pursued.

Table VIII - Recoveries of carvedilol enantiomers after ultrafiltration using the Millipore Ultrafree-CL centrifugal filters

		Recovery (%) *	
Membrane	**Cut-off (Daltons)**	**(+)-(R)-Carvedilol**	**(-)-(S)-Carvedilol**
polysulfone	10,000	n.d.	n.d.
	30,000	n.d.	n.d.
regenerated cellulose	10,000	58.6 ± 8.2	57.7 ± 7.4
	30,000	81.2 ± 15.9	82.0 ± 7.0

* mean ± s.d. of 4 determinations
n.d. not detected

3.5.4 Determination of Nonspecific Binding of Carvedilol to Equilibrium Dialysis Units

3.5.4.1 Determination of Nonspecific Binding Using Serum

The nonspecific binding of carvedilol enantiomers to the dialysis apparatus was determined at two drug serum concentrations. High recoveries of drug were obtained after 6 h in contact with the dialysis apparatus. The results are presented in Table IX and suggest that carvedilol does not bind to the dialysis cells when in the presence of serum binding proteins.

3.5.4.2 Determination of Nonspecific Binding Using Dialysis Buffer

For a highly protein bound drug, the loss of drug due to adsorption to the apparatus may not be apparent if the protein-drug solution is used to determine nonspecific binding (Kinget *et al.*, 1979, Fois and Ashley, 1991). Therefore, the binding of drug to the apparatus should be determined in the absence of protein (Fois and Ashley, 1991).

The nonspecific binding of carvedilol to the dialysis units was determined using a solution of the drug in dialysis buffer and the results are presented in Table X. Carvedilol binds to a large extent to the dialysis apparatus (about 60%) as reflected by the low recoveries of drug obtained from the buffer solutions after being in contact with the apparatus for 6 hours, as compared to buffer samples which were not in contact with the dialysis cells.

In equilibrium dialysis, the loss of drug to the apparatus is accounted for by measuring the drug concentration in the retentate and in the dialysate (Kurz *et al.*, 1977). For carvedilol very high adsorption of drug to the devices was observed. Nonetheless, for the determination of free fractions in serum, no correction for adsorption was necessary since both sides of the membrane were analyzed for carvedilol.

Table IX - Recoveries (%) of carvedilol enantiomers in serum at 24 and 240 ng/ml from the dialysis units after 6 h dialysis

	Recovery (%)	
Enantiomer	**24 ng/ml**	**240 ng/ml**
(+)-(R)	99.0 ± 3.9	98.0 ± 7.0
(-)-(S)	100.5 ± 4.6	99.6 ± 6.6

Values are mean ± s.d. of 3 determinations

Table X - Recoveries (%) of carvedilol enantiomers in dialysis buffer at 24 and 240 ng/ml from the dialysis units after 6 h dialysis

	Recovery (%)	
Enantiomer	**24 ng/ml**	**240 ng/ml**
(+)-(R)	47.4 ± 1.5	42.6 ± 0.9
(-)-(S)	48.2 ± 2.3	40.9 ± 1.7

Values are mean ± s.d. of 3 determinations

3.5.5 Determination of Dialysis Equilibrium Time for Carvedilol

The time to reach equilibrium during dialysis of carvedilol in serum against isotonic phosphate buffer was determined by measuring the free drug in the buffer compartment every 2 hours, for up to 12 hours. Figure 27 shows a plot of peak area ratios for carvedilol enantiomers/internal standard in the dialysate against time for the dialysis process. Results indicate that equilibrium is reached after about 4 hours of dialysis as can be observed by the plateau in the curve.

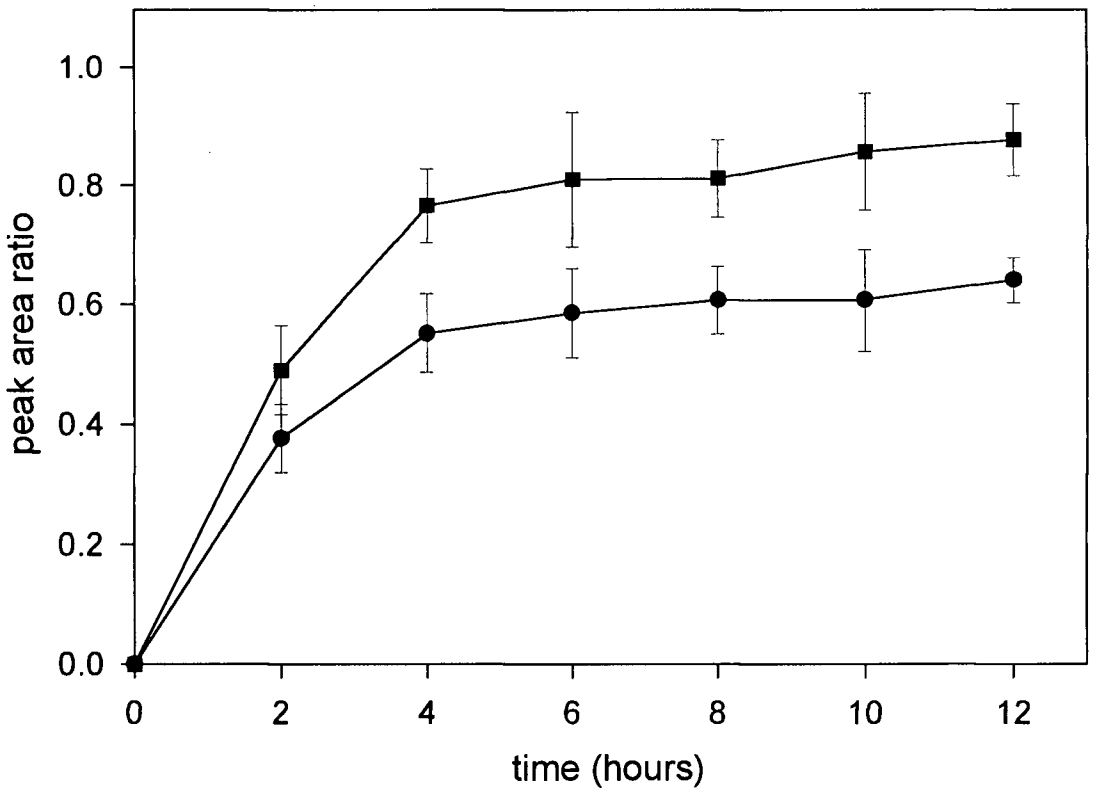

Figure 27. Time-course of equilibrium dialysis for (+)-(R)-carvedilol (●) and (-)-(S)-carvedilol (■) in serum against 0.067 M isotonic phosphate buffer, pH 7.4, at 37 °C, using a 12,000 cut-off cellulose membrane.

ANOVA of the means for all dialysis time-points showed that the means are different for both carvedilol enantiomers ($p<0.01$). A Tukey test (Zar, 1984) was subsequently performed which indicated that means from times 4, 6, 8, 10, and 12 h are the same, and are different from means at time 2 h ($p<0.05$), confirming that equilibrium is reached after 4 h of dialysis for the two enantiomers. A dialysis of 6 h, well within the equilibrium phase, was therefore selected for further experiments.

Addition of 10% TCA to the dialysate would produce turbidity due to protein precipitation if serum proteins were present in the buffer compartment due to leakage of the dialysis membrane. The presence of proteins was not detected in the dialysate up to 12 h of dialysis indicating that the membranes remained intact during the process. No significant volume shifts to the serum compartment were detected.

Long equilibration times are usually linked to slow membrane transport rates. However, it has been demonstrated with a theoretical model, that adsorption of drug to the dialysis apparatus can also significantly increase equilibration time. This is because each fractional decrease in the free drug concentration gradient occurs by transferring a larger amount of drug to the buffer compartment. This will result in longer equilibration time in order to also account for the loss of drug to the apparatus (Fois and Ashley, 1991). Carvedilol was found to be highly bound (about 60%) to the dialysis apparatus as reported in section 3.5.4.2, which could explain the somewhat long time (about 4 h) necessary to attain equilibrium.

There are two reports in the literature related to the protein binding of carvedilol enantiomers in plasma, both using equilibrium dialysis for the determinations. Fujimaki *et al.* (1990) dialyzed human plasma to which carvedilol was added against phosphate buffer for only 2 hours at room temperature. According to our results a dialysis time of

2 h would not be sufficient for the process to reach equilibrium. Therefore the results presented so far for carvedilol may be overestimating the protein binding of the drug in human plasma since the amount of free drug that passed to the buffer compartment would be lower than the amount of free drug at equilibrium. Stahl *et al.* (1993a) used rat plasma containing racemic carvedilol dialyzed at 37 °C, for 4 h. None of the reports stated if the equilibrium time for the dialysis process was determined.

3.5.6 Free Fraction of Carvedilol Enantiomers in Serum Determined by Equilibrium Dialysis

Racemic carvedilol was added to serum obtained from volunteers and the free fraction of the drug determined by equilibrium dialysis. The post-dialysis free fraction (f_u) was calculated by dividing the concentration of carvedilol enantiomers in the buffer dialysate (unbound drug) by the concentration of the enantiomers in the serum retentate (bound and free drug). The results are presented in Table XI and Table XII. Carvedilol is extensively bound to serum proteins (> 98%) as reflected by the extremely low free fractions obtained. The binding was stereoselective: the difference between the free fractions for the two enantiomers was significantly different ($p<0.02$). At a serum concentration of 2 μg/ml of each enantiomer (Table XI), (-)-(S)-carvedilol was less bound (99.1%) to serum proteins than the R-antipode (99.4%), with an R/S ratio of 0.67. In terms of free drug levels these values represent an unbound fraction of 0.9% for (-)-(S)- and 0.6% for (+)-(R)-carvedilol. At a higher serum concentration, 4 μg/ml of each enantiomer (Table XII), the percent bound was 98.6 and 99.0% for (-)-(S)- and (+)-(R)-carvedilol, respectively, with a free fraction R/S ratio of 0.71.

Table XI - Post-dialysis free fractions (f_u) of carvedilol enantiomers in serum at 2 μg/ml determined by equilibrium dialysis

Subject	Gender	Age	Weight (kg)	f_u[a] (+)-(R)	f_u[a] (-)-(S)	R/S ratio
1	F	34	53	0.007 ± <0.001	0.010 ± 0.001*	0.70
2	M	27	80	0.007 ± 0.001	0.010 ± 0.001*	0.70
3	M	27	89	0.005 ± <0.001	0.008 ± <0.001*	0.62
4	M	38	85	0.006 ± 0.001	0.009 ± 0.001*	0.67
5	M	44	80	0.008 ± 0.002	0.010 ± 0.002*	0.80
6	M	53	100	0.005 ± 0.001	0.007 ± 0.001*	0.71
Average				0.006 ± 0.001	0.009 ± 0.001*	0.67
Range				0.005 - 0.008	0.007 - 0.010	

[a] mean ± standard deviation of at least 3 determinations
* significantly different from (+)-(R) (p<0.035) (two-tailed t-test, paired samples)

Table XII - Post-dialysis free fractions (f_u) of carvedilol enantiomers in serum at 4 μg/ml determined by equilibrium dialysis

Subject	Gender	Age	Weight (kg)	f_u[a] (+)-(R)	f_u[a] (-)-(S)	R/S ratio
1	F	34	53	0.010 ± 0.001[†]	0.013 ± 0.001*[†]	0.77
2	M	27	80	0.013 ± 0.001[†]	0.019 ± 0.002*[†]	0.68
3	M	27	89	0.011 ± 0.001[†]	0.015 ± 0.001*[†]	0.73
4	M	38	85	0.008 ± 0.001	0.010 ± <0.001*	0.80
5	M	44	80	0.009 ± 0.001	0.013 ± 0.001*	0.69
6	M	53	100	0.008 ± 0.001[†]	0.012 ± 0.001*[†]	0.67
Average				0.010 ± 0.002[†]	0.014 ± 0.003*[†]	0.71
Range				0.008 - 0.013	0.010 - 0.019	

[a] mean ± standard deviation of at least 3 determinations
* significantly different from (+)-(R) (p<0.035) (two-tailed t-test, paired samples)
[†] significantly different from f_u at 2 μg/ml (p<0.01) (two-tailed t-test, unpaired samples)

The difference in binding for each enantiomer, comparing the two concentrations studied, was not significant for two of the subjects, while for 4 subjects this difference reached statistical significance ($p<0.01$).

3.5.6.1 Free Fraction of (+)-(R)-Carvedilol and (-)-(S)-Carvedilol in Serum

The binding of each enantiomer to serum proteins in the absence of the antipode was determined. The post-dialysis free fractions (mean ± s.d. of 5 determinations) obtained at 2 μg/ml were 0.003 ± <0.001 for (+)-(R)-carvedilol, and 0.006 ± 0.001 for (-)-(S)-carvedilol, with an R/S ratio of 0.50. At 4 μg/ml the free fractions obtained were 0.005 ± <0.001 for (+)-(R)-carvedilol, and 0.008 ± 0.001 for (-)-(S)-carvedilol, with an R/S ratio of 0.63. The binding was found to be stereoselective at both concentrations studied ($p<0.002$). The binding of both enantiomers at 4 μg/ml was significantly different from the binding at 2 μg/ml ($p<0.01$) suggesting that at these concentrations the binding of carvedilol enantiomers to serum proteins is concentration-dependent.

Compared to the binding of the enantiomers in the racemic mixture, the free fractions measured with each enantiomer independently were lower than those measured using the racemic mixture, suggesting that in the latter experiment the presence of one enantiomer influenced the protein binding of the optical antipode. This could be due to competition of the enantiomers for the binding sites on the serum proteins with displacement of one enantiomer by the other. The same phenomenon was observed by Evans *et al.* (1989) when studying the binding of ibuprofen enantiomers.

Fujimaki *et al.* (1990) dialyzed human plasma containing each carvedilol enantiomer against phosphate buffer for 2 hours, at room temperature. The authors reported a free fraction in plasma, at 1 μg/ml per enantiomer, of 0.0045 ± 0.002 (mean ± SD) for (+)-(R)-carvedilol, and 0.0063 ± 0.007 for (-)-(S)-carvedilol. The unbound fraction for the R-enantiomer obtained by Fujimaki *et al.* (1990) is higher than the one obtained in our experiment, although the free fraction for the S-enantiomer reported by the authors is consistent with our results. However, the results obtained by Fujimaki *et al.* should be analyzed with caution since the authors report a very high standard deviation (corresponding to C.V.s of 44 and 111 %) for the determinations. Since they dialyzed the plasma for only 2 h, at room temperature, it could be possible that the dialysis process did not reach equilibrium, resulting in high variability in the values obtained.

3.5.7 Free Fraction of Carvedilol Enantiomers in Isolated Serum Protein Solutions

In order to identify the binding proteins responsible for the stereoselective binding of carvedilol in serum, the free fractions of carvedilol enantiomers were determined by equilibrium dialysis using isolated serum protein solutions.

3.5.7.1 Free Fraction in 4% Human Serum Albumin

The free fractions obtained by equilibrium dialysis, for carvedilol enantiomers using a 4% human serum albumin (HSA) solution in isotonic phosphate buffer are reported in Table XIII and Table XIV. The unbound fractions obtained at 2 μg/ml per enantiomer in 4% HSA were 2.4% and 2.6% for (+)-(R)- and (-)-(S)-carvedilol,

respectively. The free fractions obtained were much higher (3-4 times) than those obtained in normal serum, indicating that other proteins are also involved in binding of carvedilol in serum. Similar results were obtained by Stahl *et al.* (1993a), using rat serum albumin. The free fractions obtained were 3-5 times higher than those obtained using control rat plasma (Stahl *et al.*, 1993a).

The binding to HSA was not stereoselective at 2 μg/ml per enantiomer, with an R/S ratio of 0.92. At a higher drug concentration (4 μg/ml per enantiomer) the difference in binding between the two enantiomers was statistically significant ($p<0.02$), although the R/S ratio was 0.94. The results indicate that HSA does not contribute substantially to the stereoselectivity in binding observed in serum for carvedilol enantiomers.

The free fraction of (+)-(R)- and (-)-(S)-carvedilol increased significantly (about 20%) ($p<0.02$), as the drug concentration increased from 2 to 4 μg/ml per enantiomer suggesting that the binding to HSA is saturable.

While our results show higher binding of (+)-(R)-carvedilol, both in normal serum and in 4% HSA solution, as compared to the S-antipode, inverse enantioselectivity was found by Stahl *et al.* (1993c) when comparing the binding to rat plasma (free R/S = 0.65) and to 4% rat serum albumin (free R/S = 1.27).

3.5.7.2 Free Fraction in 100 mg% and 400 mg% α_1-Acid Glycoprotein

The free fractions obtained by equilibrium dialysis, for carvedilol enantiomers using 100 mg% and 400 mg% α_1-acid glycoprotein (AAG) solutions in isotonic phosphate buffer are reported in Table XIII and Table XIV. The unbound fraction at 2 μg/ml per enantiomer in 100 mg% was 1.5% and 2.5% for (+)-(R)- and

(-)-(S)-carvedilol, respectively. The binding of carvedilol enantiomers to the acute phase protein AAG was higher than the binding to HSA, especially for (+)-(R)-carvedilol. The binding was highly stereoselective with an R/S = 0.60, indicating that AAG is probably the main protein responsible for the stereoselectivity in binding of carvedilol in serum.

As the concentration of drug in the protein solution was raised from 2 to 4 μg/ml, a dramatic increase in the free fractions for (+)-(R)- and (-)-(S)-carvedilol in 100 mg% AAG was observed, although the R/S ratio remained constant. The results suggest that the binding to AAG is saturable in the concentration range investigated.

3.5.7.3 Free Fraction in 4% Human Serum Albumin Combined with 100 mg% and 400 mg% α_1-Acid Glycoprotein

The free fractions of carvedilol enantiomers in 4% HSA combined with AAG in different concentrations was studied. In order to determine the effect of increased AAG levels simulating the changes in the reactant protein that would occur in myocardial infarction or trauma, solutions of 4% HSA containing 100 mg% or 400 mg% AAG were used for binding experiments. The results obtained are listed in Table XIII and Table XIV. As the AAG concentration increased from 100 mg% to 400 mg% the free fractions of both enantiomers decreased several fold and the R/S ratio changed from 0.82 to 0.67, at 2 μg/ml per enantiomer, and from 0.78 to 0.60, at 4 μg/ml of each carvedilol enantiomer. It can be observed that a dramatic decrease in the unbound drug concentration occurs with the rise in the AAG levels as well as a change in the ratio of unbound (+)-(R)- to (-)-(S)-carvedilol. The two enantiomers exhibit different pharmacological actions, and the antihypertensive effect produced by racemic

carvedilol is a combination of the diverse activities of (+)-(R)- and (-)-(S)-carvedilol at α_1- and β_1-adrenergic receptors. Therefore, a change in the ratio of free levels of the two enantiomers could result in a different clinical response to treatment in patients with elevated AAG levels.

3.5.8 Free Fraction of Carvedilol Enantiomers in Lipoprotein-Deficient Serum

The protein binding of carvedilol enantiomers in lipoprotein-deficient serum was determined by equilibrium dialysis. The results are listed in Table XIII and Table XIV. The free fractions obtained at the two concentrations studied were higher than those obtained for normal serum, suggesting that lipoproteins may play a role in the binding of the drug in serum.

Table XIII - Post-dialysis free fractions (f_u) for carvedilol enantiomers in serum and buffered protein solutions at a concentration of 2 μg/ml

matrix containing carvedilol	f_u[a] (+)-(R)	f_u[a] (-)-(S)	R/S ratio
human serum	0.006 ± 0.002[b]	0.009 ± 0.002[b]*	0.67
4% human serum albumin	0.024 ± 0.001	0.026 ± 0.002	0.92
100 mg% α_1-acid glycoprotein	0.015 ± 0.003	0.025 ± 0.005*	0.60
400 mg% α_1-acid glycoprotein	0.004 ± 0.001	0.006 ± 0.001*	0.67
4% human serum albumin + 100 mg% α_1-acid glycoprotein	0.013 ± 0.001	0.015 ± 0.001*	0.82
4% human serum albumin + 400 mg% α_1-acid glycoprotein	0.004 ± 0.001	0.006 ± <0.001*	0.67
lipoprotein-deficient serum	0.009 ± 0.001	0.016 ± 0.001*	0.56

[a] mean ± standard deviation of at least 3 determinations
[b] mean ± standard deviation of 6 subjects
* significantly different from (+)-(R) (p<0.02) (two-tailed t-test, paired samples)

Table XIV - Post-dialysis free fractions (f_u) for carvedilol enantiomers in serum and buffered protein solutions at a concentration of 4 μg/ml

	f_u[a]		
matrix containing carvedilol	**(+)-(R)**	**(-)-(S)**	**R/S ratio**
human serum	0.010 ± 0.002[b]	0.014 ± 0.001[b]*	0.71
4% human serum albumin	0.029 ± 0.001†	0.031 ± 0.001*†	0.94
100 mg% α_1-acid glycoprotein	0.084 ± 0.005†	0.139 ± 0.009*†	0.60
400 mg% α_1-acid glycoprotein	0.004 ± 0.001	0.007 ± 0.001*	0.57
4% human serum albumin + 100 mg% α_1-acid glycoprotein	0.014 ± 0.002	0.018 ± 0.002*	0.78
4% human serum albumin + 400 mg% α_1-acid glycoprotein	0.003 ± <0.001	0.005 ± <0.001*†	0.60
lipoprotein-deficient serum	0.019 ± 0.001†	0.029 ± 0.001*†	0.66

[a] mean ± standard deviation of at least 3 determinations
[b] mean ± standard deviation of 6 subjects
* significantly different from (+)-(R) (p<0.02) (two-tailed t-test, paired samples)
† significantly different from f_u at 2 μg/ml (p<0.02) (two-tailed t-test, unpaired samples)

3.5.9 Concentration-Dependent Binding of Carvedilol in Serum and in Isolated Serum Protein Solutions

3.5.9.1 Concentration-Dependent Binding in Serum

Concentration-dependent binding in serum of a normal volunteer was assessed over the 0.5-4 μg/ml per enantiomer serum concentration range. The results are shown in Figure 28. The binding of carvedilol enantiomers in serum was found to be independent of the concentration in the range studied. Analysis of Variance (ANOVA) of the means showed no significant difference ($p<0.05$) among the free fractions at any drug concentration.

Due to the very extensive binding of carvedilol enantiomers and therefore extremely low concentrations of drug in the dialysate on equilibrium, it was only possible to study the binding at a higher drug serum concentration. The lowest concentration examined was 500 ng/ml per enantiomer, which is beyond the therapeutic level for carvedilol since maximum concentrations in plasma were reported to be of about 74-78 ng/ml per enantiomer after an oral dose of 50 mg (Neugebauer *et al.*, 1990, Spahn *et al.*, 1990). Based on the results obtained it could be concluded that it is not likely that carvedilol will exhibit concentration-dependent binding in normal serum at therapeutic levels.

As reported previously in section 3.5.6, in 4 of the 6 volunteers examined, significant differences between the free fractions at 2 and 4 μg/ml per enantiomer were observed, whereas in 2 individuals there was no statistically significant difference in the binding at the two concentrations. It could be that the concentrations studied are approaching the binding capacity of the serum proteins resulting in saturation of the

binding sites. The different results obtained for the volunteers could also be related to variations in the protein concentrations of the individuals.

3.5.9.2 Concentration-Dependent Binding to 4% Human Serum Albumin

Concentration-dependent binding to 4% HSA in isotonic phosphate buffer was studied over the 0.5-4 μg/ml per enantiomer range. The results are shown in Figure 28. Gradual increases in the free fractions were observed, as the concentrations of carvedilol enantiomers increased. ANOVA of the results confirmed that the means are different (p>0.05). A Tukey test indicated that only above 2 μg/ml was there significant difference in the free fractions with increased drug concentration. The results suggest that at therapeutic drug concentrations (below 80 ng/ml) the albumin binding sites are not saturated and carvedilol free fractions at those levels would be independent of drug concentration.

3.5.9.3 Concentration-Dependent Binding to 100 mg% α_1-Acid Glycoprotein

Concentration-dependent binding to 100 mg% AAG in isotonic phosphate buffer was examined over the concentration range of 0.5-4 μg/ml per enantiomer. The results are presented in Figure 28. An accentuated increase in the free fractions was obtained, as the concentration of carvedilol enantiomers increased from 0.5 to 4 μg/ml, which can be observed by the slopes of the curves shown on the graph. ANOVA testing confirmed that the means are different (p>0.05). A Tukey test (Zar, 1984) showed that the means (free fractions) at 3 and 4 μg/ml are different from those at 0.5-2 μg/ml. The results indicate that the binding to AAG is saturable at higher drug concentrations.

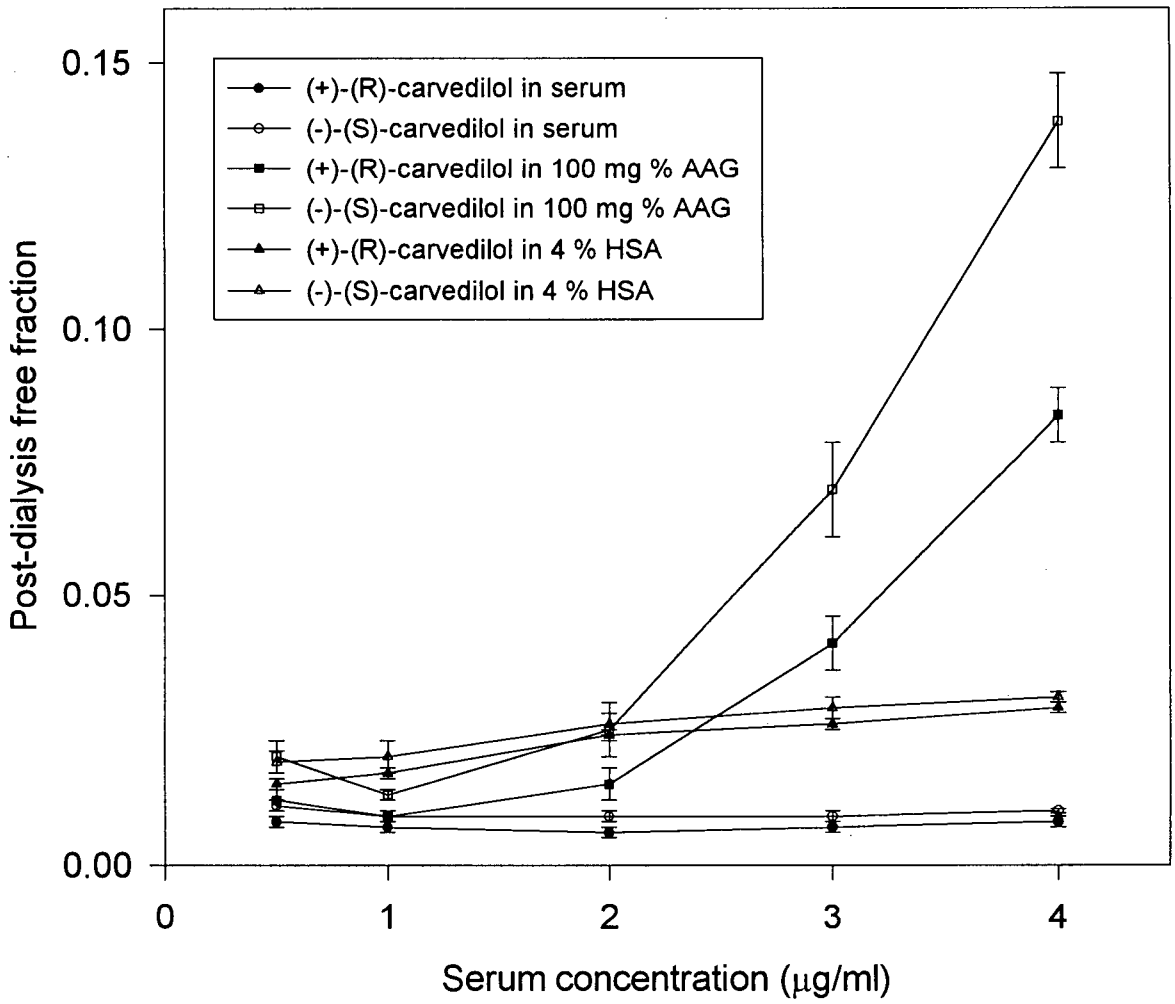

Figure 28. Concentration-dependent free fraction of carvedilol in serum, 4% human serum albumin, and 100 mg% α_1-acid glycoprotein.

4. SUMMARY AND CONCLUSIONS

An attempt to develop a very sensitive and stereoselective assay for the determination of carvedilol enantiomers in serum was undertaken. Several discrepancies in pharmacokinetic data for this drug were detected in the literature and were believed to be due to sensitivity limitations of the analytical methods employed in the studies. The approach used in the present investigation was to derivatize the enantiomers of carvedilol with a fluorogenic reagent that would produce an increase in the detection sensitivity. 2-Naphthoyl chloride and 2-anthroyl chloride have been used in our laboratory for the analysis and pharmacokinetics studies of mexiletine enantiomers. The detection limits were substantially improved after derivatization of mexiletine with these reagents.

The derivatization of carvedilol with both acid chlorides was found to be incomplete with variable amounts of carvedilol remaining after the reaction. Moreover, multiple derivatization of carvedilol with 2-naphthoyl chloride at both the secondary amine group on the side chain and the hydroxyl group was revealed by mass spectrometric analysis.

Since reaction with an acid chloride, (+)-(S)-naproxen chloride, was also used by Spahn *et al.* (1990) in one of the two studies on the stereoselective pharmacokinetics of carvedilol, an attempt to reproduce that reaction was undertaken. Using the same reaction conditions reported by these authors it was revealed by TLC that unreacted carvedilol was present after the derivatization.

An alternative reagent, (+)-(S)-1-(1-naphthyl)ethyl isocyanate (NEIC) was also tested. Variable amounts of unreacted drug were detected by HPLC analysis. Mass

spectrometric analysis of the reaction mixture revealed the formation of the mono-derivative and the di-derivative.

Since problems with the derivatization of carvedilol were encountered, the analysis of the enantiomers by direct analysis using a chiral HPLC column was investigated. A Chiralcel OD column containing a cellulose carbamate derivative-based stationary phase was tested and produced only slight separation of carvedilol enantiomers. A column containing (S)-indoline-2-carboxylic acid and (R)-1-(α-naphthyl)ethylamine as the stationary phase (Phenomenex 3022) was shown to produce separation of the enantiomers of carvedilol. The method was optimized and validated using serum samples containing carvedilol. The detection was by fluorescence utilizing the native fluorescence of carvedilol. The limit of quantitation (LOQ) of the assay was 1 ng/ml of each enantiomer in serum which was about the same as the LOQs reported in the literature. This assay was therefore used for a portion of the study of the protein binding of carvedilol enantiomers.

Capillary electrophoresis (CE) has become a very important analytical tool due to the high separation efficiencies that can be achieved as well as rapid method development and low consumption of solvents and materials. CE using cyclodextrins as chiral selectors was investigated for the analysis of carvedilol enantiomers in serum. Several types and concentrations of cyclodextrins were tested. Baseline resolution of the enantiomers was obtained with the use of 10 mM hydroxypropyl-β-cyclodextrin as the chiral selector in the run buffer. Other parameters such as separation voltage, buffer concentration, and cartridge temperature were optimized. The method was validated using carvedilol added to serum. The LOQ obtained was 50 ng/ml. This is a much higher value than the one obtained with the chiral HPLC method. However, it

has to be considered that the volumes injected in CE are only of the order of 20 nl as compared to 10 μl with the stereoselective HPLC method.

The chiral HPLC and CE methods were compared by analyzing a series of calibration curves of carvedilol enantiomers in serum by the two methods. The concentrations obtained by the two assays were not found to be significantly different. Therefore the CE method could be used as an alternative to HPLC in the study of the protein binding of carvedilol to serum proteins.

Several aspects of the stereoselective binding of carvedilol enantiomers to serum proteins have not yet been addressed. These include the extent of binding of the drug in serum and the identity of the binding proteins. The applicability of ultrafiltration methods was examined for the determination of the free levels of carvedilol in serum. The occurrence of extensive nonspecific binding of the drug to the ultrafiltration devices made the use of such techniques unreliable. The free fraction of carvedilol in human serum was therefore determined by equilibrium dialysis. Because carvedilol is highly bound to serum proteins (> 98%) the unbound drug concentrations are extremely low. The CE method did not provide enough sensitivity for the determination of free drug levels in the buffer dialysate. Therefore, unbound carvedilol was determined using the developed chiral HPLC method, while the total levels in the serum retentate (bound and free) were measured by the stereoselective CE assay.

The post-dialysis free fractions of the enantiomers at a concentration of 2 μg/ml in serum were significantly different ($p<0.035$) and were determined to be 0.6% for (+)-(R)-carvedilol and 0.9% for (-)-(S)-carvedilol with an R/S ratio of 0.67. When the enantiomers were examined separately, the free fractions obtained were 0.3% for (+)-(R)-carvedilol and 0.6% for (-)-(S)-carvedilol with an R/S ratio of 0.50. The

unbound fractions were lower than those obtained when the racemic mixture was tested, suggesting that competitive binding with displacement of one enantiomer by the other from the binding sites may occur with the racemic mixture.

The binding of carvedilol to isolated protein solutions was also examined. The free fractions of carvedilol at 2 μg/ml per enantiomer in 4% human serum albumin (HSA) were higher than those obtained from human serum. Unbound fractions of 2.4% and 2.6% were obtained for (+)-(R)-and (-)-(S)-carvedilol, respectively, with an R/S ratio of 0.92. The difference in binding to HSA was not found to be statistically significant. The binding of carvedilol at 2 μg/ml per enantiomer to 100 mg% α_1-acid glycoprotein (AAG) was found to be highly stereoselective. The unbound fraction of (-)-(S)-carvedilol was 2.5%, similar to the free fraction in HSA (2.6%). However, the free fraction of (+)-(R)-carvedilol was much lower, 1.5%, indicating preferential binding of that enantiomer to AAG (R/S ratio = 0.60). The results suggest that AAG is the major serum protein responsible for the stereoselective binding of carvedilol enantiomers in serum.

Concentration-dependent binding of carvedilol to serum and isolated proteins solutions was also determined. The binding to serum was found to be independent of concentration over the range of concentrations studied (0.5-4 μg/ml per enantiomer). The binding to 4% HSA over the same concentration range was also tested. There was significant difference in the free fractions for enantiomer concentrations higher than 2 μg/ml. When examining the binding of carvedilol to 100 mg% AAG at 0.5-4 μg/ml per enantiomer, the free fractions were significantly different at drug concentrations above 3 μg/ml per enantiomer. Because of the extremely low unbound drug concentrations, a result of the extensive protein binding of carvedilol, it was only possible to study the

protein binding of the drug at concentrations higher than those observed after therapeutic doses of carvedilol. Therefore, based on the results obtained, it is not likely that at therapeutic levels (below 80 ng/ml) the enantiomers of carvedilol will exhibit concentration-dependent binding.

In order to assess the changes in binding of carvedilol enantiomers to altered levels of the acute phase reactant protein AAG that would occur in myocardial infarction or surgery, the binding to a 4% HSA solution combined with 400 mg% AAG was tested. As compared to the binding to normal protein levels (4% HSA and 100 mg% AAG) the free fractions of the enantiomers decreased several fold and the R/S ratio changed from 0.82 to 0.67. Since carvedilol enantiomers differ in their pharmacological action, the change in the ratio of the unbound fractions could result in different response to treatment in these patients.

5. FUTURE RESEARCH PLANS

Because of its high efficiency, CE could provide an excellent method for the separation of closely related compounds such as drug metabolites. The excellent resolving power of CE using cyclodextrins could therefore be very valuable in stereoselective drug metabolism studies.

We have recently received several chiral metabolites of carvedilol donated by Boehringer Mannheim. Figure 29 shows the electropherograms of ethanolic solutions of 4 of the metabolites using the CE assay developed for carvedilol enantiomers. The enantiomers of two of the metabolites (BM 91.0228, 3-hydroxycarvedilol and BM 91.0162, 8-hydroxycarvedilol) were completely resolved. Partial separation was obtained for the enantiomers of BM 91.0183 (1-hydroxycarvedilol). The enantiomers of *O*-desmethylcarvedilol (BM 12.242) were only slightly separated. Although further method development was not undertaken, it is reasonable to predict that complete resolution could be easily achieved with the use of buffer additives such as methylcellulose or organic modifiers (methanol, acetonitrile). Two of the metabolites (5'-hydroxycarvedilol and 6-hydroxycarvedilol) were not resolved using 10 mM hydroxypropyl-β-cyclodextrin and were detected as single peaks. There are several types of substituted cyclodextrins, including charged cyclodextrins, which could alter the selectivity of the interaction with these metabolites and provide enantiomer resolution.

Using the chiral HPLC system developed for carvedilol the enantiomers of only 3 metabolites (8-hydroxycarvedilol, 6-hydroxycarvedilol, and 5'-hydroxycarvedilol) were slightly resolved. Two metabolites, 1-hydroxycarvedilol and 3-hydroxycarvedilol, did

not produce any signal and may have been retained by the column. Due to the restrictions in mobile phase inherent with chiral HPLC columns, it is likely that mobile phase adjustments would not result in resolution and that other chiral stationary phase would be required in order to provide enantiomer separation for these metabolites.

Therefore the use of chiral CE using cyclodextrins appears as an attractive alternative for the study of the stereoselective metabolism of carvedilol.

In addition to the benefits of CE for metabolic studies, future investigations using a tunable laser-induced fluorescence detector would be particularly valuable since the major short fall of the UV detector available for the present study did not have the sensitivity of fluorometric detection methods. Alternatively, the use of a CE-MS interface would also increase sensitivity and in addition would allow for the unambiguous determination of the structure of the metabolites. Such methods would allow, for the first time, a complete stereoselective, pharmacokinetic and metabolism study for the enantiomers of carvedilol.

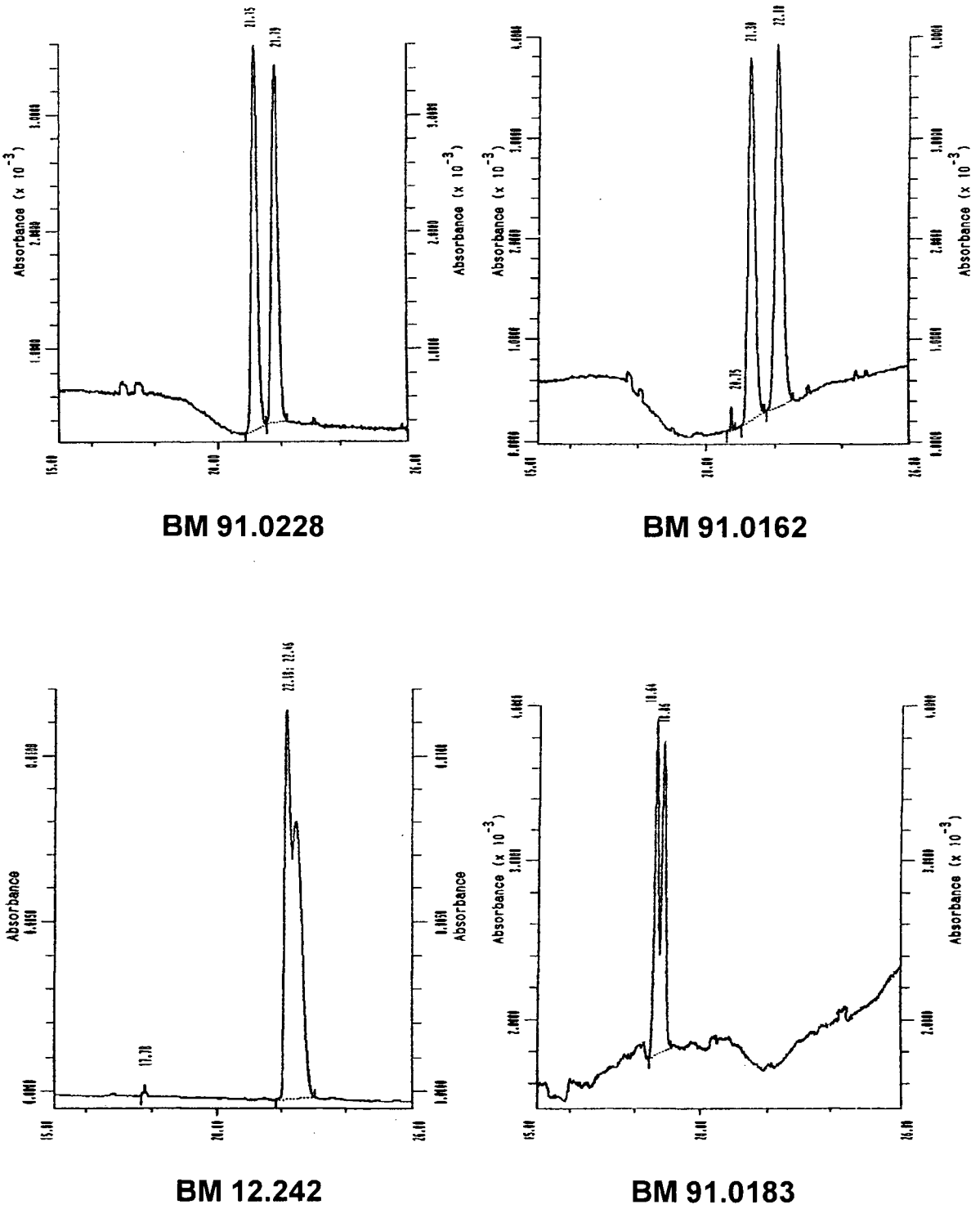

Figure 29. Electropherograms of carvedilol metabolites. **Electrophoretic conditions:** background electrolyte: 25 mM sodium phosphate buffer, pH 2.5, 10 mM hydroxypropyl-β-cyclodextrin; capillary: fused silica, uncoated, 57 (50) cm length, 75 μm I.D.; voltage: 18 kV; temperature: 20 °C; injection: pressure, 4 s; detection: UV at 200 nm.

6. REFERENCES

Albani, F., Riva, R., Contin, M., Baruzzi, A. Stereoselective binding of propranolol enantiomers to human α_1-acid glycoprotein and human plasma. *Br. J. Clin. Pharmacol.* **18:**244-246, 1984.

Albin, M., Weinberger, R., Sapp, E., Moring, S. Fluorescence detection in capillary electrophoresis: evaluation of derivatizing reagents and techniques. *Anal. Chem.* **63:**417-422, 1991.

Altria, K.D. (Ed.) Capillary Electrophoresis Guidebook. Principles, Operation, and Applications. Humana Press Inc., Totowa, 1996.

Altria, K.D. Essential peak area normalization for quantitative impurity content determination by capillary electrophoresis. *Chromatographia* **35:**177-182, 1993.

Altria, K.D., Goodall, D.M., Rogan, M.M. Quantitative applications and validation of the resolution of enantiomers by capillary electrophoresis. *Electrophoresis* **15:**824-827, 1994.

Altria, K.D., Goodall, D.M., Rogan, M.M. Chiral separation of β-amino alcohols by capillary electrophoresis using cyclodextrins as buffer additives. I. Effect of varying operating parameters. *Chromatographia* **34:**19-24, 1992.

Armstrong, D.W., Rundlett, K.L., Chen, J.-R. Evaluation of the macrocyclic antibiotic vancomycin as a chiral selector for capillary electrophoresis. *Chirality* **6:**496-509, 1994.

Armstrong, D.W., Ward, T.J., Armstrong, R.D., Beesley, T.E. Separation of drug stereoisomers by the formation of β-cyclodextrin inclusion complexes. *Science* **232:**1132-1135, 1986.

Baeyens, W., Weiss, G., Van der Weken, G., Van den Bossche, W. Analysis of pilocarpine and its *trans* epimer, isopilocarpine, by capillary electrophoresis. *J. Chromatogr.* **638:**319-326, 1993.

Barre, J., Didey, F., Delion, F., Tillement, J.-P. Problems in therapeutic drug monitoring: free drug level monitoring. *Ther. Drug Monit.* **10:**133-143, 1988.

Bartsch, W., Sponer, G., Strein, K., Müller-Beckmann, B., Kling, L., Böhm, E., Martin, U., Borbe, H.O. Pharmacological characteristics of the stereoisomers of carvedilol. *Eur. J. Clin. Pharmacol.* **38:**S104-S107, 1990.

Behm, H.L., Wagner, J.G. Errors in interpretation of data from equilibrium dialysis protein binding experiments. *Res. Commun. Chem. Pathol. Pharmacol.* **26:**145-160, 1979.

Bhatti, M.M., Foster, R.T. Stereospecific high-performance liquid chromatographic assay of metoprolol. *J. Chromatogr.* **579:**361-365, 1992.

Birnbaum, S., Nillson, S. Protein based capillary affinity gel electrophoresis for the separation of optical isomers. *Anal. Chem.* **64:**2872-2874, 1992.

Bowers, W.F., Fulton, S., Thompson, J. Ultrafiltration *vs* equilibrium dialysis for determination of free fraction. *Clin. Pharmacokinet.* **9(Suppl.1):**49-60, 1984.

Brunner, L.J., DiPiro, J.T., Feldman, S. High-performance capillary electrophoresis in the pharmaceutical sciences. *Pharmacotherapy* **15:**1-22, 1995.

Brunner, L.J., DiPiro, J.T., Feldman, S. Metabolic cage isolation reduces antipyrine clearance in rats. *J. Pharm. Pharmacol.* **46:**581-584, 1994.

Brunner, L.J., DiPiro, J.T., Feldman, S. Serum antipyrine concentrations determined by micellar electrokinetic capillary chromatography. *J. Chromatogr.* **622:**98-102, 1993.

Chankvetadze, B., Endresz, G., Blaschke, G. About some aspects of the use of charged cyclodextrins for capillary electrophoresis enantioseparation. *Electrophoresis* **15:**804-807, 1994.

Chen, F.-T.A., Liu, C.-M., Hsieh, Y.-Z., Sternberg, J.C. Capillary electrophoresis - a new clinical tool. *Clin. Chem.* **37:**14-19, 1991.

Cheng, Y.-F., Dovichi, N.J. Subattomole amino acid analysis by capillary zone electrophoresis and laser-induced fluorescence. *Science* **242:**562-564, 1988.

Colangelo, P., Chandler, M., Blouin, R., McNamara, P. Stereoselective binding of propranolol in the elderly. *Br. J. Clin. Pharmacol.* **27:**519-522, 1989.

Cruzado, I., Vigh, G. Chiral separations by capillary electrophoresis using cyclodextrin-containing gels. *J. Chromatogr.* **608:**421-425, 1992.

Darmon, A., Thenot, J.P. Determination of betaxolol enantiomers by high-performance liquid chromatography. Application to pharmacokinetic studies. *J. Chromatogr.* **374:**321-328, 1986.

de Mey, C., Breithaupt, K., Schloos, J., Neugebauer, G., Palm, D., Belz, G.G. Dose-effect and pharmacokinetic-pharmacodynamic relationships of the β_1-adrenergic receptor blocking properties of various doses of carvedilol in healthy humans. *Clin. Pharmacol. Ther.* **55:**329-337, 1994.

Dethy, J.M., De Broux, S., Lesne, M., Longstreth, J., Gilbert, P. Stereoselective determination of verapamil and norverapamil by capillary electrophoresis. *J. Chromatogr. B* **654:**121-127, 1994.

Dette, C., Ebel, S., Terabe, S. Neutral and anionic cyclodextrins in capillary zone electrophoresis: enantiomeric separation of ephedrine and related compounds. *Electrophoresis* **15:**799-803, 1994.

D'Hulst, A., Verbeke, N. Quantitaion in chiral capillary electrophoresis: theoretical and practical considerations. *Electrophoresis* **15:**854-863, 1994.

D'Hulst, A., Verbeke, N. Chiral separation by capillary electrophoresis with oligosaccharides. *J. Chromatogr.* **608:**275-287, 1992.

Donato, M.G., Van den Eeckhout, E., Van den Bossche, W., Sandra, P. Capillary zone electrophoresis and micellar electrokinetic capillary chromatography of some non-steroidal antiinflammatory drugs (NSAIDs). *J. Pharm. Biomed. Anal.* **11:**197-201, 1993.

Eap, C.B., Cuendet, C., Baumann, P. Binding of d-methadone, l-methadone, and dl-methadone to proteins in plasma of healthy volunteers: role of the variants of α_1-acid glycoprotein. *Clin Pharmacol. Ther.* **47:**338-346, 1990.

Eisenberg, E.J., Patterson, W.R., Kahn, G.C. High-performance liquid chromatographic method for the simultaneous determination of the enantiomers of carvedilol and its O-desmethyl metabolite in human plasma after chiral derivatization. *J. Chromatogr.* **493:**105-115, 1989.

Evans, A.M., Nation, R.L., Sanson, L.N., Bochner, F., Somogyi, A.A. Stereoselective plasma protein binding of ibuprofen enantiomers. *Eur. J. Clin. Pharmacol.* **36:**283-290, 1989.

Evenson, M.A., Wiktorowicz, J.E. Automated capillary electrophoresis applied to therapeutic drug monitoring. *Clin. Chem.* **38:**1847-1852, 1992.

Fanali, S. Use of cyclodextrins in capillary zone electrophoresis. Resolution of terbutaline and propranolol enantiomers. *J. Chromatogr.* **545:**437-444, 1991.

Fanali, S. Separation of optical isomers by capillary zone electrophoresis based on host-guest complexation with cyclodextrins. *J. Chromatogr.* **474:**441-446, 1989.

Fanali, S., Bocek, P. Enantiomer resolution by using capillary zone electrophoresis: resolution of racemic tryptophan and determination of the enantiomer composition of commercial pharmaceutical epinephrine. *Electrophoresis* **11:**757-760, 1990.

Fanali, S., Cristalli, M., Nardi, A., Ossicini, L., Shukla, S.K. Capillary zone electrophoresis in pharmaceutical analysis. *Farmaco (Pavia)* **45(Suppl. 6):**693-702, 1990.

Fantucci, P., Mattioli. E., Villa, A.M., Villa, L. Conformational behaviour and molecular similarity of some β_1-adrenergic ligands. *J. Comput.-Aided Mol. Des.* **6:**315-330, 1992.

Fernandez, C., Martin, C., Gimenez, F., Farinotti, R. Clinical pharmacokinetics of zopiclone. *Clin. Pharmacokinet.* **29:**431-441, 1995.

Fois, R.A., Ashley, J.J. Drug binding to apparatus: a factor controlling time to equilibrium in equilibrium dialysis studies. *J. Pharm. Sci.* **80:**300-302, 1991.

Frenz, J., Wu, S.-L., Hancock, W.S. Characterization of human growth hormone by capillary electrophoresis. *J. Chromatogr.* **480:**379-391, 1989.

Fujimaki, M. Oxidation of R(+)- and S(-)-carvedilol by rat liver microsomes. Evidence for stereoselective oxidation and characterization of the cytochrome P450 isozymes involved. *Drug Metab. Dispos.* **22:**700-708, 1994.

Fujimaki, M. Stereoselective disposition and tissue distribution of carvedilol enantiomers in rats. *Chirality* **4:**148-54, 1992.

Fujimaki, M., Hakusui, H. Identification of two major biliary metabolites of carvedilol in rats. *Xenobiotica* **20:**1025-1034, 1990.

Fujimaki, M., Murakoshi, Y., Hakusui, H. Assay and disposition of carvedilol enantiomers in humans and monkeys: evidence of stereoselective presystemic metabolism. *J. Pharm. Sci.* **79:**568-72, 1990.

Fujimaki, M., Shintani, S., Hakusui, H. Stereoselective metabolism of carvedilol in the rat. Use of enantiomerically radiolabeled pseudoracemates. *Drug Metab. Dispos.* **19:**749-753, 1991.

Fujimura, K., Suzuki, S., Hayashi, K., Masuda, S. Retention behavior and chiral recognition mechanism of several cyclodextrin-bonded stationary phases for dansyl amino acids. *Anal. Chem.* **62:**2198-2205, 1990.

Gareil, P., Gramond, J.P., Guyon, F. Separation and determination of warfarin enantiomers in human plasma samples by capillary zone electrophoresis using a methylated beta-cyclodextrin-containing electrolyte. *J. Chromatogr.* **615:**317-325, 1993.

Gaus, H.-J., Treumann, A., Kreis, W., Bayer, E. Separation of cardiac glycosides by micellar electrokinetic capillary electrophoresis. *J. Chromatogr.* **635:**319-327, 1993.

Goodall, D.M., Lloyd, D.K., Williams, S.J. Current trends in capillary electrophoresis. *LC-GC* **8:**788-799, 1990.

Goodall, D.M., Williams, S.J., Lloyd, D.K. Quantitative aspects of capillary electrophoresis. *Trends Anal. Chem.* **10:**272-279, 1991.

Gross, A.S., Heuer, B., Eichelbaum, M. Stereoselective protein binding of verapamil enantiomers. *Biochem. Pharmacol.* **37:**4623-4627, 1988.

Guttman, A. Novel separation scheme for capillary electrophoresis of enantiomers. *Electrophoresis* **16:**1900-1905, 1995.

Guttman, A., Brunet, S., Jurado, C., Cooke, N. Rapid chiral separation methods development by cyclodextrin-mediated capillary electrophoresis for acidic and basic compounds. *Chirality* **7:**409-414, 1995.

Guttman, A., Cooke, N. Practical aspects of chiral separations of pharmaceuticals by capillary electrophoresis. I. Separation optimization. *J. Chromatogr. A* **680:**157-162, 1994.

Guttman, A., Paulus, A., Cohen, A.S., Grinberg, N., Karger, B.L. Use of complexing agents for selective separation in high performance capillary electrophoresis. Chiral resolution via cyclodextrins incorporated within polyacrylamide gels. *J. Chromatogr.* **448:**41-53, 1988.

Guzman, N.A., Moschera, J., Iqbal, K., Malick, A.W. Effect of buffer constituents on the determination of therapeutic proteins by capillary electrophoresis. *J. Chromatogr.* **608:**197-204, 1992.

Hadwiger, M.E., Torchia, S.R., Park, S., Biggin, M.E., Lunte, C.E. Optimization of the separation and detection of the enantiomers of isoproterenol in microdialysis samples by cyclodextrin-modified capillary electrophoresis using electrochemical detection. *J. Chromatogr. B* **681:**241-249, 1996.

Hakusui, H., Fujimaki, M. Pharmacokinetics of carvedilol in hypertensive patients with renal failure. *Drugs* **36(Suppl. 6):**144-147, 1988.

Heiger, D.N., Kaltenbach, P., Sievert, H.-J.P. Diode array detection in capillary electrophoresis. *Electrophoresis* **15:**1234-1247, 1994.

Hervé, F., Urien, S., Albengres, E., Duché, J.-C., Tillement, J.-P. Drug binding in plasma. A summary of recent trends in the study of drug and hormone binding. *Clin. Pharmacokinet.* **26:**44-58, 1994.

Hinderling, P.H., Bres, J., Garrett, E.R. Protein binding and erythrocyte partitioning of disopyramide and its monodealkylated metabolite. *J. Pharm. Sci.* **63:**1684-1690, 1974.

Honda, S., Taga, A., Kakehi, K., Koda, S., Okamoto, Y. Determination of cefixime and its metabolites by high-performance capillary electrophoresis. *J. Chromatogr.* **590:**364-368, 1992.

Hsyu, P.-O., Giacomini, K.M. High performance liquid chromatographic determination of the enantiomers of β-adrenoceptor blocking agents in biological fluids. I: Studies with pindolol. *J. Pharm. Sci.* **75:**601-605, 1986.

Hu, T., Zuo, H., Riley, C.M., Stobaugh, J.F., Lunte, S.M. Determination of alpha-difluoromethylornithine in blood by microdialysis sampling and capillary electrophoresis with UV detection. *J. Chromatogr. A* **716:**381-388, 1995.

Jellum, E., Thorsrud, A.K., Time, E. Capillary electrophoresis for diagnosis and studies of human disease, particularly metabolic disorders. *J. Chromatogr.* **559:**455-465, 1991.

Johansson, I.M., Gron-Rydberg, M.B., Schmekel, B. Determination of theophylline in plasma using different capillary electrophoretic systems. *J. Chromatogr. A* **652:**487-493, 1993.

Jorgenson, J.W., Lukacs, K.D. Zone electrophoresis in open-tubular glass capillaries. *Anal. Chem.* **53:**1298-1302, 1981.

Kinget, R., Bontinck, A.-M., Herbots, H. Problems of dialysis techniques in the study of macromolecule binding of drugs. *Int. J. Pharm.* **3:**65-72, 1979.

Knox, J.H. Terminology and nomenclature in capillary electroseparation systems. *J. Chromatogr. A* **680:**3-13, 1994.

Koike, Y., Magnusson, A., Steiner, E., Rane, A., Sjöqvist, F. Ultrafiltration compared with equilibrium dialysis in the determination of unbound phenytoin in plasma. *Ther. Drug Monit.* **7:**461-465, 1985.

Krämer, B.K., Ress, K.M., Erley, C.M., Risler, T. Pharmacokinetic and blood pressure effects of carvedilol in patients with chronic renal failure. *Eur. J. Clin. Pharmacol.* **43:**85-88, 1992.

Krstulovic, A.M. (Ed.) Chiral Separations by HPLC. Applications to pharmaceutical compounds. Ellis Horwood, Chichester, 1989.

Krull, I.S., Mazzeo, J.R. Capillary electrophoresis: the promise and the practice. *Nature* **357:**92-94, 1992.

Kurz, H. Methodological problems in drug-binding studies. In: Reidenberg, M.M., Erill, S. (Eds.) Drug-protein binding, Praeger, New York, 1986.

Kurz, H., Trunk, H., Weitz, B. Evaluation methods to determine protein-binding of drugs. *Arzneim.-Forsch.* **27:**1373-1380, 1977.

Kwok, D.K.W., Igwemezie, L., Kerr, C.R., McErlane, K.M. High-performance liquid chromatographic analysis using a highly sensitive fluorogenic reagent, 2-anthroyl chloride, and stereoselective determination of the enantiomers of mexiletine in human serum. *J. Chromatogr. B* **661:**271-280, 1994.

Kwong, T.C. Free drug measurements: methodology and clinical significance. *Clin. Chim. Acta* 151:193-216, 1985.

Landers, J.P. Capillary electrophoresis: pioneering new approaches for biomolecular analysis. *Trends Biochem. Sci.* **18:**409-414, 1993.

Landers, J.P., Oda, R.P., Spelsberg, T.C., Nolan, J.A., Ulfelder, K.J. Capillary electrophoresis: a powerful microanalytical technique for biologically active molecules. *BioTechniques* **14:**98-111, 1993.

Lave, T., Efthymiopoulus, C., Koffel, J.C., Jung, L. Determination of tertatolol enantiomers in biological fluids by high-performance liquid chromatography. *J. Chromatogr.* **572:**203-210, 1991.

Lessem, J.N., Lukas, M.A. Development of a multiaction beta blocker. *Cardiology* **82(Suppl. 3):**50-58, 1993.

Li, S., Lloyd, D.K. Direct chiral separations by capillary electrophoresis using capillaries packed with α_1-acid glycoprotein chiral stationary phase. *Anal. Chem.* **65:**3684-3690, 1993.

Lima, J.J., Jungbluth, G.L., Devine, T., Robertson, L.W. Stereoselective binding of disopyramide to human plasma protein. *Life Sci.* **35:**835-839, 1984.

Lima, J.J., MacKichan, J.J., Libertin, N., Sabino, J. Influence of volume shifts on drug binding during equilibrium dialysis: correction and attenuation. *J. Pharmacokinet. Biopharm.* **11:**483-497, 1983.

Lindsay, S. High Performance Liquid Chromatography. 2nd ed., John Wiley, Chichester, 1992.

Liu, H., Montoya, J.L., Forman, L.J., Eggers, C.M., Barham, C.F., Delgado, M. Determination of free valproic acid: evaluation of the Centrifree system and comparison between high-performance liquid chromatography and enzyme immunoassay. *Ther. Drug Monit.* **14:**513-521, 1992.

Liu, J., Shirota, O., Wiesler, D., Novotny, M. Ultrasensitive fluorometric detection of carbohydrates as derivatives in mixtures separated by capillary electrophoresis. *Proc. Nat. Acad. Sci. USA* **88:**2302-2306, 1991.

Lloyd, D.K. Capillary electrophoretic analyses of drugs in body fluids: sample pretreatment and methods for direct injection of biofluids. *J. Chromatogr. A* **735:**29-42, 1996.

Lloyd, D.K., Cypess, A.M., Wainer, I.W. Determination of cytosine-beta-D-arabinoside in plasma using capillary electrophoresis. *J. Chromatogr.* **568:**117-124, 1991.

Lockwood, F., Wagner, J.G. Plasma volume changes as a result of equilibrium dialysis. *J. Pharm. Pharmacol.* **35:**387-388, 1983.

Louis, W.J., McNeil, J.J., Workman, B.S., Drummer, O.H., Conway, E.L. A pharmacokinetic study of carvedilol (BM 14.190) in elderly subjects: preliminary report. *J. Cardiovasc. Pharmacol.* **10(Suppl. 11):**S89-S93, 1987.

Lukkari, P., Sirén, H., Pantsar, M., Riekkola, M.-L. Determination of ten β-blockers in urine by micellar electrokinetic capillary chromatography. *J. Chromatogr.* **632:**143-148, 1993.

Luksa, J., Josic, D. Determination of cimetidine in human plasma by free capillary zone electrophoresis. *J. Chromatogr. B* **667:**321-327, 1995.

Lurie, I.S. Micellar electrokinetic capillary chromatography of the enantiomers of amphetamine, metamphetamine and their hydrohyphenethylamine precursors. *J. Chromatogr.* **605:**269-275, 1992.

Lysko, P.G., Feuerstein, G.Z., Ruffolo Jr., R.R. Carvedilol - A novel multiple action antihypertensive drug. *Pharm. News* **2:**12-16, 1995.

Ma, Y., Zhang, R., Cooper, C.L. Indirect photometric detection of polyamines in biological samples separated by high-performance capillary electrophoresis. *J. Chromatogr.* **608:**93-96, 1992.

MacKichan, J.J. Influence of protein binding and use of unbound (free) drug concentrations. In: Evans, W.E., Schentag, J.J., Jusko, W.J. (Eds.): Applied Pharmacokinetics. Principles of Therapeutic Drug Monitoring. Applied Therapeutics, Inc., Vancouver, WA, 1992.

Mayer, S., Schurig, V. Enantiomer separation by electrochromatography in open tubular columns coated with chirasil-dex. *J. Liq. Chromatogr.* **16:**915-931, 1993.

McCormick, R.M. Capillary zone electrophoretic separation of peptides and proteins using low pH buffers in modified silica capillaries. *Anal. Chem.* **60:**2322-2328, 1988.

McErlane, K.M., Igwemezie, L., Kerr, C.R. Stereoselective analysis of the enantiomers of mexiletine by high-performance liquid chromatography using fluorescence

detection and study of their stereoselective disposition in man. *J. Chromatogr.* **415:**335-346, 1987.

McLaughlin, G.M., Nolan, J.A., Lindahl, J.L., Palmieri, R.H., Anderson, K.W., Morris, S.C., Morrison, J.A., Bronzert, T.J. Pharmaceutical drug separations by HPCE: practical guidelines. *J. Liq. Chromatogr.* **15:**961-1021, 1992.

McTavish, D., Campoli-Richards, D., Sorkin, E.M. Carvedilol: a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy. *Drugs* **45:**232-258, 1993.

Meier, P., Thormann, W. Determination of thiopental in human serum and plasma by high-performance capillary electrophoresis-micellar electrokinetic chromatography. *J. Chromatogr.* **559:**505-513, 1991.

Mikkers, F.E.P., Everaerts, F.M., Peek, J.A.F. Isotachophoresis: the concepts of resolution, load capacity and separation efficiency. *J. Chromatogr.* **168:**293-315, 1979.

Morgan, T. Clinical pharmacokinetics and pharmacodynamics of carvedilol. *Clin. Pharmacokinet.* **26:**335-346, 1994.

Morgan, T., Anderson, A., Cripps, J., Adam, W. Pharmacokinetics of carvedilol in older and younger patients. *J. Human Hypertens.* **4:**709-715, 1990.

Naylor, S., Tomlinson, A.J., Benson, L.M., Gorrod, J.W. Capillary electrophoresis and capillary electrophoresis-mass spectrometry in drug and metabolite analysis. *Eur. J. Drug Metab. Pharmacokinet.* **3:**235-240, 1994.

Neugebauer, G., Akpan, W., Kaufmann, B., Reiff, K. Stereoselective disposition of carvedilol in man after intravenous and oral administration of the racemic compound. *Eur. J. Clin. Pharmacol.* **38:**S108-S111, 1990.

Neugebauer, G., Akpan, W., vonMöllendorff, E., Neubert, P., Reiff, K. Pharmacokinetics and disposition of carvedilol in humans. *J. Cardiovasc. Pharmacol.* **10(Suppl. 11):**S85-S88, 1987.

Neugebauer, G., Gabor, M., Reiff, K. Disposition of carvedilol enantiomers in patients with liver cirrhosis: evidence for disappearance of stereoselective first-pass extraction. *J. Cardiovasc. Pharmacol.* **19(Suppl. 1):**S142-S146, 1992.

Neugebauer, G., Gabor, M., Reiff, K. Pharmacokinetics and bioavailability of carvedilol in patients with liver cirrhosis. *Drugs* **36(Suppl. 6):**148-154, 1988.

Neugebauer, G., Neubert, P. Metabolism of carvedilol in man. *Eur. J. Drug Metab. Pharmacokinet.* **16:**257-260, 1991.

Nichols, A.J., Gellai, M., Ruffolo Jr, R.R. Studies on the mechanism of arterial vasodilation produced by the novel antihypertensive agent, carvedilol. *Fundam. Clin. Pharmacol.* **5:**25-38, 1991.

Nichols, A.J., Sulpizio, A.C., Ashton, D.J., Hieble, J.P., Ruffolo Jr, R.R. The interaction of the enantiomers of carvedilol with α_1- and β_1-adrenoceptors. *Chirality* **1:**265-270, 1989.

Nielen, M.W.F. (Enantio-)separation of phenoxy acid herbicides using capillary zone electrophoresis. *J. Chromatogr.* **637:**81-90, 1993.

Nishi, H., Terabe, S. Application of micellar electrokinetic chromatography to pharmaceutical analysis. *Electrophoresis* **11:**691-701, 1990.

Ofori-Adjei, D., Ericsson, Ö., Lindström, B., Sjöqvist, F. Protein binding of chloroquine enantiomers and desethylchloroquine. *Br. J. Clin. Pharmacol.* **22:**356-358, 1986.

Ohshima, T., Johno, I., Kitazawa, S. Comparative evaluation of microultrafiltration devices for determination of protein binding. *Ther. Drug Monit.* **10:**310-315, 1988.

Olefirowicz, T.M., Ewing, A.G. Capillary electrophoresis with indirect amperometric detection. *J. Chromatogr.* **499:**713-719, 1990.

Ong, C.P., Ng, C.L., Lee, H.K., Li, S.F.Y. Determination of antihistamines in pharmaceuticals by capillary electrophoresis. *J. Chromatogr.* **588:**335-339, 1991.

Oravcová, J., Bystricky, S., Trnovec, T. Different binding of propranolol enantiomers to human alpha$_1$-acid glycoprotein. *Biochem. Pharmacol.* **38:**2575-2579, 1989.

Oravcová, J., Lindner, W., Szalay, P., Bohácik, L., Trnovec, T. Interaction of propafenone enantiomers with human α_1-acid glycoprotein. *Chirality* **3:**30-34, 1991a.

Oravcová, J., Mlynárik, V., Bystricky, S., Soltés, L., Szalay, P., Bohácik, L., Trnovec, T. Interaction of pirprofen enantiomers with human serum albumin. *Chirality* **3:**412-417, 1991b.

Pacifici, G.M., Viani, A. Methods of determining plasma and tissue binding of drugs. Pharmacokinetic consequences. *Clin. Pharmacokinet.* **23:**449-468, 1992.

Packer, M., Bristow, M.R., Cohn, J.N., Colucci, W.S., Fowler, M.B., Gilbert, E.M., Shusterman, N.H. The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. *N. Engl. J. Med.* **334:**1349-1355, 1996.

Parsons, D.L., Fan, H.F. Loss of propranolol during ultrafiltration in plasma protein binding studies. *Res. Commun. Chem. Pathol. Pharmacol.* **54:**405-408, 1986.

Patil, P.N., Miller, D.D., Trendelenburg, U. Molecular geometry and adrenergic drug activity. *Pharmacol. Rev.* **26:**323-392, 1975.

Paulus, A. Oligonucleotide separations with capillary gel electrophoresis. *Boll. Chim. Farm.* **131:**299-301, 1992.

Peterson, T.E., Trowbridge, D. Quantitation of *l*-epinephrine and determination of the *d-ll*-epinephrine enantiomer ratio in a pharmaceutical formulation by capillary electrophoresis. *J. Chromatogr.* **603:**298-301, 1992.

Pfeffer, M.A., Stevenson, L.W. β-Adrenergic blockers and survival in heart failure. *N. Engl. J. Med.* **334:**1396-1397, 1996.

Pfiasky, K.M. Disease-induced changes in the plasma binding of basic drugs. *Clin. Pharmacokinet.* **5:**246-262, 1980.

Pfiasky, K.M., Borgå, O., Odar-Cederlöf, I., Johansson, C., Sjöqvist, F. Increased plasma protein binding of propranolol and chlorpromazine mediated by disease-induced elevations of plasma α_1-acid glycoprotein. *N. Engl. J. Med.* **299:**1435-1439, 1978.

Piquette-Miller, M., Foster, R.T. High-performance liquid chromatographic determination of diacetolol enantiomers. *J. Chromatogr.* **533:**300-303, 1990.

Piquette-Miller, M., Foster, R.T., Pasutto, F.M., Jamali, F. Stereospecific high-performance liquid chromatographic assay of acebutolol in human plasma and urine. *J. Chromatogr.* **526:**129-137, 1990.

Pirkle, W.H., Pochapsky, T.C. Considerations of chiral recognition relevant to the liquid chromatographic separation of enantiomers. *Chem. Rev.* **89:**347-362, 1989.

Pleasance, S., Ayer, S.W., Laycock, M.V., Thibault, P. Ionspray mass spectrometry of marine toxins. III. Analysis of paralytic shellfish poisoning toxins by flow-injection analysis, liquid chromatography/mass spectrometry and capillary electrophoresis/mass spectrometry. *Rapid Commun. Mass Spectrom.* **6:**14-24, 1992a.

Pleasance, S., Thibault, P., Kelly, J. Comparison of liquid-junction and coaxial interfaces for capillary electrophoresis-mass spectrometry with application to compounds of concern to the aquaculture industry. *J. Chromatogr.* **591:**325-339, 1992b.

Prichard, B.N.C. Combined α- and β-receptor inhibition in the treatment of hypertension. *Drugs* **28(Suppl. 2):**51-68, 1984.

Pruñonosa, J., Obach, R., Diez-Cascón, A., Gouesclou, L. Comparison of high-performance liquid chromatography and high-performance capillary electrophoresis for the determination of cicletanine in plasma. *J. Chromatogr.* **581:**219-226, 1992a.

Pruñonosa, J., Obach, R., Diez-Cascón, A., Gouesclou, L. Determination of cicletanine enantiomers in plasma by high-performance capillary electrophoresis. *J. Chromatogr.* **574:**127-133, 1992b.

Rabel, S.R., Stobaugh, J.F. Applications of capillary electrophoresis in pharmaceutical analysis. *Pharm. Res.* **10:**171-186, 1993.

Rawjee, Y.Y., Vigh, G. A peak resolution model for the capillary electrophoretic separation of the enantiomers of weak acids with hydroxypropyl β-cyclodextrin-containing background electrolytes. *Anal. Chem.* **66:**619-627, 1994.

Rawjee, Y.Y., Williams, R.L., Vigh, G. Capillary electrophoretic chiral separations using cyclodextrin additives. Part III: peak resolution surfaces for ibuprofen and homatropine as a function of the pH and the concentration of β-cyclodextrin. *J. Chromatogr. A* **680:**599-607, 1994.

Reiff, K. High-performance liquid chromatographic method for the determination of carvedilol and its desmethyl metabolite in body fluids. *J. Chromatogr.* **413:**355-362, 1987.

Reinhoud, N.J., Tjaden, U.R., Irth, H., van der Greef, J. Bioanalysis of some anthracyclines in human plasma by capillary electrophoresis with laser-induced fluorescence detection. *J. Chromatogr.* **574:**327-334, 1992.

Rogan, M.M., Altria, K.D. Introduction to the theory and applications of chiral capillary electrophoresis. Beckman Instruments, Fullerton, 1993.

Rogan, M.M., Drake, C., Goodall, D.M., Altria, K.D. Enantioselective enzymatic biotransformation of 2'-deoxy-3'-thiacytidine (BCH 189) monitored by capillary electrophoresis. *Anal. Biochem.* **208:**343-347, 1993.

Routledge, P.A., Stargel, W.W., Wagner, G.S., Shand, D.G. Increased plasma propranolol binding in myocardial infarction. *Br. J. Clin. Pharmacol.* **9:**438-440, 1980.

Ruffolo Jr, R.R. Stereochemical requirements for activation and blockade of α_1- and α_2-adrenoceptors. *TiPS* **5:**160-164, 1984.

Ruffolo Jr, R.R., Boyle, D.A., Brooks, D.P., Feuerstein, G.Z., Venuti, R.P., Lukas, M.A., Poste, G. Carvedilol: a novel cardiovascular drug with multiple actions. *Cardiovasc. Drug Rev.* **10:**127-157, 1992.

Ruffolo Jr, R.R., Boyle, D.A., Venuti, R.P., Lukas, M.A. Carvedilol (Kredex®): a novel multiple action cardiovascular agent. *Drugs Today* **27:**465-492, 1991.

Ruffolo Jr, R.R., Gellai, M., Hieble, J.P., Willette, R.N., Nichols, A.J. The pharmacology of carvedilol. *Eur. J. Clin. Pharmacol.* **38:**S82-S88, 1990.

Sänger-van de Griend, C.E., Gröningsson, K., Westerlund, D. Chiral separation of local anaesthetics with capillary electrophoresis. Evaluation of the inclusion complex of the enantiomers with Heptakis (2,6-di-O-methyl)-β-cyclodextrin. *Chromatographia* **42:**263-268, 1996.

Schaefer, W. Formation of a carbamoyl glucuronide conjugate of carvedilol *in vitro* using dog and rat liver microsomes. *Drug Metab. Dispos.* **20:**130-133, 1992.

Schaefer, W.H., Goalwin, A., Dixon, F., Hwang, B., Killmer, L., Kuo, G. Structural determination of glucuronide conjugates and a carbamoyl glucuronide conjugate of carvedilol: use of acetylation reactions as an aid to determine positions of glucuronidation. *Biol. Mass Spectrom.* **21:**179-188, 1992.

Schutzner, W., Fanali, S. Enantiomers resolution in capillary zone electrophoresis by using cyclodextrins. *Electrophoresis* **13:**687-690, 1992.

Senderoff, S.G., Villani, A.J., Landvatter, S.W., Garnes, K.T., Heys, J.R. Synthesis of the enantiomers and three racemic metabolites of carvedilol labeled to high specific activity with tritium. *J. Labelled Compd. & Radiopharm.* **33:**1091-1105, 1993.

Sepaniak, M.J., Cole, R.O., Clark, B.K. Use of native and chemically modified cyclodextrins for the capillary electrophoretic separation of enantiomers. *J. Liq. Chromatogr.* **15:**1023-1040, 1992.

Shihabi, Z.K. Clinical applications of capillary electrophoresis. *Ann. Clin. Lab. Sci.* **22:**398-405, 1992.

Snopek, J., Soini, H., Novotny, M.V., Smolkova-Keulemansova, E., Jelinek, I. Selected applications of cyclodextrin selectors in capillary electrophoresis. *J. Chromatogr.* **559:**215-222, 1991.

Soini, H., Riekkola, M.-L., Novotny, M.V. Chiral separations of basic drugs and quantitation of bupivacaine enantiomers in serum by capillary electrophoresis with modified cyclodextrin buffers. *J. Chromatogr.* **608:**265-274, 1992.

Spahn, H. S-(+)-Naproxen chloride as acylating agent for separating the enantiomers of chiral amines and alcohols. *Arch. Pharm.* (Weinheim) **321:**847-850, 1988.

Spahn, H., Henke, W., Langguth, P., Schloos, J., Mutschler, E. Measurement of carvedilol enantiomers in human plasma and urine using S-naproxen chloride for chiral derivatization. *Arch. Pharm.* (Weinheim) **323:**465-469, 1990.

St. Pierre, L.A., Sentell, K.B. Cyclodextrins as enantioselective mobile phase modifiers for chiral capillary electrophoresis. Effects of pH and cyclodextrin concentration. *J. Chromatogr. B* **657:**291-300, 1994.

Stagni, G., Davis, P.J., Ludden, T.M. Human pharmacokinetics of betaxolol enantiomers. *J. Pharm. Sci.* **80:**321-324, 1991.

Stahl, E., Baumgartner, U., Henke, D., Schölmerich, J., Mutschler, E., Spahn-Langguth, H. Rats with portacaval shunt as a potential experimental pharmacokinetic model for liver cirrhosis: application to carvedilol stereopharmacokinetics. *Chirality* **5:**1-7, 1993a.

Stahl, E., Baumgartner, U., Krauss, D., Spahn, H., Schölmerich, J. Pharmacokinetics of (R)- and (S)-carvedilol and their glucuronides in rats with portacaval shunt after racemate dosage. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **341(Suppl.):**R4 (Abstract 14), 1990.

Stahl, E., Henke, D., Mutschler, E., Spahn-Langguth, H. Saturable enantioselective first-pass effect for carvedilol after high oral racemate doses in rats. *Arch. Pharm.* (Weinheim) **326:**123-125, 1993b.

Stahl, E., Mutschler, E., Baumgartner, U., Spahn-Langguth, H. Carvedilol stereopharmacokinetics in rats: affinities to blood constituents and tissues. *Arch. Pharm.* (Weinheim) **326:**529-533, 1993c.

Stegehuis, D.S., Irth, H., Tjaden, U.R., Van der Greef, J. Isotachophoresis as an on-line concentration pretreatment technique in capillary electrophoresis. *J. Chromatogr.* **538:**393-402, 1991.

Steuer, W., Grant, I., Erni, F. Comparison of high-performance liquid chromatography, supercritical fluid chromatography and capillary zone electrophoresis in drug analysis. *J. Chromatogr.* **507:**125-140, 1990.

Stevenson, D., Wilson, I.D. (Eds.) Chiral separations. Plenum Press, New York, 1988.

Sudor, J., Foret, F., Bocek, P. Pressure refilled polyacrylamide columns for the separation of oligonucleotides by capillary electrophoresis. *Electrophoresis* **12:**1056-1058, 1991.

Tagliaro, F., Poiesi, C., Aiello, R., Dorizzi, R., Ghielmi, S., Marigo, M. Capillary electrophoresis for the investigation of illicit drugs in hair: determination of cocaine and morphine. *J. Chromatogr.* **638:**303-309, 1993.

Taylor, D.R., Maher, K. Chiral separations by high-performance liquid chromatography. J. Chromatogr. Sci. **30:**67-85, 1992.

Tenero, D.M., Boyle, D.A., Cyronak, M.J., Kahn, C.G., Singh, K.P., Stote, R.M., Ileson, B., Gombar, C.T., DiCristo, M.B. Intersubject and intrasubject variability in the pharmacokinetics of carvedilol (SK&F 105517, BM 14190). *Pharmacotherapy* **10:**245, 1990.

Terabe, S. Selectivity manipulation in micellar electrokinetic chromatography. *J. Pharm. Biomed. Anal.* **10:**705-715, 1992.

Terabe, S., Otsuka, K., Ichikawa, K., Tsuchiya, A., Ando, T. Electrokinetic separations with micellar solutions and open-tubular capillaries. *Anal. Chem.* **56:**111-113, 1984.

Thibault, P., Paris, C., Pleasance, S. Analysis of peptides and proteins by capillary electrophoresis/mass spectrometry using acidic buffers and coated capillaries. *Rapid Commun. Mass Spectrom.* **5:**484-490, 1991a.

Thibault, P., Pleasance, S., Laycock, M.V. Analysis of paralytic shellfish poisons by capillary electrophoresis. *J. Chromatogr.* **542:**483-501, 1991b.

Thormann, W., Lienhard, S., Wernly, P. Strategies for the monitoring of drugs in body fluids by micellar electrokinetic capillary chromatography. *J. Chromatogr.* **636:**137-148, 1993.

Thormann, W., Meier, P., Marcolli, P., Binder, F. Analysis of barbiturates in human serum and urine by high-performance capillary electrophoresis-micellar electrokinetic capillary chromatography with on-column multi-wavelength detection. *J. Chromatogr.* **545:**445-460, 1991.

Tomlinson, B., Prichard, B.N.C., Graham, B.R., Walden, R.J. Clinical pharmacology of carvedilol. *Clin. Investig.* **70:**S27-S36, 1992.

Tozer, T.N., Gambertoglio, J.G., Furst, D.E., Avery, D.S., Holford, N.H.G. Volume shifts and protein binding estimates using equilibrium dialysis: application to prednisolone binding in humans. *J. Pharm. Sci.* **72:**1442-1446, 1983.

Tran, A.D., Blanc, T., Leopold, E.J. Free solution capillary electrophoresis and micellar electrokinetic resolution of amino acid enantiomers and peptide isomers with L- and D-Marfey's reagent. *J. Chromatogr.* **516:**241-249, 1990.

Tsuji, K., Baczynskyj, L., Bronson, G.E. Capillary electrophoresis-electrospray mass spectrometry for the analysis of recombinant bovine and porcine somatotropins. *Anal. Chem.* **64:**1864-1870, 1992.

Tsukiyama, H., Otsuda, K., Horii, M. Haemodynamic effects of new β-blockers with vasodilatory properties in essential hypertension. *Drugs* **36(Suppl. 6):**48-54, 1988.

Varin, F., Cubeddu, L.X., Powell, J.R. Liquid chromatographic assay and disposition of carvedilol in healthy volunteers. *J. Pharm. Sci.* **75:**1195-1197, 1986.

Vespalec, R., Bocek, P. Chiral separations by capillary electrophoresis: present state of the art. *Electrophoresis* **15:**755-762, 1994.

vonMöllendorff, E., Reiff, K., Neugebauer, G. Pharmacokinetics and bioavailability of carvedilol, a vasodilating beta-blocker. *Eur. J. Clin. Pharmacol.* **33:**511-513, 1987.

Wainer, I.W., Doyle, T.D. Application of high-performance liquid chromatographic chiral stationary phases to pharmaceutical analysis: structural and conformational effects in the direct enantiomeric resolution of α-methylarylacetic acid anti-inflammatory agents. *J. Chromatogr.* **284:**117-124, 1984.

Walle, U.K., Walle, T., Bai, S.A., Olanoff, L.S. Stereoselective binding of propranolol to human plasma, α_1-acid glycoprotein, and albumin. *Clin. Pharmacol. Ther.* **34:**718-723, 1983.

Wätzig, H., Lloyd, D.K. Effect of pH and sodium dodecyl sulfate concentration on the analytical window in the direct-injection analysis of plasma samples by capillary electrophoresis. *Electrophoresis* **16:**57-63, 1995.

Whitlam, J.B., Brown, K.F. Ultrafiltration in serum protein binding determinations. *J. Pharm. Sci.* **70:**146-150, 1981.

Wolfisberg, H., Schmutz, A., Stotzer, R., Thormann, W. Assessment of automated capillary electrophoresis for therapeutic and diagnostic drug monitoring: determination of bupivacaine in drain fluid and antipyrine in plasma. *J. Chromatogr. A* **652:**407-416, 1993.

Wren, S.A.C. Theory of chiral separation in capillary electrophoresis. *J. Chromatogr.* **636:**57-62, 1993.

Wren, S.A.C., Rowe, R.C. Theoretical aspects of chiral separation in capillary electrophoresis. III. Application to β-blockers. *J. Chromatogr.* **635:**113-118, 1993.

Wren, S.A.C., Rowe, R.C., Payne, R.S. A theoretical approach to chiral capillary electrophoresis with some practical implications. *Electrophoresis* **15:**774-778, 1994.

Yao, X.-W., Wu, D., Regnier, F.E. Manipulation of electroosmotic flow in capillary electrophoresis. *J. Chromatogr.* **636:**21-29, 1993.

Yue, T.-L., Mckenna, P.J., Lysko, P.G., Gu, J.-L., Lysko, K.A., Ruffolo Jr., R.R., Feuerstein, G.Z. SB 211475, a metabolite of carvedilol, a novel antihypertensive agent, is a potent antioxidant. *Eur. J. Pharmacol.* **251:**237-243, 1994.

Zar, J.H. Biostatistical analysis. 2nd ed., Prentice Hall, Englewood Cliffs, 1984.

Zhirkov, Y.A., Piotrovskii, V.K. On the usefulness of ultrafiltration in drug-protein binding studies. *J. Pharm. Pharmacol.* **36:**844-845, 1984.

Zhou, H.-H., Wood, A.J.J. Stereoselective disposition of carvedilol is determined by CYP2D6. *Clin. Pharmacol. Ther.* **57:**518-524, 1995.

Zief, M., Crane, L.J. (Eds.) Chromatographic chiral separations. Marcel Dekker, New York, 1988.