DEVELOPMENT OF A SENSITIVE AND STEREOSELECTIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY METHOD FOR PROPAFENONE ENANTIOMERS IN HUMAN PLASMA

BY

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ABSTRACT

Propafenone is a new class 1C antiarrhythmic agent with additional calcium antagonistic and beta-blocking activities. Clinically it is effective in the treatment of supraventricular and ventricular tachycardia, atrial and ventricular fibrillation, ventricular premature contractions and for the management of Wolf-Parkinson-White syndrome. In North America it is still an investigational drug.

Propafenone is a chiral drug and is used clinically in the racemic form. The enantiomers of numerous chiral drugs have been shown to differ in their disposition kinetics in the body due to their stereoselective pharmacokinetics and/or pharmacodynamic properties. Two enantiomers are thus often considered as two different entities. The relative antiarrhythmic activities of individual enantiomers of propafenone have not been studied, nor their pharmacokinetic parameters have been elucidated. In order to study the possible enantioselective role of propafenone in the body, a stereoselective assay method would be required. The present study describes the development of a sensitive and stereoselective chromatographic assay method for the simultaneous determination of the two enantiomers of propafenone in human plasma.

Attempts for direct separation of the enantiomers of propafenone included several GLC and HPLC chiral stationary phases. The chiral stationary phases were a Chirasil-Val^{R} GLC stationary phase, a Pirkle 2,4 dinitro-(D)-phenylglycine HPLC stationary phase and a β-cyclodextrin HPLC stationary phase. Unfortunately, these did not resolve the enantiomers of propafenone.

Formation of the diastereomers with R(+)-α-methylbenzylisocyanate

and racemic propafenone were partially resolved on a reverse phase HPLC using a 5 u, 25 x 0.45 cm i.d. ODS column and methanol/water (70:30) as the mobile phase. However, due to the long retention time (42 min), incomplete resolution (R_S=1.15) and poor sensitivity for detection (500 ng of each enantiomer injected) this method was not deemed suitable for the pharmacokinetic studies planned, since the therapeutic plasma concentration range of propafenone is 64-1044 ng/mL.

The second chiral derivatizing reagent, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylisothiocyanate (GITC), was synthesized in our laboratory. This reagent gave better resolution of the enantiomers (R_S=1.4) within 15 minutes with enhanced sensitivity for detection (150 ng of each enantiomer injected).

To further optimize the limit of detection for future pharmacokinetic studies of propafenone, R(-)-1-(naphthyl) ethylisocyanate, a chiral derivatizing agent, was employed. This reagent reacted with racemic propafenone and permitted the resolution of both enantiomers within 24 minutes (R_S=1.25) and the minimum level of detection was 100 ng (at the detector) for each enantiomer of propafenone. Using this method, linearity was established over the concentration range, 125-1000 ng for each enantiomer (injected) with a coefficient of determination (r^2) of greater than 0.99.

Reproducibility and precision of this assay method was obtained with an average coefficient of variability of 4.5% for the R(-) enantiomer and 7.2% for S(+) enantiomer at concentrations of 125-1000 ng/mL. Below the lower quantity, the NEIC-propafenone reaction virtually stopped at the conditions set for derivatization. A similar lack of reactivity at low concentrations was also observed with the

GITC-propafenone reaction.

The absence of an autocatalysing effect of propafenone at lower nanogram levels, as well as two possible conformational forms of propafenone were also investigated. The existence of two conformational isomers of propafenone, due to intramolecular hydrogen bonding in aprotic solvents, was chromatographically verified. In addition, chromatographic separation of all the proposed conformers was obtained, indicating that enantiomeric separation and quantitation of propafenone enantiomers as their urea derivatives is substantially hindered.

To eliminate hydrogen bonding interactions, the carbonyl group of propafenone was blocked with dansylhydrazine and subsequently derivatized with the chiral R(-)NEIC reagent. The HPLC resolution (R_S=1.35) of this dual derivative was better than that using the R(-) NEIC reagent alone, and the minimum level of detection was 2.5 ng for each enantiomer. Unfortunately, this procedure still did not provide adequate assay precision and accuracy at the lower levels required for single dose pharmacokinetic studies.

TABLE OF CONTENTS

LIST OF FIGURES

LISTS OF FIGURES cont'd

LIST OF TABLES

LIST OF SCHEMES

SYMBOLS AND ABBREVIATIONS

AAG	α_1-acid glycoprotein
AV	atrio-ventricular
CNS	central nervous system
CV	coefficient of variation
DOPA	3-(3,4-dihydroxyphenyl)alanine
dp/dt	rate of development of left ventricular pressure
ECD	electron capture detector
EI	electron impact
EI-MS	electron impact mass spectrum (spectra)
Em	emission
Ex	excitation
FID	flame ionization detector
GC/GLC	Gas-liquid chromatography
GITC	2,3,4,6 tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate
HFBA	heptafluorobutyric anhydride
HPLC	high-performance liquid chromatography
hr	hour
HV	His-ventricular, His-bundle
HSA	human serum albumin
i.d.	internal diameter
IPA	isopropanol, 2-propanol
I.S.	internal standard

k	reaction rate constant
MBIC	methylbenzylisocyanate
mL	millilitre
M	molarity, molar
u	micron
ug	microgram
N	normality, normal
NEIC	naphthylethylisocyanate
ng	nanogram
N-TPC	N-trifluoro-acetyl-L-proloylchloride
PR	atreoventricular conduction period of electrocardiogram
PVC	premature ventricular contraction
PTFE	polytetrafluoroethylene
QRS	intraventricular conduction period of electrocardiogram
QT	interval of the electrocardiogram representing ventricular depolarization and repolarization
r	correlation coefficient
r^2	coefficient of determination
RP	reverse phase
R_S	resolution factor
R_t	retention time
SA	sino-atrial
$\pm$ S.D.	$\pm$ standard deviation

TCA	trichloroacetic acid
TEA	triethylamine
TLC	thin-layer chromatography
uv	ultraviolet
VPB	ventricular premature beats

This thesis is dedicated to the loving memory of my father.

ACKNOWLEDGEMENT

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1. INTRODUCTION

Propafenone, 2'-[3-(propylamino)-2-(hydroxy)-(propoxy)]- 3-phenylpropiophenone is a new class 1C antiarrhythmic agent. The drug was synthesized in 1970 and its hydrochloride salt has been commercially available in West Germany (Knoll, AG) since 1977 as Rytmonorm^{R}. Propafenone has also been approved for marketing in Spain, Italy and Portugal for the treatment of cardiac arrhythmias of varying origin. In North America it is still an investigational drug.

A number of studies of this new antiarrhythmic drug have appeared over the past ten years and have established its pharmacological (Keller et al., 1978, Kohlhardt, 1977, Palma et al., 1982) electrophysiological (Seipel et al., 1977, Walleffe et al., 1981) and antiarrhythmic (Durante et al., 1980, Meyer-Estorff et al., 1978) properties as well as its toxicities (Kretzschmer et al., 1983a, Von. Philipsborn, 1984).

The drug may be administered either orally or intravenously. With oral administration, dose dependent systemic bioavailability of 12% to 50% has been reported (Hollmann et al., 1983, Palma et al., 1982, Connolly et al., 1983). The mean therapeutic plasma concentrations of propafenone have been reported to be 64 ng/ml to 1044 ng/ml and the mean half-life of the drug is from 3 to 6 hours (Hollmann et al., 1983, Connolly et al., 1983). Propafenone structurally resembles propranolol for its aryloxy propanolamine moiety, with a chiral center in its molecular arrangement. The drug is used as a racemic mixture of R and S enantiomers.

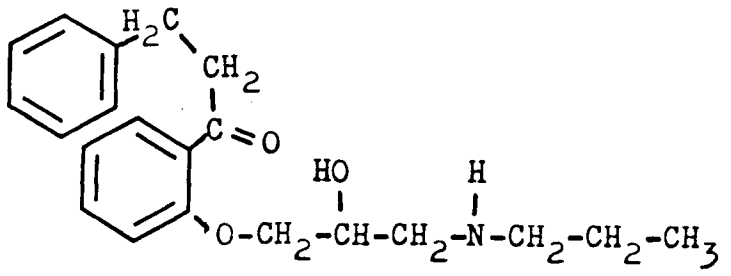
About 25% of the drugs in use today contain chiral centres (Simonyi, 1984). The fact that drug enantiomers differ chemically and

exhibit differing biological activities, has led to the suggestion that they be considered as different compounds (Simonyi, 1984, Ariens, 1986). The differences in intrinsic biological activity of enantiomers are due mainly to enantioselectivity in protein binding, storage, and/or transport processes in the body. Thus, the plasma levels and pharmacokinetics of racemic drugs do not always reflect those of the individual active enantiomers. Despite this knowledge, most chiral drugs, including propafenone, are used therapeutically as racemic mixtures. The relative activity of the individual enantiomers of propafenone have not, as yet, been determined nor have their pharmacokinetic parameters have been elucidated. The present study was therefore aimed at developing a sensitive high-performance liquid chromatographic assay method for the simultaneous determination of both enantiomers of propafenone in human biological fluids.

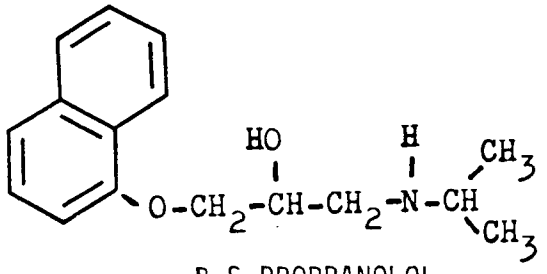
2. BACKGROUND

2.1 Chemistry

Propafenone structurally resembles the beta-adrenoceptor blockers which include propranolol. Its molecular formula is $C_{21}H_{27}NO_3$ and its molecular weight is 341.46. Propafenone Hydrochloride (pK_a=9) is a crystalline white powder with a melting point of 172^{o}C. It is only slightly soluble in cold water. Propafenone base is also crystalline with a melting point of 69^{o}C (observed).



R,S PROPAFENONE



R,S PROPRANOLOL

Propafenone has three reactive functional groups. Due to the proximity of the ortho aryl carbonyl group to the active alcoholic and amino hydrogens in the side chain, propafenone is believed to have intramolecular hydrogen bonding, resulting in two conformational isomers. The relative abundance of the two isomers are expected to be dependent on the pH of the solvent and the conformational flexibility of the two isomers, as has been shown for other such molecular arrangements (Kuhn et al., 1964). Except for chemical properties, the thermodynamic and solubility parameters of the two conformational isomers are assumed to be almost identical. Propafenone, being a chiral drug, consists of two enantiomers. The absolute configurations of the two enantiomers have very recently been reported by Blaschke et al.,1987, and were determined to be the R(-) and S(+) propafenone respectively.

2.2 Clinical Indications of Propafenone

Propafenone has been shown to be an effective antiarrhythmic agent due to its blockade of the fast inward sodium current (Kohlhardt, 1984).

In patients with Wolf-Parkinson-White syndrome, Breithardt, et al., 1984, reported that propafenone was an effective agent for long-term management of this disease. The drug is useful in decreasing the multiple episodes of recurrent ventricular tachycardia or ventricular fibrillation (Heger et al., 1984). Podrid et al., 1982, demonstrated that propafenone suppressed complex ventricular ectopy without depressing the ejection fraction in patients with normal left ventricular function, but it reduced the ejection fraction in those exhibiting myocardial dysfunction. Propafenone was evaluated as a potent antiarrhythmic agent in arrhythmias that appear to depend on sympathetic drive (Coumel et al., 1984). The drug decreases the anterograde conduction in the accessory pathway as well as in the normal pathway, thereby protecting the heart against rapid ventricular rates during circus movement tachycardia, atrial fibrillation or during atrial flutter (Waleffe et al., 1981).

2.3 Comparative Clinical Trials against other Antiarrhythmic Agents

Asshauer and Vorpahl, 1977, compared the oral doses of propafenone (300 mg) with procainamide (500 mg) in patients with stable ventricular extrasystoles. Propafenone was found to significantly reduce the number of extrasystoles, while they remained refractory with procainamide. Propafenone was established to be superior to disopyramide, perhexiline and quinidine in patients with history of myocardial infarction and ectopic ventricular arrhythmias (De Brito et al.,1979). However, Klempt and his co-workers, 1982, evaluated propafenone (900 mg) against mexiletine (600 mg) and flecainide (400 mg) in patients following

myocardial infarction. The percent reduction in single and repetitive ventricular premature beats (VPB) were 80 and 97 for propafenone, 53 and 83 for mexiletine and 94 and 99 for flecainide. Both flecainide and propafenone lengthened the duration of PQ and QRS intervals seen in the electrocardiogram, while mexiletine did not change these parameters. The subjective side effects of headache and dizziness were similar for all three drugs.

2.4 Plasma Levels and Therapeutic Effectiveness

The plasma concentration-effect relationship of propafenone on atrioventricular conduction time (PR interval) in patients with cardiac arrhythmias revealed that a significant correlation between serum propafenone concentration and changes in PR interval was present (Keller, et al.,1978). The maximum increase in PR interval was achieved between two to three hours at the highest plasma concentration of propafenone and returned to baseline as the drug concentration fell. A similar concentration-effect relationship was observed in the suppression of ventricular premature beats when propafenone concentrations in plasma reached levels of 64 to 1044 ng/mL (Connolly et al.,1983). However, marked inter-subject variations in the effective plasma concentrations were also noted. A potent antiarrhythmic effect has also been demonstrated for 5-hydroxy propafenone, a major metabolite of propafenone, in rats, and was correlated with greater antiarrhythmic response than the parent drug (Kretzschman et al., 1983, Siddoway et al., 1983b, 1987).

2.5 Selected Pharmacodynamic Studies

2.5.1 Pharmacology

Racemic Propafenone has been shown to be an effective drug in the treatment of supraventricular and ventricular arrhythmias and ectopic beats (Hollmann et al, 1983). In both in vitro and in vivo experiments in animal models, it has been found to be a membrane stabilizing antiarrhythmic drug which inhibits the fast Sodium (Na^+) inward current (Ledda et al., 1981). The local anesthetic effect of propafenone corresponds to that of procaine, and at higher concentrations calcium antagonistic effects were also noted. It is, however, 100 times less potent than verapamil (Scholz, 1982) in this later action. The effect of oral and intravenous propafenone in patients with ventricular arrhythmias has been evaluated. Oral propafenone was found to be more effective than the intravenous formulation in that a complete regression of PVC was noted in 85% of cases; whereas the intravenous drug was found to be effective in 50% of cases in suppressing the severe ventricular arrhythmias (Ledain et al., 1982).

Propafenone contains an aryloxy propanolamine structure, which is common to beta adrenergic blockers. The weak beta sympatholytic effect of the drug has been observed in inhibiting the isoprenaline-induced positive inotropic and chronotropic effects in isolated guinea pig atria (Ledda et al., 1981). The degree of inhibition of these beta sympatholytic effects was found to be 50 times greater in tracheal muscles than in myocardium (Scholz, 1982). The beta sympatholytic effect seen in human volunteers was estimated to be 2 to 5% of that of other typical beta blocking agents (Von.Philipsborn, 1984). Propafenone

was found to influence exercise-induced tachycardia as well as the resting heart rate, and to a certain extent, the systolic and diastolic blood pressure (Muller-Peltzer et al., 1983).

Anticonvulsant effects have also been demonstrated for propafenone at a dose seven times greater than that required for the suppression of arrhythmias (Kretzschmer et al., 1983a). The normal and dose-induced ECG pattern in rabbits indicated that at higher doses some CNS side effects occur, such as dizziness and fatigue (Von. Philipsborn, 1984).

2.5.2 Electrophysiological Effects on the Heart

The antiarrhythmic effect of propafenone is based on an inhibition of the rapid transmembrane Na^+ influx by depressing the maximal rate of depolarization (V_{max}) in ventricular myocardium and Purkinje fibres without modifying the resting potential (Ledda et al., 1981). Propafenone was graded as a class 1C antiarrhythmic agent, although it looses its virtual specificity for the Na^+ system and produces additional changes in the plateau and repolarization phase of the action potential, consistent with a decrease of slow calcium-induced inward current (Ledda et al., 1981). At higher concentrations of the drug, it has an inhibiting effect on SA node and gains the property of a class 4 antiarrhythmic agent (Kohlhardt, 1984).

In patients with ventricular arrhythmias, the drug causes a 16% increase in the PR interval of the electrocardiogram and a prolongation of the QRS interval by 18%. A slight increase of the QT interval has also been reported at higher doses (Salerno et al., 1984). The drug thus prolongs the atrioventricular and intraventricular conduction times and left ventricular systolic function (Hollmann, 1983).

2.5.3 Haemodynamic Effect

Bachour et al., 1977, first reported a change in the haemodynamic parameters after intravenous administration of propafenone to patients with coronary heart disease. A significant rise of right atrial and pulmonary artery pressure was observed between five to twenty minutes, with an instantaneous increase in peripheral resistance and decrease in cardiac output. Fill et al., 1977, reported that heart rate and left ventricular end-systolic pressure were relatively unchanged after intravenous administration of propafenone but with a significant decrease in left ventricular dp/dt ratio within 10 minutes. In patients with low, grade II - V arrhythmias, propafenone demonstrated a negative chronotropic effect of 16% of the patients. Mexiletine, tocainide and disopyramide showed similar cardiac effects in 17, 19, and 30% of patients, respectively (Wester et al., 1982).

2.6 Arrhythmia Suppression

The concentration-response relation for suppression of arrhythmias by propafenone shows substantial interindividual variability. Salerno et al., 1984, reported minimum effective trough plasma concentrations in patients ranging from 91 to 3271 ng/mL. Similarly, Connolly et al., 1983, observed that the effective concentrations ranged from 64 to 1044 ng/ml, and that the highest concentration required in their patients for 90% suppression of ectopic beats was approximately 800 ng/mL. Siddoway and his associates, 1984, reported that the mean concentration ranges for effective dosing were 143 to 1992 ng/mL.

2.7 Adverse Effects

Propafenone is considered to be relatively safe as compared to other antiarrhythmic agents. The common side effects after prolonged use of the drug are bitter taste, mild nausea and dry mouth. Moreover, central nervous system side effects, including visual blurring, dizziness and paraesthesias, were also noted when the serum propafenone concentrations were 900 ng/mL or more (Connolly et al., 1983). These effects were more pronounced in poor metabolizers than extensive metabolizers of propafenone (Siddoway et al., 1987). Cardiovascular side effects are rare but drug-induced transient bradycardia and ventricular fibrillation were observed in a few cases (Beck et al., 1980).

2.8 Drug Interactions

Limited drug interaction studies have been reported in the literature for propafenone. However, Beck et al., 1982, reported that co-administration of propafenone and propranolol increased the PR interval by an average of 25%, whereas administration of propafenone and propranolol separately increased the PR interval by 13 and 19%, respectively. This indicates that the additive effect of both substances can lead to an inhibition of atrioventricular conduction and of sinus node function. Similar increases in effect were observed after the co-administration of propafenone and lidoflazine (Beck et al., 1980). Due to the high level of protein-binding of propafenone (97%), a possible drug interaction would be expected with warfarin. While this has not been determined, phenprocoumon did not interfere with

propafenone disposition. A 37% increase in serum digoxin levels was observed after co-administration of propafenone in digitalized patients (Knoll AG report on propafenone, 1984, Belz et al., 1982).

2.9 Metabolism of Propafenone

Propafenone, like most antiarrhythmic drugs, undergoes extensive hepatic oxidative metabolism and less than 1% of the drug is excreted unchanged in urine and bile (Marchensini et al., 1983). The glucuronide and sulphate conjugates of 5-hydroxy propafenone are the main metabolites in plasma, urine and bile, followed by the conjugates of hydroxy-methoxy propafenone and propafenone itself (Hege et al., 1984, Kretzschman et al., 1983). 5-hydroxy propafenone was found to be twice as potent as propafenone in its antiarrhythmic efficacy in rats (Hege et al., 1984). A minor metabolite, N-depropyl propafenone was also identified in plasma, although its pharmacological and antiarrhythmic activities are still uncertain (Philipsborn, 1984, Kates et al., 1985). Like lorcainide, hepatic metabolism of propafenone involves a saturable process which accounts for the non-linear characteristics of drug metabolism (Connolly et al., 1983). Siddoway et al., 1983b, 1987, classified arrhythmic patients as either extensive or poor metabolizers of propafenone on the basis of their ability to metabolize debrisoquine by cytochrome P-450 isozyme(s). The steady state concentration of 5-hydroxy propafenone exceeded those of the parent drug in extensive metabolizers, while poor metabolizers accumulated low concentrations of metabolite and accrued higher concentrations of the parent drug.

Metabolism of chiral drugs is generally stereospecific and it could be that, like propranolol, the racemic drug propafenone may also

exhibit stereospecific metabolism.

2.10 Pharmacokinetics of Propafenone

Propafenone is readily absorbed from the gastrointestinal tract and reaches a peak plasma concentration within 2 to 3 hours with dose dependent bioavailability ranging from 12 to 50% (Hollmann et al., 1983, and Connolly et al., 1983). The change in bioavailability with variation of dose is nonlinear which indicates that propafenone is subject to saturable processes during presystemic liver passage. Food also increases the bioavailability of propafenone. A 147 % increase of bioavailability has been reported by Axelson et al., 1987, when propafenone was taken orally with a standard breakfast.

The disposition kinetics after intravenous administration of the drug shows that propafenone is rapidly distributed with a distribution half-life of 4.7 minutes. The volume of the central compartment is approximately 0.7 to 1.1 L/kg (Hollmann et al., 1983). The plasma concentration decays rapidly in a bi-exponential manner. The steady-state volume of distribution is approximately 1.9 to 3 L/kg (Seipel et al., 1980). More than 97% of the drug is protein-bound and the largest concentration of propafenone was found in lungs, which is 10-fold higher than that found in heart muscle (Seipel et al., 1980).

It has been reported that propafenone single-dose pharmacokinetics, based on total serum concentrations, are non-linear (Connolly et al., 1984). The same authors reported a 10-fold increase in steady-state serum concentrations for a 3-fold increase in dose from 300 mg to 900 mg of propafenone. The apparent terminal elimination half-life of propafenone ranges from 3.6 to 4.6 hours, however,

variations in half-lives from 1.8 to 17.2 hours have also been reported due to interindividual variability (Keller et al., 1978, Hollmann et al., 1983, and Connolly et al., 1983). In patients with complex ventricular arrhythmias, half-lives ranging from 1.8 to 32.3 hours have also been reported (Siddoway et al., 1983). There was also great variability in the clearance following oral administration, with a range of 192 to 4918 mL/min., and dose-corrected mean plasma concentration ranges of 0.13 to 3.32 ng/mL per milligram of dose. Clearance also decreases during long-term therapy (Siddoway et al., 1983). Very recently, Siddoway and his associates, 1987, reported that propafenone disposition kinetics differ between poor metabolizers and extensive metabolizers and the authors speculated that the nonlinear relationship between dose and plasma concentration is likely due to saturation of the cytochrome P-450 isozyme(s) involved.

2.11 Protein-Binding of Propafenone

Neumann, (1978) first studied the plasma protein-binding of ^{3}H-propafenone in man using an equilibrium dialysis technique. At plasma concentrations up to 1600 ng/mL, plasma protein-binding was about 97%. Protein- binding was observed to decrease slightly at higher concentrations of the drug. Similar protein-binding results have been reported in the dog and the rat (Neumann, 1979). Propafenone, in the concentration range of 50 to 1250 ng/mL, equilibrates rapidly between human erythrocytes and plasma in the ratio of 0.66 to 0.74 which indicated that the drug is extensively bound to plasma proteins at therapeutic concentrations (Hollmann et al., 1983). Higuichi et al., 1985, determined the plasma protein binding of the drug in both in vitro

and *in vivo* and reported that plasma protein- binding of propafenone was 69 to 88% in rats, 94 to 95% in dogs and 76 to 89% in humans. In another study, Steinbach *et al.*, 1983, reported that about 98% of propafenone is bound to plasma proteins in humans.

Basic drugs usually bind to AAG, and this binding can also be stereoselective. The S(+) enantiomer of disopyramide was reported to bind preferentially with AAG as compared to the R(-) enantiomer (Lima *et al.*, 1984,), and S propranolol was noted to bind preferentially to that of R propranolol (Albani *et al.*, 1984). Gillis *et al.*, 1985, reported the presence of two binding sites on AAG for both racemic propranolol and propafenone. From non-linear Scatchard plots the authors pointed out that stereoselective binding characteristics for both drugs are expected on AAG.

2.12 Stereoselectivity in Drug Action

An asymmetric molecule and its mirror image are characterized by many identical physical and chemical properties, but when the molecule finds itself in the chiral environment afforded by the body, physiological manifestations of differences between enantiomers are frequently observed. The enantioselective behavior is due to the association of the chiral drugs to the chiral structures in organisms resulting in diastereomeric properties of drug enantiomers, which contribute to differences in drug transport, binding, drug-receptor interaction and drug biotransformation.

Numerous studies have been reported with isolated tissues and intact animals to verify the enantioselective nature of the interaction of drugs with cellular and tissue compartments (Ariens *et al.*, 1983;

Smith, 1984; Patil et al., 1970). An example is R(-) norepinephrine, which is 300 times more potent than its antipode as an adrenergic receptor agonist on the rabbit aorta, and has higher binding affinity for the receptors, or preferred intrinsic activity at the binding site. The differences between the pharmacological activity of enantiomers may be of pharmacokinetic as well as of pharmacodynamic origin. However, the relative differences in absorption, distribution, metabolism, protein- or tissue-binding may confer stereoselectivity of drug enantiomer interactions in a chiral environment.

2.12.1 Absorption

Due to similar lipid and aqueous solubilities, absorption of drug enantiomers in the body should be identical and follow first-order kinetics - except for the manner in which the drug enantiomers show enantioselectivity and are absorbed by active transport processes. For example, in studies in rabbits by means of an in situ ligated loop and an in-vitro tissue accumulation technique, Shindo et al., 1973, demonstrated that L-DOPA is absorbed from rat intestine by an active transport mechanism, which proceeds independently from the decarboxylation process. Such a carrier-mediated active process of absorption is saturable and leads to dose-dependent absorption of the substrate. Further examples of enantioselective absorption include the L-isomers of methotrexate and ascorbic acid, which are preferentially absorbed in the body as compared to their antipodes (Wade et al., 1973; Handel et al., 1984).

2.12.2 Presystemic Elimination

Enzymes are asymmetric in nature and exhibit differences in affinities between drug enantiomers for reactive sites. The drug enantiomers of high extraction ratio exhibit differences in bioavailability for first-pass extraction by both liver and gut. It has been found that propranolol undergoes stereoselective presystemic clearance in dogs (Walle and Walle, 1979) and in man (Silber and Reiglemen, 1980; Von Bahr et al., 1982) and that intrinsic clearance of R(+) propranolol exceeds that of the more pharmacologically active S(-) propranolol enantiomer. Similarly, a significant stereoselective hepatic extraction has been demonstrated for metoprolol. The bioavailability of the S(-) enantiomer was found 1.4 times higher than that of R(+) enantiomer in extensive metabolizers (Dayer et al., 1985). Another example of stereoselective first-pass metabolism is verapamil; in which the systemic availability of the more active (-) enantiomer was 1/2 to 1/3 that of the (+) enantiomer (Eichelbaum et al., 1984).

2.12.3 Distribution

Stereoselective distribution may arise due to carrier-mediated transport or selective uptake of drugs by various organs and by binding to tissue and plasma proteins. Selective tissue-binding would increase the elimination half-life of the more highly bound enantiomer and may contribute to differences in enantiomer concentrations at relevant receptors as well as in plasma. The liver/plasma concentration ratios of S and R phenprocoumon in rats were found to be significantly different; the values being 6.9 $\pm$ 0.5 and 5.2 $\pm$ 0.2, respectively (Schmidt and Jahnchen, 1977), indicating a preferred uptake of the pharmacologically more potent enantiomer into the target organ.

Cardiac uptake of the beta-adrenergic blocker, propranolol, is enantioselective. Kawashima et al., 1976, in a study in rats, reported that the serum and heart concentrations of the individual enantiomers of propranolol were different. After 5 minutes, the serum levels of the pharmacologically active S isomer were three times lower than those of its antipode. During the following 4 hours, however, the R form disappeared faster from the serum ($t_{1/2}$ = 24 min.) than the S form ($t_{1/2}$ = 52 min.). The initial lower serum levels of S isomer were interpreted as indicative of a highly selective cardiac uptake. However, most of the propranolol found in heart tissue was the S isomer (82 $\pm$ 3% up to 90 min. and 100% after 2 hours). It appears, therefore, that the pharmacologically most active enantiomer is taken up by liver and rapidly metabolized. These facts are similar to the findings of George et al., 1972, in which the plasma half-lives of S and R propranolol in man were 3.2 $\pm$ 0.42 hours and 2.02 $\pm$ 0.55 hours respectively. These results also closely parallel those observed in rats, suggesting a comparable and therapeutically important stereoselective uptake by cardiac tissue. Similarly, (-) timolol in the rat was taken up much more avidly by particulate fractions of heart, lung and brain than the (+) enantiomer, which was bound only to non-specific sites and eliminated faster (Tocco et al., 1976). Selective transport into rat brain has been documented for the active S enantiomer of α-methyldopa (Ames et al., 1977).

Due to the chiral nature of human serum albumin (HSA) and α_1-acid glycoprotein (AAG), stereoselective interactions of drug enantiomers are to be expected. The stereoselective binding interaction of L tryptophan with HSA has been well documented. L Tryptophan was found to bind to a

single site with an affinity of about 100 times than that for D tryptophan (McMenany et al., 1958). Studies of the stereoselective binding of drug enantiomers to plasma proteins have been reviewed by Alebic-Kolbah et al., 1979. In 1975, Muller and Wolhert found that the S(+) enantiomer of oxazepam hemisuccinate had 35-fold higher binding affinity to human serum albumin than its antipode.

2.12.4 Metabolism

The two enantiomers of a chiral substrate react differently with enzymes, which are themselves chiral and hence enantioselectivity can be expected.

Stereoselective metabolism was observed after the sequential administration of R and S enantiomers of mephenytoin to dogs (Kupfer and Bircher, 1979). This study showed that aromatic hydroxylation to form 4-hydroxymephenytoin was highly selective for the S enantiomer. Conversely the R enantiomer, was metabolised to 5-phenyl 5-ethylhydantoin (PEH) via N-demethylation. The long elimination half-life (> 80 hr.) for PEH, coupled with the short elimination half-life (4 hr.) for 4-hydroxy-mephenytoin led to the supposition that the major therapeutic effect from chronic administration of racemic mephenytoin is due to the R enantiomer.

Enantioselective biotransformation was observed with the anticoagulant drug warfarin. In man, the biologically more potent enantiomer, S warfarin, was metabolized and inactivated primarily by oxidation to 7-hydroxy warfarin (Lewis et al., 1974). Conversely, a major route of biotransformation for R warfarin was via a highly stereoselective reduction to R/S warfarin alcohol (Lewis et al., 1974).

The metabolic fate of the sedative hypnotic, glutethimide, presents an even more dramatic example of enantioselective pathways for biotransformation. In the dog, 4-hydroxy-glutethimide appeared to arise exclusively from the R enantiomer, while the 1-hydroxyethyl metabolite appeared to be formed from the S isomer. However, the same degree of stereoselectivity was not maintained in man (Kennedy and Fischer, 1979).

Enantioselectivity has been shown in genetic polymorphic oxidation of several chiral drugs. The (-) enantiomer of the beta adrenergic antagonist, bufurolol, was more extensively ring hydroxylated in position 4 than its (+) antipode, while the reverse is true for 1'-hydroxylation. In liver microsomes from normal subjects (extensive metabolizers), the (+)/(-) ratio for the rate of 1'-hydroxylation favoured the (+) enantiomer by 2-fold. The metabolism of the anticonvulsant, mephenytoin, offers another example of a stereoselective hydroxylation defect. While in humans, S mephenytoin ($t_{1/2}$ = 1 hour) was rapidly p-hydroxylated, the R enantiomer ($t_{1/2}$ = 70 hours) is slowly N-methylated to PEH, an active metabolite which tends to accumulate due to very slow elimination ($t_{1/2}$ = 150-200 hour) (Wedlung et al., 1985).

Propafenone is mainly metabolized by cytochrome P-450 isozyme(s) to yield 5-hydroxy propafenone. This oxidative metabolic route was found to be genetically controlled by an autosomal recessive trait (determined by debrisoquine administration) and a bimodal distribution in patients was noted (Siddoway et al., 1987). The poor metabolizers showed higher drug levels than extensive metabolizers. Beta-adrenergic drugs, including bufuralol and metoprolol, showed stereoselective biotransformation and also exhibited genetic polymorphism (Lennard et al., 1983). Being structurally related to beta-adrenergic drugs,

stereoselective biotransformation of propafenone could also be a logical possibility.

The most intriguing aspect of the metabolism of chiral drugs is that of enantiomeric inversion of a number of active derivatives of 2-arylpropionic acids by such processes. It has been observed that one isomer of these compounds becomes configurationally more stable than the other, with a unidirectional configurational inversion (Kripalani et al., 1976). A representative example is that of the administration of racemic ibuprofen or its enantiomers to several species, including man, in which an extensive inversion of the pharmacologically less potent R enantiomer was observed (Kaiser et al., 1976). Additional evidence has been reported for the R to S inversion of other related antiinflamatory drugs such as clidanac (Tamura et al., 1981).

2.12.5 Renal Clearance

Renal clearance is not usually stereoselective unless a drug is actively secreted and/or reabsorbed. Stereo-selectivity in filtration at the glomerulous would depend mainly on stereoselective differences in the non protein-bound levels of one the enantiomers of the drug (Williams and Lee, 1985).

2.13 Stereoselective Drug Analysis

Conventional methods for measuring optical activity of isomers include polarimetry, optical rotatory dispersion and circular dichroism. Chromatographic methods offer distinct advantages for the analyses and separation of optical isomers over other methods due to the small sample size required, independence from the magnitude of specific rotation, and

most importantly, independence from other optically active species present (Konig et al., 1977).

Both GLC and HPLC have been widely used for the separation of diverse enantiomeric compounds. In general, the resolution of enantiomers by chromatographic means has been achieved by one of the two methods. The first involves the conversion of the racemate to a mixture of diastereomers using a suitable chemical reaction with a chiral reagent, followed by resolution of the diastereomers on a non-chiral stationary phase (Konig et al., 1977 and Parr et al., 1971). The second method involves direct separation of the enantiomers on a chiral stationary phase (Pirkle et al., 1979).

Earlier attempts for the separation of enantiomers as their diastereomers were reported by Gil-Av et al., 1966, who resolved a series of racemic secondary alcohols as their (+) lactic acid derivatives on a GLC capillary column coated with polypropylene glycol. Other investigators have used N-trifluoroacetyl-L-proloylchloride (N-TPC) as a chiral derivatizing agent for the GLC assay of amino acids (Westley et al., 1968, Dabrowiak et al., 1971), and primary and secondary alcohols. Koreeda et al., 1973, used an HPLC technique to resolve the enantiomers of abscisic acid derivatized with (+)-α-methoxy-α-trifluoromethylphenyl acetychloride (MTP). Helmchen and Strubent, 1974, showed that diastereomeric amides formed from the reaction of racemic amines with optically pure methylmandelylchloride were separable by HPLC. Hermansson et al., 1982, employed the anhydrides formed with tert-butoxycarbonyl-L-alanine and tert-butoxy-L-leucine with R,S alanine for the determination of the ratio of the R enantiomer in presence of the S enantiomer of several alkanolamines with beta-adrenergic activity.

With S(-)-1-phenylethylisocyanate as a chiral derivatizing reagent, Thompson and his associates, 1982, were able to separate the enantiomers of propranolol as their urea derivatives on a reverse phase HPLC system. An extension of this method was achieved by Sedman and his associate, 1983, to separate the enantiomers of eleven beta-blocking agents derivatized with 2,3,4-tri-O-acetyl-α-D-arabinopyranosylisothiocyanate (AITC) or 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylisothiocyanate (GITC). Recently, Belanger et al., 1985, resolved the enantiomers of mexiletine as their thiourea derivatives with GITC.

Despite the fact that diastereomeric separation of enantiomers has been widely used as a technique, there are basic requirements that must be considered. The optically active reagent (chiral selector, SE) must be available in the purified form as this has a direct influence on accuracy of the maximum detectable optical purity of the chiral solutes (selectands, SA). The reaction must be mild so that virtually no racemization of the chiral centres of the SE and SA occur. Finally, the derivatization reaction must be quantitative for each enantiomer because the constants (K_1 and K_2) of the reactions, as shown below, may not be equal.

$$\text{(R)-SE} + \text{(R)-SA} \xrightarrow{K_1} \text{(R)-SE-(R)-SA}$$

$$\text{(R)-SE} + \text{(S)-SA} \xrightarrow{K_2} \text{(R)-SE-(S)-SA}$$

Perhaps the most important requirement is the steric conformation of the chiral centre(s) and the distance(s) to the reactive functional group(s) from chiral centers in the SE and SA molecules, since

favourable and unfavourable spatial arrangements of the chiral substituents with respect to each other in the resulting diastereomeric (R)-SE-(R)-SA, and (R)-SE-(S)-SA derivatives, reflect their different lipophilicity or polarity. These arrangements have a direct influence on adequate resolution by diverse chromatographic systems. Finally,for sensitive and selective analytical purposes, SE reagents should have a chromophore or fluorophore to enhance the detectibility of the derivatives (Linder et al, 1984).

The direct separation of enantiomers, although a valuable technique, is strongly dependant on structural elements of the SE and SA molecules. It involves the formation of transient diastereomeric complexes between the chiral solute and the chiral selector/sorbent stationary phase which does not involve covalent bonds. The relative stability of these diastereomeric complexes results in different rates of elution of the enantiomers. The direct resolution of racemic amino acids was reported by Kotake et al., 1951. Dalgliesh, 1952, subsequently studied the structural features necessary for resolution to occur with aromatic amino acids using cellulose paper chromatography. He postulated that for direct separation, at least three points of attachment were required. These included the formation of hydrogen bonds, a dipole-dipole interaction, and a π-π stereochemical interaction and/or dispersion forces between the solute and the sorbent. Gil-Av et al., 1966, first introduced the use of an N-trifluoroacetyl-L-isoleucine-lauryl ester chiral stationary phase for the separation of N-trifluoracetyl-amino acid esters on wall-coated capillary columns. Recognizing the essential role of the -NH-COCH-(R)-NH-CO- group (where, R is the chiral center) in these types of peptide ester phases, the

search for structural features of amides which might increase their selectivity and thermal stability, led to the development of the N-lauroyl-L-valyl-tert-butylamide phase and, subsequently, the diamide phases which exhibited greater efficiency, higher resolution factors and reduced retention times (Feibush et al., 1971,). Frank and his co-workers, 1978, later coupled L-valine-tert-butylamide to the carboxyl groups of the co-polymer of dimethylsiloxane and carboxyalkylmethylsiloxane to produce a highly versatile GLC chiral stationary phase (Chirasil-Val^R) which is now commercially available. It has been used to separate enantiomeric drugs and metabolites of amino acids of high and low volatility and some amino alcohols (Frank et al., 1978) and primary amines (McErlane et al., 1983). In addition, the high thermal stability of Chirasil-Val^R made it possible, for the first time, to employ a mass-spectrometer coupled to a GLC system for the analysis of enantiomers (Frank et al., 1978, Weiss et al., 1978). Another class of potentially active stationary phases that have shown great promise for enantiomer resolution, are the carbonyl bis-(amino acid)-esters. Enantiomeric amine derivatives could be separated using these phases in liquid, solid and mesophase, depending on the operating temperature (Liu et al., 1982, Feibush et al., 1971, Corbin et al., 1970, Lochmuller et al., 1974).

Chiral resolution of enantiomers by HPLC has included the use of chiral columns such as the Pirkle^R chiral column which is an N-(3,5-dinitrobenzoyl)-D-phenylglycine phase bonded to silica. This stationary phase is capable of separating the enantiomers of primary amines, amino alcohols, and amino acids (McErlane et al., 1987, Pirkle et al., 1981).

Cyclodextrin bonded stationary phases and protein bonded phases

(AAG or bovine serum albumin attached to silica), which are also commercially available, have been used for the HPLC separation of variety of compounds including amides, amino acids and amino alcohols (Armstrong, 1984). The three point interaction for chiral recognition in cyclodextrin phases is achieved through a tight-fit inclusion complex formation between the solute and stationary phase.

The use of ion-pair reagents, such as quaternary ammonium compounds or alkyl sulphonic acids, has been proposed for chiral HPLC applications. The method is based on ion-pair chromatography with a chiral counter ion added to the mobile phase. Petterson and Schill, 1981, demonstrated the usefulness of (+)-10-camphorsulfonic acid as a chiral ion-pair reagent for the direct resolution of amino alcohols of the beta-blocker series. The authors were also able to resolve the ephedrine analogues by ion-pair formation of chiral amines by using a non-chiral lipophilic counter-ion. These ion-pairs could be stereoselectively extracted and/or partitioned into a chiral stationary phase created by adsorption of a lipophilic chiral mobile phase additive, (R,R-di-N-butyltartarate), on a reverse phase system.

Another technique, called ligand exchange chromatography was developed by Davenkov et al., 1980, to separate the enantiomers of amino and/or carboxyl compounds. In this method, an optically active ligand is bonded covalently to an insoluble carrier. After charging this adsorbant with a metal ion, the racemate to be resolved forms diastereomeric complexes with the metal-ion and the adsorbent, which lead to resolution of the enantiomers.

Other methods reported for the resolution of enantiomers include the use of antiserum with the ability to discern the stereochemical

differences in drug molecules. Several authors have used this procedure to develop sensitive and stereoselective radioimmunoassay procedures. Findley et al., 1981, developed a stereospecific radioimmunoassay for (+) pseudoephedine in humans. Using an identical approach, Midha et al., 1983, studied (+) and (-) ephedrine in human plasma after an oral dose of the racemate.

2.14 Analytical Methods for Propafenone

2.14.1 Analytical Method for R,S Propafenone

Several HPLC and GLC methods have been reported for the analysis of racemic propafenone in biological fluids. The method described by Keller et al., 1978, was not optimal since sensitivity was low and large volumes of plasma (4 to 8 mL) were required. The HPLC methods described by Harapat and Kates, 1982, and Brode et al., 1982, fulfilled sensitivity and reproducibility requirements for clinical monitoring. Both methods involved extraction from alkalinized plasma and back extraction from the organic phase to an acid phase. A highly sensitive HPLC method with fluorometric detection has been reported by Brode et al., 1984. This method was used to monitor the plasma concentrations of racemic propafenone and its 5-hydroxy metabolite down to 0.2 ng/ml, but large differences in the coefficient of variation at low drug levels were reported. Moreover, the procedure was time-consuming and there was difficulty in removing the excess fluorogenic reagent.

GLC methods with electron capture detection are very sensitive. Marchensini and his associates, 1982, developed a GLC method which could monitor propafenone down to 10 ng/mL in plasma. Chan et al., 1986,

developed a similar GLC method, using HFBA as the derivatizing reagent. Applicability of this method has been reported by Axelson et al., 1978, to measure the bioavailability of propafenone while the drug was taken with food. This method was also aimed at monitoring the plasma protein-binding of total and unbound fraction of the racemic drug in plasma.

2.14.2 Analytical Method for Propafenone Enantiomers

Thus far, one analytical study has been published for the separation of the R and S isomers of propafenone by fractional crystallization of the diastereomeric tartaric acid derivatives (Blaschke et al., 1987). The same authors also used (+) phenylethylisocyanate reagent to determine the purity of the enantiomers by HPLC. This procedure was used to obtain the pure individual enantiomers for the study of their absolute configuration, but not for their measurement in biological samples. At the present time a stereoselective assay for the enantiomers of propafenone is lacking. Thus, for the elucidation of the pharmacokinetic parameters of propafenone enantiomers, a sensitive and stereoselective assay is required.

2.15 Objective of the Study

To develop a sensitive and stereoselective chromatographic assay method for propafenone enantiomers in human biological fluids.

3. EXPERIMENTAL

3.1 Supplies

3.1.1 Drug and Internal Standards

R,S-2'-[3-(propylamino)-2-(hydroxy)-propoxy]-3-phenylpropiophenone hydrochloride (Propafenone hydrochloride), and R,S-2'-[3-(ethylamino)-2(hydroxy)-propoxy]-3-phenylpropiophenone hydrochloride (Li-1115 hydrochloride) - Knoll Pharmaceuticals, Markham, Ont., Canada.

α-Bromonaphthalene - ICN Pharmaceuticals, Inc., Plainview, N.Y. USA.

Desipramine hydrochloride - Sigma Chemical Co. ST.Louis, Mo., USA.

(+)Naproxen - Syntex Inc. Mississauga, Ont. Canada.

3.1.2 Chemicals and Reagents

R(+)-α-Methyl benzylisocyanate, R-(-)-1-(naphthyl)ethyl isocyanate, dansylhydrazine - Aldrich Chemical Co. Milwaukee, Wis. USA.

Acetobromo-α-D-glucose - Sigma Chemical Co.

Silver thiocyanate - Eastman Kodak Co. Rochester, N.Y. USA.

2.3,4,6 Tetra-O-acetyl-β-D-glucopyranosylisothiocyanate (GITC) - Synthesized in our laboratory.

Phenytoin, dicyclohexylcarbodiimide (DCCI) - Sigma Chemical Co.

Urea - Mallinckrodt Inc. Paris, France.

Heptafluorobutyric anhydride (HFBA), trifluoroacetic anhydride (TFAA) - Pierce Chemical Company, Rockford, Il., USA.

Piperidine, pyridine, trichloroacetic acid (TCA) - BDH, Poole, England.

Triethylamine, N-trifluoroacetyl-L-proloylchloride - Aldrich Chemical Co.

Sodium hydroxide - American Scientific and Chemicals, Portland, Or. USA.

Mono- and disodium phosphate - Mallinckrodt Chemical Works, St. Louis, Mo. USA.

Hydrochloric acid, perchloric acid, sodium sulphate (granular), dimethylformamide - BDH Chemicals, Vancouver, B.C. Canada.

3.1.3 Solvents

Acetonitrile, benzene, chloroform, dichloromethane, n-hexane, methanol, 2-propanol, toluene, water, acetone (HPLC grade) - Fisher Scientific, Vancouver, B.C. Canada.

3.2 Chromotographic Stationary Phases

5u Ultrasphere ODS column (25 X 0.46 cm i.d.) - Beckman Instruments Inc., Berkeley, Ca. USA.

HPLC disposable guard column (1.5 X 0.32 cm i.d.) with C_{18} cartridge and reusable holder - Rainin Instrument Co. Inc., Woburn, USA.

3% Silar 10 C GLC, and 3% OV-17 packed columns (2 m X 2 mm i.d.) coated on Cromosorb W - HP, 80/100 mesh - prepared in our laboratory.

SE-30 fused-silica capillary column (50 m X 0.25 mm i.d. - Hewlett-Packard, Avondale, Pa., USA.

Chirasil-Val^R fused silica capillary column (50 m X 0.25 mm i.d.) - Applied Science. Rockwood, Ont.,Canada.

Pirkle^R ionic (type 1-A) hi-chrom reversible HPLC column (25 cm X 4.6 mm i.d.) containing 3,5 dinitrobenzoyl-(D)-phenylglycine bonded to-γ-amino silanized silica - Regis Chemical Co., Morton Grove, Il., USA.

Cyclobond (I) -β-cyclocdextrin bonded HPLC column (25 X 0.46 cm i.d.) - Advanced Separation Technologies, Inc. Whippany, N.J. USA.

3.3 Equipment

A Hewlett Packard model 1082B high-performance liquid chromatograph equipped with a Hewlett Packard model 79850B data terminal - Hewlett Packard, Palo Alto, Ca. USA.

Holochrome variable uv detector - Gilson Medical Electronics, Middletown, Wi, USA.

Schoeffel model 970 LC fluorometer - Kratos, Westwood, N.J. USA.

A Hewlett Packard model 5830 gas chromatograph, equipped with flame ionization, and electron Capture (^{63}Ni) detectors - Hewlett Packard.

A Hewlett Packard 5700A gas chromatograph; interfaced to a Varian Mat-111 Mass spectrometer. A Varian model 620/L computer to record electron impact spectra on a chart recorder.

A Unicam SP-1000 IR spectrometer, Pye Unicam Ltd. Cambridge, England, UK.

A Beckman model 24 uv spectrophotometer, Beckman Scientific Instruments Division, Irvine, Ca., USA.

A Perkin-Elmer model 141 Polarimeter, Perkin-Elmer and Co. GMBH., Uberlingen, West Germany.

Miscellaneous

Vortex mixer - Sybron Co., Dubuque, Io, USA.

pH meter - Orion Research Inc., Ma., USA.

Thomas Hoover Capillary Melting point apparatus - Arthur H. Thomas Company, Philadelphia, Pa., USA.

Direct connect universal column prefilter - Alltech Associates Inc., Deerfield, Il., USA.

Millipore filters - Millipore Corp., Bedford, Ma. USA.

3.4 Stock Solutions

3.4.1 R,S Propafenone Hydrochloride

R,S propafenone hydrochloride (equivalent to 0.1 mg/mL of the free base) was prepared in deionized distilled water. The solution was further diluted with deionized distilled water to the desired concentrations. The stock and dilute solutions were kept at $4^{o}C$ for up to 3 months.

3.4.2 R,S Propafenone Free Base

R,S propafenone hydrochloride in water was basified with sodium hydroxide at pH > 12 and extracted into benzene. The organic extract was evaporated to dryness under a gentle stream of clean nitrogen in a $40^{o}C$ water bath. The dried crystals of propafenone base were accurately weighed and dissolved in known volumes of organic solvents.

3.4.3 R,S-2'-[3-(ethylamino)-2-(hydroxy)-propoxy]-3 phenylpropiophenone Hydrochloride (Li-1115) and Desipramine Hydrochloride (Internal Standards)

Stock solutions equivalent to 0.1 mg/mL of the free base were prepared by dissolving these compounds in deionized distilled water and were kept at $4^{o}C$ until used.

3.4.4 (0.2 M) Phosphate Buffer Solution of pH 2.8

Monosodium phosphate (27.8 g) and disodium phosphate (28.4 g) were dissolved separately in 1 L of HPLC grade water to give solutions A and B respectively. An aliquot of 93.5 mL of solution A was added to 6.5 mL

of solution B in a 100 mL volumetric flask and perchloric acid was added dropwise to the mixture to adjust the pH to 2.8. The buffer was kept at 4°C until used.

3.4.5 Trichloroacetic Acid Solution (10% w/v)

Trichloroacetic acid (10 g) was dissolved in deionized distilled water to a final volume of 100 mL.

3.5 Synthesis of 2,3,4,6 Tetra-O-Acetyl-β-D-Glucopyranosylisothiocyanate (GITC)

3.5.1 Purification of Acetobromo-α-D-glucose

The commercial product, acetobromo-α-D-glucose, was purified by rapidly cooling a hot saturated solution in absolute alcohol. The crystalline material which separated was filtered under suction and then was thoroughly washed with light petroleum (30°- 60°) to remove traces of alcohol (Ber, 49, 584, 1916). The purified material was stored at -20°C until required.

3.5.2 Synthesis of GITC

Purified acetobromo-α-D-glucose (1.025 g) was heated at 103 ± 2°C in dry, freshly distilled xylene (5 mL) with dried silver thiocyanate (1.25 g) under efficient stirring. After one hour, additional silver thiocyanate (0.85 g) was added in two equal portions at intervals of 30 minutes. The reaction mixture was then filtered while hot through a fritted glass funnel by suction. The precipitate of silver bromide was washed twice with 5 mL portions of hot xylene. About 20 mL of cold

petroleum ether (30^0- 60^0) was added to the filtrate and the mixture was kept at 4^0C for 16 hours. The resulting crystals were recovered and recrystallized three times with mixture of xylene-petroleum ether (1:4) and dried over phosphorous pentoxide. The purity of the product was determined by reverse phase HPLC with 65:35 methanol/water as mobile phase.

M.P. 110-111^{0}C (lit. m.p. 112-114^{0}C, Nimura et al.,1982) IR (stretching frequency): N = C = S, 2100 cm^{-1}, CH_3O - C = O, 1750 cm^{-1}. (Prepared by a modification of the method of Nimura et al., 1980)

3.6 Preparation of the Derivatives of R,S Propafenone

3.6.1 Heptafluorobutyric Anhydride (HFBA) Derivative

To 0.5 mg of R,S propafenone free base in dry benzene (1 mL) were added 25 uL of HFBA and 1 uL of TEA. The reaction mixture was agitated for 1 minute and then heated at 65^{0}C for 1 hour. After cooling, the reaction mixture was evaporated to dryness under a gentle stream of clean nitrogen at 40^{0}C in a water bath. The residue was reconstituted with 1 mL of dry benzene

3.6.2 N-Trifluoroacetyl (L) Proloylchloride [N-TPC] Derivative

To 1 mg of R,S propafenone free base in dry dichloromethane (1 mL) were added, 10 uL of N-TPC and 5 uL of dry pyridine. The reaction mixture was stirred by vortex for 1 minute and then heated at 45^{0}C for

20 minutes. After cooling, the reaction mixture was evaporated to dryness under a stream of clean nitrogen in a 40°C water bath. The residue was reconstituted with 1 mL of dry dichloromethane.

3.6.3 β-Naphthoylchloride Derivative

To 0.5 mg of R,S propafenone free base in 1 mL of dry dichloromethane were added, 0.5 mg of β-naphthoylchloride and 1 uL of dry TEA. The reaction mixture was heated at 45°C for 20 minutes and then evaporated to dryness under a stream of clean nitrogen at 40°C in a water bath. The residue was reconstituted with 1 mL of methanol.

3.6.4 R(+)-α-Methylbenzylisocyanate [R(+)MBIC] Derivative

To 1 mg of R,S propafenone free base in 1 mL of dry acetonitrile:dimethylformamide (8:2) were added, 5 uL of R(+)MBIC and 1 uL of TEA. The reaction mixture was held at room temperature for 30 minutes and then evaporated to dryness under a stream of clean nitrogen in a 40°C water bath. The residue was reconstituted with 1 mL of chloroform for GLC-FID experiments. For HPLC experiments the derivative was reconstituted with 1 mL of methanol.

3.6.5 Isopropylisocyanate Derivative

To 0.5 mg of propafenone free base in 0.5 mL of dichloromethane were added, a 50 molar excess of isopropyl isocyanate and 1 uL of dry TEA. The reaction mixture was stirred by vortex for 1 minute and was heated at 45°C for 20 minutes in a dry heating block. The contents of the tube were then evaporated to dryness under a gentle stream of

nitrogen at 40^{0}C in a water bath. The residue was reconstituted with 1 mL of dichloromethane.

3.6.6 2,3,4,6-Tetra-O-acetyl-β-D-Glucopyranosyl isothiocyanate (GITC) Derivative

To 0.1 mg of R,S propafenone free base was added, a 50 molar excess of 0.5% w/v GITC reagent in dry acetonitrile:dimethylformamide (8:2). A solution of 5 uL of 1% w/v diphenhydantoin (phenytoin) in chloroform, or 1 uL of TEA were added as catalysts. The reaction mixture was stirred by vortex for 1 minute and was held for 30 minutes at room temperature before evaporating to dryness under a stream of clean nitrogen. The residue was reconstituted with 1 mL of methanol.

3.6.7 R(-)-1-(Naphthyl)ethylisocyanate [R(-)NEIC] Derivative

To 2 ug of propafenone free base in a 10 mL screw-capped culture tube were added, 100 uL of 0.01% v/v R(-)NEIC in acetonitrile and 400 uL of dry acetonitrile containing 0.003 (M) TEA. The reaction mixture was heated at 75^{0}C for 30 minutes in a dry heating block. The tube was cooled, and 1 uL of piperidine was added. The tube was reheated at 75^{0}C for 30 minutes to destroy the excess reagent. After cooling, the mixture was evaporated to dryness and reconstituted in 150 uL of 2-propanol.

3.6.8 Propafenone-Dansylhydrazine-R(-)NEIC Derivative

To propafenone base (20 - 1000 ng) was added, 100 uL of 0.2% w/v dansylhydrazine in the presence of 0.48% w/v TCA in dry toluene. The reaction mixture was diluted with 400 uL of dry benzene and stirred by

vortex for one minute in a screw-capped culture tube. The tube was heated at 75°C for 20 minutes and the excess reagent was destroyed by adding 100 uL of acetone and heating for another 20 minutes at 75°C. After cooling, the contents of the tube were evaporated to dryness under a gentle stream of clean nitrogen in a 40°C water bath. The residue was basified with 2 mL of 2 M sodium hydroxide. The free base was extracted with 6 mL of benzene which was subsequently evaporated to dryness under a stream of clean nitrogen. The residue was reacted with 150 uL of 0.01% v/v R(-)NEIC in dry acetonitrile at room temperature for 30 minutes. The excess R(-)NEIC reagents were destroyed by adding 1 uL of piperidine and storing the samples at room temperature for 20 minutes.

3.6.9 Derivatization of Propafenone with (+)Naproxen

An aliquot of 10 ug of propafenone base and a 50 molar excess of (+) naproxen in 1 mL of dry dichloromethane were allowed to react at room temperature for one hour in the presence of excess of dicyclohexylcarbodimide (DCCI). The reaction mixture was filtered and sequentially washed with 5% HCl, 5% NaOH, and water, and reconstituted with 1 mL of 2-propanol. An aliquot of the reaction mixture was injected onto the HPLC column, employing methanol/water (75:25) as mobile phase delivered at a flow rate of 1 mL/min.

3.7 Preliminary GLC and HPLC Analysis of Propafenone using Chiral and Achiral Stationary Phases

3.7.1 Chiral GLC Phase

Underivatized propafenone did not elute from the chirasil-Val^R column. The following derivatization procedure were used to increase the volatility and / or selectivity of the chiral phase for direct resolution of various derivatives of propafenone.

a) An aliquot of 1 uL of the freshly prepared HFBA derivative of propafenone (0.5 mg/mL) in benzene was injected onto the Chirasil-Val^R capillary column in the GC system coupled with electron capture detector. Chromatographic conditions - column temperature: 220^{o}C, injection temperature: 250^{o}C, carrier gas (He) flow rate: 1.2 mL/min, make-up gas (argon/methane, 95:5) flow rate:
30 mL/min, detector temperature: 350^{o}C, injection mode: split (1:30).

b) An aliquot of 2 uL of the freshly prepared isopropyl isocyanate derivative of propafenone (0.5 mg/mL) in dichloromethane was injected onto the chirasil-Val^R column in the GC system. The detector used was flame ionization. . Chromatographic conditions - column temperature: 200^{o}C, injection temperature: 250^{o}C, detector temperature: 275^{o}C, carrier gas (He) flow rate: 1.3 mL/minute, make-up gas (He) flow rate: 20 mL/minute, injection mode: split (1:30).

3.7.2 Achiral GLC Stationary Phase

a) An aliquot of 1 uL of the freshly prepared R(+)MBIC derivative of

propafenone (1 mg/mL) in dry chloroform was injected on to SE-30 capillary column in the GC system. Chromatographic conditions - column temperature: 190°C, injection temperature: 240°C, detector (FID) temperature: 275°C, carrier gas (He) flow rate: 1.2 mL/minute, make-up gas (N_2) flow rate: 30 mL/minute, injection mode: split (1:20).

b) An aliquot of 4 uL of freshly prepared R(+)MBIC derivative of propafenone (1.0 mg/mL) in dry chloroform was injected onto an OV-17 packed column in the GC. Chromatographic conditions - injection temperature: 300°C, column temperature: 280°C, FID temperature: 275°C, carrier gas (He) flow rate: 40 mL/minute.

c) An aliquot of 1.5 uL of freshly prepared N-TPC derivative of propafenone (1 mg/mL) in dichloromethane was injected onto the SE-30 capillary column in the GC. Chromatographic conditions - column temperature, 180°C, injection temperature: 225°C, detector temperature: 275°C, carrier gas (He) flow rate: 1.2 mL/min, make-up gas flow rate: 25 mL/min, injection mode: split (1:30).

3.7.3 Chiral HPLC Phases

a) Propafenone free base (100 ug) was dissolved in 500 uL of methanol and 20 uL of this solution was injected onto a β-cyclodextrin HPLC column (25 cm X 0.45 cm i.d.). Mobile phase: Methanol/water (60:40) delivered at a flow rate of 0.8 mL/min. Detection: 254 nm.

b) An aliquot of 10 uL of the freshly prepared 2-naphthoyl chloride derivative of propafenone (0.5 mg/mL) was injected onto a (25 X 0.4 cm i.d.) Pirkle ionic (type- 1A) HPLC column. Mobile phase: 9% 2-propanol in hexane delivered at a flow rate of 1 mL/min. Detection: 254 nm.

3.7.4 Achiral HPLC Phases

a) An aliquot of 20 uL of the R(+)MBIC derivative of propafenone (1 mg/mL) in methanol was injected onto a (25 X 0.45 cm i.d.) 5u ultrasphere, ODS HPLC column. Mobile phase:) Methanol/water(70:30), delivered at a flow rate of 1 mL/min. Detection: 254 nm.

b) A 20 uL aliquot of the freshly prepared GITC derivative of propafenone (0.1 mg/mL) was injected onto a (25 X 0.45 cm i.d.) 5u ODS column. Mobile phase Methanol/ phosphate buffer (0.2 M) of pH 2.8 (75:25), delivered at a flow rate of 1 mL/min. Detection: 250 nm.

3.8 HPLC Resolution of the Enantiomers of Propafenone

3.8.1 Resolution of the Diastereomeric R(+)MBI Derivatives

Duplicate samples, each containing 20 ug of R,S propafenone hydrochloride in 1 mL of water, were placed in 10 mL PTFE-lined screw-capped culture tubes, and 0.5 mL of 3 M NaOH and 6 mL of benzene were added. The tubes were tightly capped and tumbled for 20 minutes on a rotating tumbler. The tubes were then centrifuged for 10 minutes at 2500 r.p.m. and the upper benzene layers were transferred to clean dry

test tubes. The solvent was evaporated to dryness following the procedure described before. The residues were reconstituted in 100 uL of dry dichloromethane and 5 uL of R(+)-α-methylbenzylisocyanate was added to each tube. The tubes were stirred by vortex for 1 minute and kept at room temperature for 30 minutes. After reaction, 5 uL of HPLC grade water was added to the reaction mixtures to destroy the excess reagent. After a further 20 minutes, 20 uL of the derivative was injected onto the HPLC-ODS column (5u. 25 X 0.45 cm i.d.). The mobile phase, methanol/water (71:29) was delivered at a flow rate of 0.8 mL/min. Detection:
254 nm.

3.8.2 Resolution of Diastereomeric GITC Derivatives

Duplicate samples, each containing 10 ug of R,S propafenone hydrochloride (1 mL of stock solution) were pipeted into 10 mL PTFE-lined screw-capped culture tubes and 0.5 mL of 3 M NaOH was added to each tube. Extraction was carried out with 6 mL of benzene following the procedure described above.

To the residue was added, 95 uL of 0.5% w/v GITC in dry acetonitrile. The mixture was stirred by vortex and held at room temperature for 30 minutes. To each tube was added,
5 uL of HPLC grade water and the tubes were held at room temperature for a further 20 minutes. A 20 uL aliquot was injected onto the HPLC ODS column (5u, 25 X 0.45 cm i.d.). The mobile phase was methanol/phosphate buffer (0.2 M) of pH 2.8 (74:26), delivered at a flow rate of 1 mL/min. Detection: 250 nm.

3.8.3 Resolution of the Diastereomeric R(-)NEIC Derivatives

Duplicate samples, each containing 1 ug of R,S propafenone hydrochloride (in 1 mL of distilled and deionized water) were placed in 10 mL PTFE-lined screw-capped culture tubes, and 0.5 mL of 3 M NaOH was added. The free base was extracted with 6 mL of benzene as described above. The dried residue was reconstituted with 150 uL of dry acetonitrile and then reacted with 5 uL of 0.1% v/v R(-)NEIC in dry acetonitrile. The reaction mixture was stirred by vortex for one minute and then heated for 30 minutes at 75^{o}C. The excess reagent was destroyed with 1 uL of piperidine by allowing the samples to sit for a further 20 minutes. The reaction mixture was evaporated to dryness and then reconstituted with 150 mL of 2-propanol. An aliquot of this was injected onto the HPLC ODS column (5u, 25 X 0.45 cm i.d.). The mobile phase was methanol/water (76:24) delivered at flow rate 1 mL/min. Detection: 230 nm.

3.8.4 Resolution of diastereomeric Propafenone-Dansylhydrazine -R(-)NEIC Derivative

A sample of 1 ug of propafenone base (extracted as described in sections 3.8.1 - 3.8.3) was reconstituted with 0.9 mL of dry benzene. To this, 0.1 mL of 0.2% w/v dansylhydrazine in the presence of 0.48% w/v trichloroacetic acid (TCA) in toluene was added (Brode et al., 1984). The reaction mixture was stirred by vortex for 1 minute and then heated for 20 minutes at 65^{o}C in a dry heating block. After cooling, excess acetone was added and the sample was heated for a further 20 min. at 65^{o}C to destroy the excess dansylhydrazine. The contents of the tube were cooled and

evaporated to dryness under clean nitrogen at 40^{0}C in a water bath. The residue was basified with 0.5 mL of 3 M NaOH and extracted with 6 mL of benzene. After evaporating the organic solvent, 100 uL of 0.1% v/v R(-) NEIC in acetonitrile was added to the residue and the samples were held for 30 min. at room temperature. An additional 0.9 mL of acetonitrile was added to bring the volume to 1 mL. The tube was stirred by vortex and 10 to 20 uL was injected onto the HPLC ODS column (5u, 25 X 0.45 cm i.d.), Mobile phase, methanol water (74:26) delivered at 1 mL/min. Detection: Fluorescence, Ex: 220 mm. Em: 418 nm (cut-off filter).

3.9 Quantitative Analysis of Propafenone Enantiomers

3.9.1 Selection of Internal Standard

2'-[2-(Hydroxy)-3-(ethylamino)-propoxy]-3-phenylpropriophenone, Li-1115 was initially chosen as an internal standard based on its structural similarity to that of propafenone. Desipramine, an achiral secondary amine was also chosen as a second internal standard required for complete derivatization.

3.9.2 Selection of External Standard

α-Bromonaphthalene was chosen as an external standard to measure the recovery of propafenone from plasma as well as to assess the stoichiometric ratio of reagent required for complete derivatization of propafenone.

3.9.3 Selection of Catalyst

Phenytoin and Urea were initially chosen as bifunctional catalysts

for the propafenone-GITC reaction.

Triethylamine was chosen as a catalyst for the propafenone-R(-) NEIC and Propafenone-HFBA reactions.

3.9.4 Extraction Solvents

Four organic solvents: benzene, dichloromethane, a mixture of benzene, dichloromethane and 2-propanol (7:3:1), and chloroform, were evaluated to assess their efficiency for extraction of propafenone base and the internal standards from plasma. The phase volume ratio of V_{org}/V_{aqu} was 2.

3.9.5 Plasma Protein Precipitation

Propafenone is highly bound to plasma proteins and it was therefore necessary to precipitate the proteins before extraction. For this purpose, 0.4 mL of 10% trichloroacetic acid in water was added to 1 mL of plasma containing propafenone hydrochloride (1 to 2 ug/mL). The plasma was shaken for one minute and then the pH was adjusted above 12 with 0.5 mL of 3 M NaOH, followed by extraction, derivatization and HPLC analysis.

3.9.6 Optimization of Reaction Conditions for Derivatization

a) Optimum Reaction Time

Optimum reaction times for propafenone and the internal standard was studied with four, triplicate samples, each containing 3 ug of R,S propafenone hydrochloride and 1 ug of the internal standard in 1 mL of

distilled deionized water. Extraction was carried out as described in section 3.8. Derivatization was carried out separately with R(+)MBIC, GITC and R(-)NEIC at room temperature, at different time intervals of 15, 30, 45 and 60 minutes. A 5 uL portion of 0.002% v/v of α-bromonaphthalene, as external standard was added to each sample and stirred by vortex. The samples were analyzed by a plot of peak height ratio against time.

b) Optimum Reaction Temperature for Derivatization of Propafenone with R(-)NEIC

Five sets of tubes, each set in triplicate, containing 2 ug of R,S propafenone hydrochloride and 1 ug of the internal standard were prepared in 1 mL of distilled deionized water. Extraction with 6 mL of benzene was carried out as described before. To each tube, 100 uL of 0.01% v/v R(-)NEIC in dry acetonitrile was added. A volume of 50 uL of 0.003 M TEA and 350 uL of dry acetonitrile was added to each tube and the tubes were stirred by vortex. Each set of tubes were held for 30 minutes at room temperature, 45^{o}C, 60^{o}C, 75^{o}C and 90^{o}C. The tubes were cooled, evaporated to dryness, and reconstituted with 100 uL of 2-propanol.

An aliquot of 20 uL from each tube was injected onto the HPLC ODS column. The samples were analyzed by plotting peak-height ratio against temperature using a 5 uL portion of 0.002% v/v of α-bromonaphthalene as external standard added to each sample before injection onto the column.

3.9.7 Stoichiometric Ratio of R(-)NEIC Reagent & Drug

Five aliquots of triplicate samples, each containing

2 ug of propafenone base, were subjected to derivatization with a 1.8, 4.5, 9, 45 or 90 molar excess of R(-)NEIC at 75°C for 30 minutes. The samples were evaporated to dryness under nitrogen at 40°C in a water bath and reconstituted with 150 uL of 2-propanol. 5 ul of 0.002% v/v of α-bromonaphthalene was used as the external standard. An aliquot of 20 uL from each tube was injected onto the HPLC ODS column for analysis.

3.9.8 Optimizing the Sensitivity for Detection

The uv sensitivity of propafenone was determined in solvent systems of (a) acetonitrile/phosphate buffer (0.2 M) of pH 2.8 (65:35), (b) acetonitrile, (c) methanol/phosphate buffer (0.2 M) of pH 2.8 (75:25),and (d) methanol/water (75:25),. The uv sensitivity was also determined for the propafenone GITC and propafenone R(-)NEIC derivatives.

All uv scans of propafenone and its derivatives were done on a Beckman model 25, uv-spectrophotometer over the range, 180 nm to 350 nm. Respective solvent blanks were used as the reference in each case.

3.9.9 Polarimetric Measurement of R(-)NEIC Derivatives of R,S Propafenone

Racemic propafenone hydrochloride (1 mg) was converted to its free base following the procedure described before. The free base was reacted with a 5 molar excess of R(-)NEIC in dry acetonitrile at 75°C for 30 minutes to yield diastereomeric urea derivatives of the enantiomers. A volume of 20 uL of the diastereomeric mixture was repeatedly injected on to the HPLC ODS column (5u , 25 X 0.45 cm i.d.) using methanol/water (75:25) as the mobile phase delivered at 0.9

mL/min. The two diastereomeric fractions from the HPLC eluant, which corresponded to two resolved peaks of the enantiomers of propafenone were separately collected into two clean flasks. The purity of each fraction was ascertained by injection of 200 uL of each fraction onto the same column. The mobile phase was removed under distillation at reduced pressure at room temperature and the residues were reconstituted in equal volumes of chloroform. Optical rotation studies of each fraction were conducted on a Perkin-Elmer Model 141 polarimeter in a 1 mL tube at $25^{o}C$. The rotation of the fraction which eluted earlier was -22^{o}, and that of the fraction which eluted later was -5^{o}.

3.9.10 Structural Identity of the Derivatives of R,S Propafenone

The two diastereomeric fractions containing the enantiomers, isolated as described above, were evaporated to dryness in PTFE-lined screw-capped culture tubes and reconstituted with 2 mL of methanol. A 2 uL aliquot of each fraction was injected onto the GC-MS (HP 5700A GC, interfaced with a Varian Mat-111 Mass Spectrometer) using EI mode. Conditions - filament current: 300 uA, electron beam energy: 70 ev, ion source pressure: 8×10^{-6} torr, column: 3% silar 10 C on chromosorb W-HP (2 m X 2mm i.d.), injection port temp.: $250^{o}C$, oven temperature: $150^{o}C$ to $185^{o}C$ at $8^{o}C$/min, column helium flow rate: 20 mL/min.

3.9.11 Efficiency of Recovery of Propafenone Enantiomers from plasma

Samples of 1000 ng and 2000 ng (equivalent to the free base) of R,S propafenone hydrochloride were added to two 1 mL aliquots of plasma in two PTFE-lined screw-capped culture tubes. The samples were extracted with benzene as described before. In two separate tubes,

identical amounts of R,S propafenone free base were taken and all four tubes containing the samples were reacted with R(-)NEIC at 75°C for 30 minutes. The samples were reconstituted with 0.25 mL of 2-propanol and stirred by vortex for 1 minute. To each tube, 5 uL of 0.002% v/v of α-bromonaphthalene were added and the samples were assayed by HPLC. Recovery analyses were done as the percentage of peak height ratios of identical concentrations of enantiomers extracted, to that of the corresponding enantiomers unextracted.

3.10 Assay of Propafenone Enantiomers by High-Performance Liquid Chromatography with UV Detection

3.10.1 Extraction, Derivatization and HPLC Analysis

Four aliquots of 1 mL of blank human plasma in triplicate, in 10 mL PTFE-lined screw-capped culture tubes were spiked with 250, 500, 1000 and 2000 ng (equivalent to the free base) of R,S propafenone hydrochloride and 1000 ng of Li-1115. Additional distilled water was added to adjust to equal volumes. Plasma proteins were precipitated with 0.4 mL of 10% trichloroacetic acid as described in section 3.9.5. The pH of the samples was adjusted above 12 by the addition of 0.5 mL of 3 M sodium hydroxide and were extracted with 6 mL of benzene. Anhydrous sodium sulphate (1 g) was added to remove trace quantities of water from the organic extract. After centrifugation for 5 minutes at 2500 r.p.m., the organic portions were transferred to clean, dry test tubes. The extracts were evaporated to dryness at 40°C in a water bath and the residue in the culture tubes was reacted with 5 uL of R(+)MBIC in 150 uL of dry acetonitrile at room temperature for 30 minutes. The excess

reagent was destroyed by the addition of 2 uL of water and the samples were held at room temperature for an additional 30 minutes. A 20 uL aliquot was injected onto the HPLC ODS column (25 X 0.45 cm i.d.). Mobile phase, methanol/water (70:30) delivered at 1 mL/min. Detection: 254 nm.

3.10.2 Extraction, Derivatization and HPLC Analysis of R,S Propafenone using GITC as a Chiral Derivatizing Reagent

Four aliquots of 1 mL of blank human plasma in triplicate, in 10 mL PTFE-lined screw-capped culture tubes were spiked with 250, 500, 1000 and 2000 ng (equivalent to the free base) of R,S propafenone hydrochloride and 300 ng of desipramine hydrochloride. Plasma protein precipitation and extraction steps were similar to section (3.10.1). Derivatization was carried by the addition of 150 uL of 0.5% w/v GITC solution in dry acetonitrile at room temperature for 30 minutes. The excess reagent was destroyed with 2 uL of water and held for an additional 30 minutes at room temperature. A 20 uL aliquot was injected onto the HPLC ODS column. For very low concentrations, (less than 500 ng/mL) the whole volume of derivatized product was injected. Mobile phase: methanol/phosphate buffer (0.2 M) of pH 2.8 (75:25) delivered at 1 mL/min. Detection: 250 nm.

3.10.3 Extraction, Derivatization and HPLC Analysis of R,S Propafenone using R(-)NEIC as a Chiral Derivatizing Reagent

Four aliquots of 1 mL of blank human plasma each in triplicate, in 10 mL PTFE-lined screw-capped culture tubes were spiked with 250, 500, 1000 and 2000 ng (equivalent to free base) of R,S propafenone

hydrochloride and 300 ng of Li-1115 hydrochloride (or, desipramine hydrochloride). Additional distilled water was added to adjust to equal volumes before plasma precipitation. After extraction, as outlined in section (3.10.1), derivatization was carried out with 100 uL of 0.01% v/v R(-)NEIC in 400 uL of dry acetonitrile containing 0.003 M TEA. The tubes were heated at 75^{o}C in a dry heating block for 30 minutes. After cooling to room temperature, 1 uL of piperidine was added to each tube to destroy the excess reagent. The contents of the tubes were evaporated to dryness and reconstituted with 200 uL of 2-propanol. A 20 uL aliquot was injected onto the HPLC ODS column. A larger volume was injected for samples of concentrations below 300 ng/mL of plasma. Mobile phase: methanol/water (76:24) delivered at 1 mL/min. Detection, 230 nm.

3.11 Calibration Curve and Precision of Assay of R,S Propafenone R(-)NEIC Derivatives.

Five aliquots containing 250, 500, 1000 and 2000 ng (equivalent to the free base) of R,S propafenone hydrochloride solutions, in triplicate, were placed in PTFE-lined screw-capped culture tubes containing 1 mL of blank plasma. To each tube, 300 ng of R,S Li-1115 (internal standard) was added. Additional distilled water was added to adjust to equal volumes. Extraction and derivatization with R(-)NEIC were done as described in section 3.10.3. The calibration curves were constructed by plotting peak-height ratios of each enantiomer to those of the known concentrations of propafenone and the internal standard.

Inter-assay variability was determined from the triplicate preparations, whereas intra-assay variability was determined by

triplicate injection of four of the samples containing 250, 500, 1000 and 2000 ng of R,S propafenone.

3.12 Reverse Phase Thin Layer Chromatography of Propafenone-GITC and Propafenone-Dansylhydrazine-GITC Derivatives

An aliquot of 0.1 mg of propafenone base was reacted with GITC in 100 uL acetonitrile at room temperature for 30 min. as described in section 3.6.6. The reaction mixture, propafenone free base in acetonitrile, and 0.5% GITC in acetonitrile were spotted on a KC_{18} reverse phase TLC plate and developed with methanol/phosphate buffer (0.2 M) of pH 2.8 (76:24). An unreacted propafenone spot (R_f=0.29, a propafenone-GITC derivative spot (R_f=0.21) and a spot for excess GITC (R_f=0.46) were observed in the reaction mixture.

Another test tube containing 0.1 mg of propafenone base was reacted with an excess of dansylhydrazine in the presence of trichloroacetic acid in toluene at 75^{o}C for 15 min. After dansylation the reaction mixture was spotted on a KC_{18} TLC plate along with propafenone and dansylhydrazine. Propafenone-dansylhydrazone (R_f=0.53) and excess dansylhydrazine (R_f=0.93) were identified. No residual propafenone spot was detectable on the plate. After extracting the propafenone-hydrazone derivative with 5 mL of benzene at pH >12, followed by evaporation to dryness as before, the residue was reacted with an excess of 0.5% GITC in 100 mL of acetonitrile at room temperature for 30 minutes. TLC analysis of the reaction mixture indicated three spots. Among these, one fluorescent spot of propafenone-dansylhydrazine-GITC (R_f=0.2) and excess GITC (R_f=0.45) spot were identified. A third spot (R_f=0.91), which eluted with the solvent

front was also fluorescent and was identified as the GITC-dansyl hydrazine adduct.

4. RESULTS AND DISCUSSION

4.1. Analytical Development for Chromatographic Resolution of Propafenone Enantiomers

4.1.1 Gas-Liquid Chromatographic Studies on Chiral and Achiral Stationary Phases

Initial GLC experiments for the resolution of enantiomers of propafenone (Fig. 1) were carried out based upon the principles of direct separation of the enantiomers on commercially available chiral stationary phases. A Chirasil-Val^R capillary column, which was coated with optically active, N-isobutyryl-L-valine tert. butylamide as the stationary phase, was used at its maximum recommended temperature of 220^oC coupled with an electron capture detector. The heptafluorobutyryl derivatives of propafenone enantiomers eluted in R_t=29.34 minutes without enantiomeric separation (Fig. 2A). However, underivatized propafenone did not elute from the column under the similar conditions but using FID. The trifluoroacetyl derivatives of the enantiomers of propafenone likewise were unresolved under the conditions employed. This chiral phase has been demonstrated to be effective for the resolution of primary amines, amino acids and amino alcohols (McErlane and Pillai, 1983, Frank et al., 1978) but was not found suitable for the chiral separation of enantiomeric secondary amines such as propafenone. It was concluded that the lack of an amino hydrogen in the substituted amide derivatives of propafenone as depicted in fig. 3A, reduced the interactions with the chiral stationary phase and thus prevented chiral resolution.

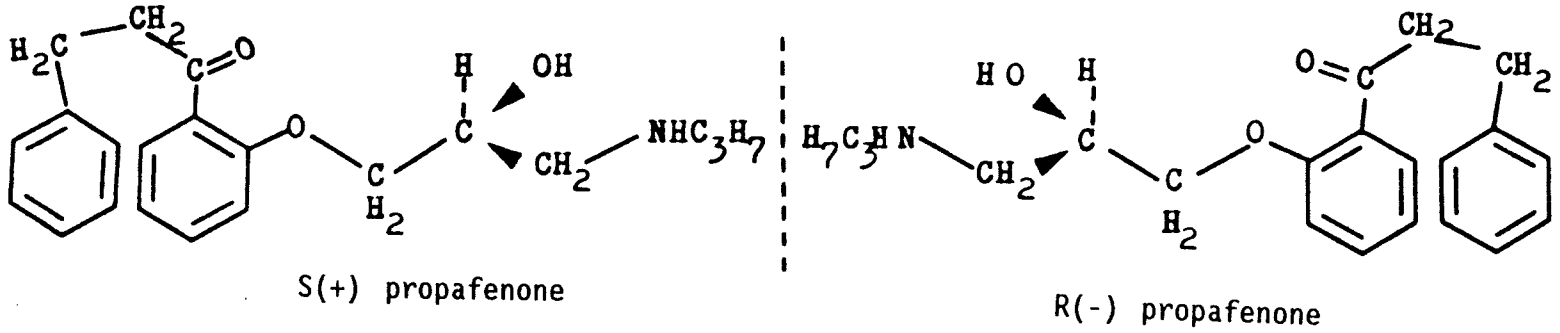


Figure 1. Structure of Propafenone Enantiomers

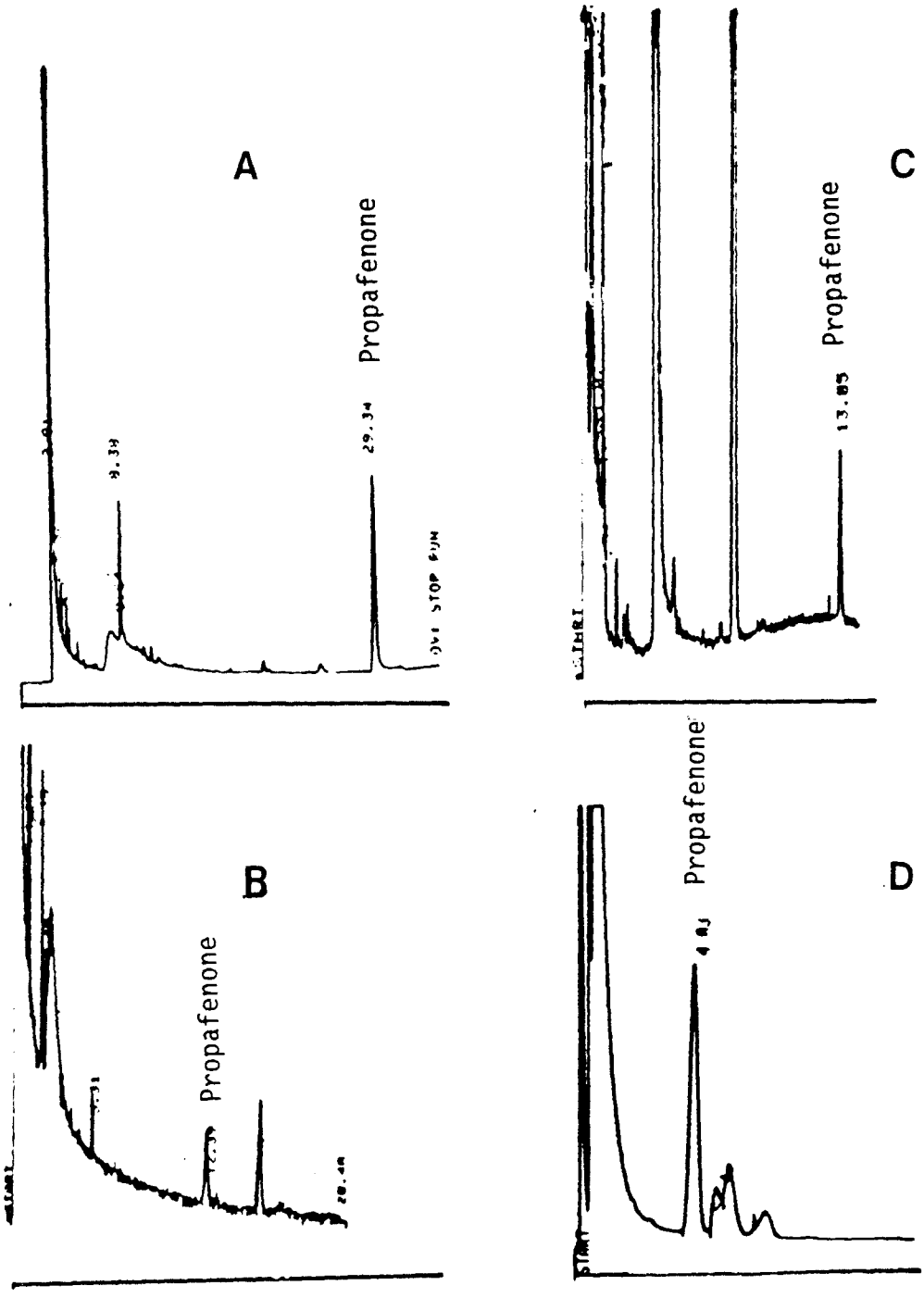


Figure 2.

Representative Chromatograms Showing Unresolved Peaks of R,S Propafenone Under Various GLC Conditions.

A) HFBA derivative of propafenone chromatographed on Chirasil-Val^{R} capillary column at 220^{o}C, detection ECD.

B) N-TPC derivative of propafenone chromatographed on SE-30 capillary column at 180^{o}C, detection FID.

C) R(+)MBIC derivative of propafenone chromatographed on SE-30 capillary column at 190^{o}C, detection FID.

D) R(+)MBIC derivative of propafenone chromatographed on OV-17 packed column at 280^{o}C, detection FID.

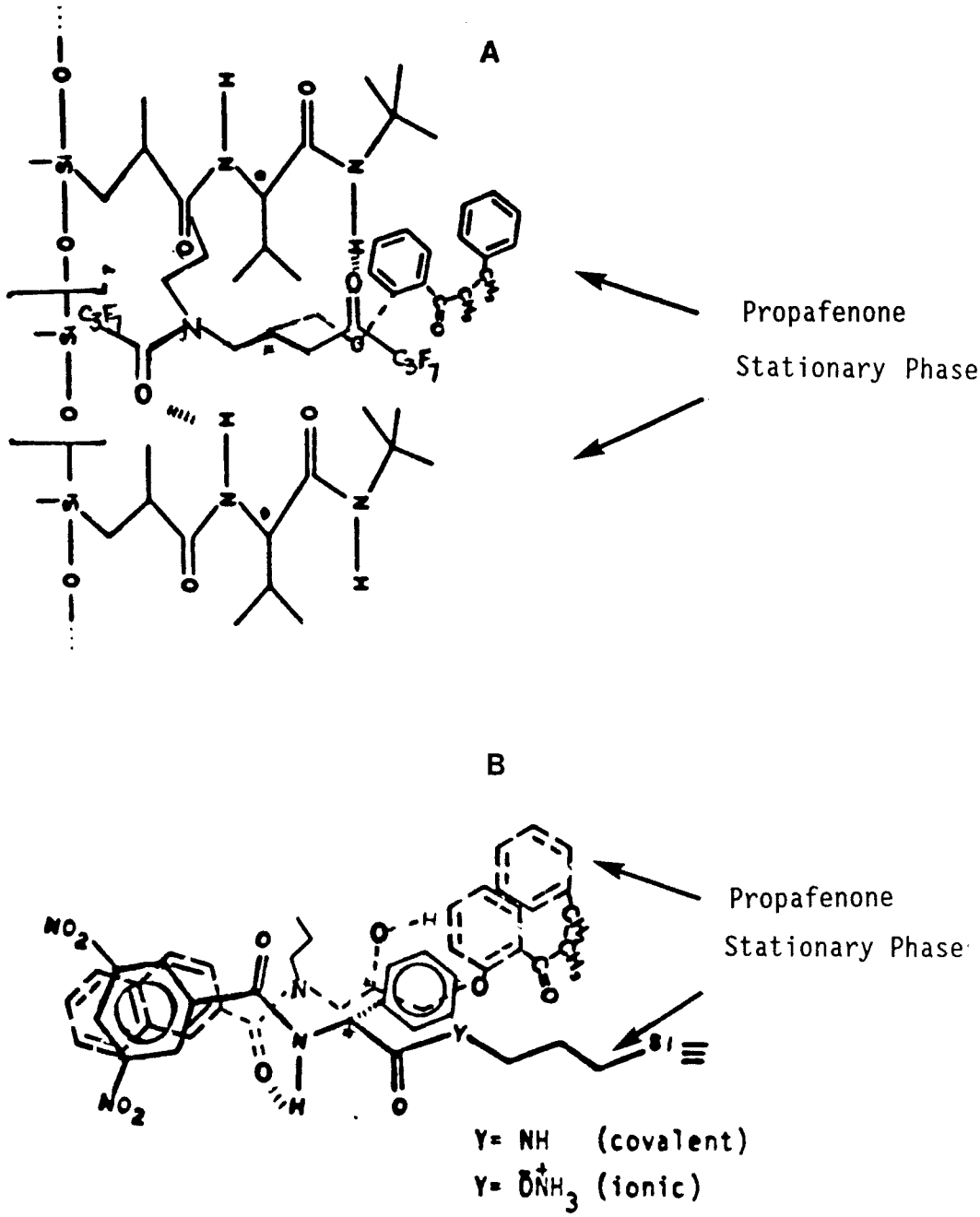


Figure 3.

Stereochemical Interaction Between HFBA Derivative of Propafenone Enantiomers and Chirasil-Val^{R} Stationary Phase (A) and Between Naphthoyl Chloride Derivative of Propafenone and Pirkle^{R} Stationary Phase (B).

To increase the hydrogen bonding capacity of propafenone for the Chirasil-Val^{R} stationary phase, racemic propafenone was reacted with isopropylisocyanate to form the urea derivatives. However, GLC analysis of the enantiomer derivatives on this chiral column still failed to resolve the enantiomers (figures not shown).

Alternative GLC methods were chosen based on the principles of diastereomeric separation of the enantiomers of propafenone on suitable achiral phases. The differential thermodynamic stability aided by the conformational rigidity of the diastereomer molecules (Rose et al., 1966) have been noted to facilitate resolution of enantiomers. Fig. 2B is a representative chromatogram of the diastereomeric N-trifluoro-acetyl-L-proloyl chloride (N-TPC) derivatives of propafenone. An SE-30 capillary GLC column was used at a temperature of 180^{o}C coupled with a flame ionization detector. An unresolved peak of the enantiomer derivatives eluted in R_t=12.39 minutes. A few extra peaks appeared which were thought to be due to the reported (Silber et al., 1980) degradation of the reagent during storage, even at low temperature. A second reagent that was employed for the diastereomer formation was R(+)α-methylbenzylisocyanate. However, the enantiomers of propafenone, as their diastereomeric urea derivatives, could not be resolved by the SE-30 capillary column nor by an OV-17 packed column. The columns were operated at 190^{o}C and 280^{o}C, respectively (Fig. 2C and 2D). The unresolved peaks in the chromatograms eluted in 13.85 and 4.03 min. respectively (Fig. 2C and 2D). Parallel blank experiments, without propafenone, were carried out to identify the propafenone peaks in each of the chromatograms discussed.

4.1.2 High-Performance Liquid Chromatographic Studies on Chiral and Achiral Stationary Phases

A major emphasis was given to HPLC as a method for the direct separation of propafenone enantiomers. The first chiral HPLC column employed was a Pirkle (1-A) column, containing N-3,5-dinitrobenzoyl-D-phenylglycine ionically bonded to γ-amino silanized silica. Due to the strong interaction of the propafenone base with this stationary phase, propafenone did not elute from this column. The β-naphthoyl chloride derivative of propafenone was chromatographically examined using 9% 2-propanol in hexane as the eluting solvent (Fig. 4A). The polarity of the mobile phase was varied by changing the proportion of 2-propanol in the mixture, however, there was no resolution of the enantiomers with any of the mobile phases employed. In order to alter the π-π interactions, the α-naphthoyl and 2,4 dinitrobenzoyl derivatives were examined. However, no resolution was observed with either of the derivatives.

Direct HPLC separation of the propafenone enantiomers was also attempted using a β-cyclodextrin bonded column and methanol/water (65:35) as the mobile phase, delivered at a flow rate of 0.8 mL/min. In the experimental study performed, as shown in fig. 4B, no separation of enantiomers was observed by the fact that an unresolved peak of propafenone eluted in 60 min. in the chromatogram. This β-cyclodextrin stationary phase usually facilitates the separation of solutes, including chiral ones, by the principle of exclusion chromatography. The exact fitting of the solute in the desired clatherate, hydrogen bonding interactions between the solute and the stationary phase at the mouth of the cyclodextrin cavity and hydrophobic interactions (depicted

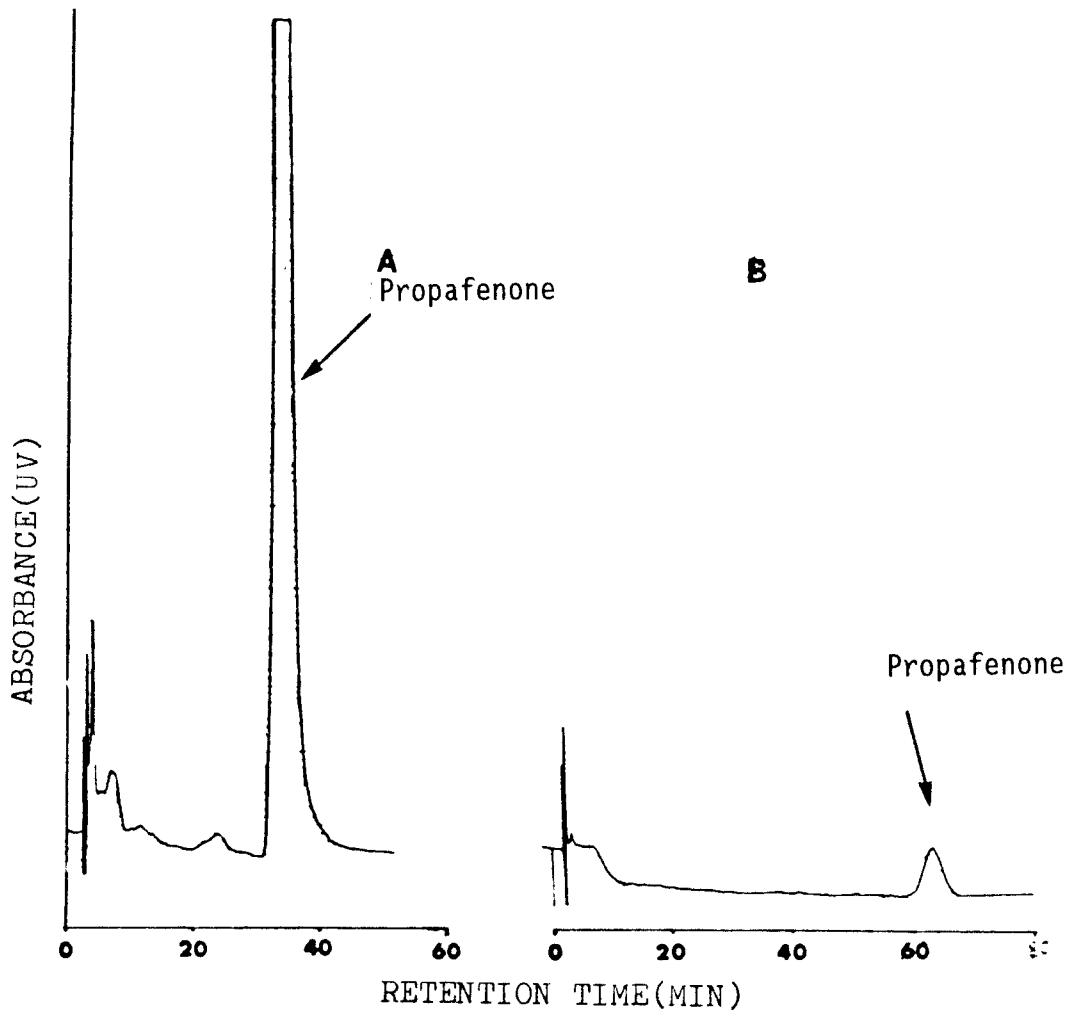


Figure 4 .
Representative Chromatograms Showing Unresolved Peaks of R,S-Propafenone by HPLC.
A) β-Naphthoyl derivative of propafenone chromatographed on Pirkle^{R} column. HPLC conditions: mobile phase; 2-propanol/hexane (9:100) delivered at 1 mL/min; detection 254nm.
B) Underivatized propafenone chromatographed on β-cyclodextrin column. HPLC conditions: mobile phase; methanol/water (65:35) delivered at 0.8 mL/min; detection 254nm.

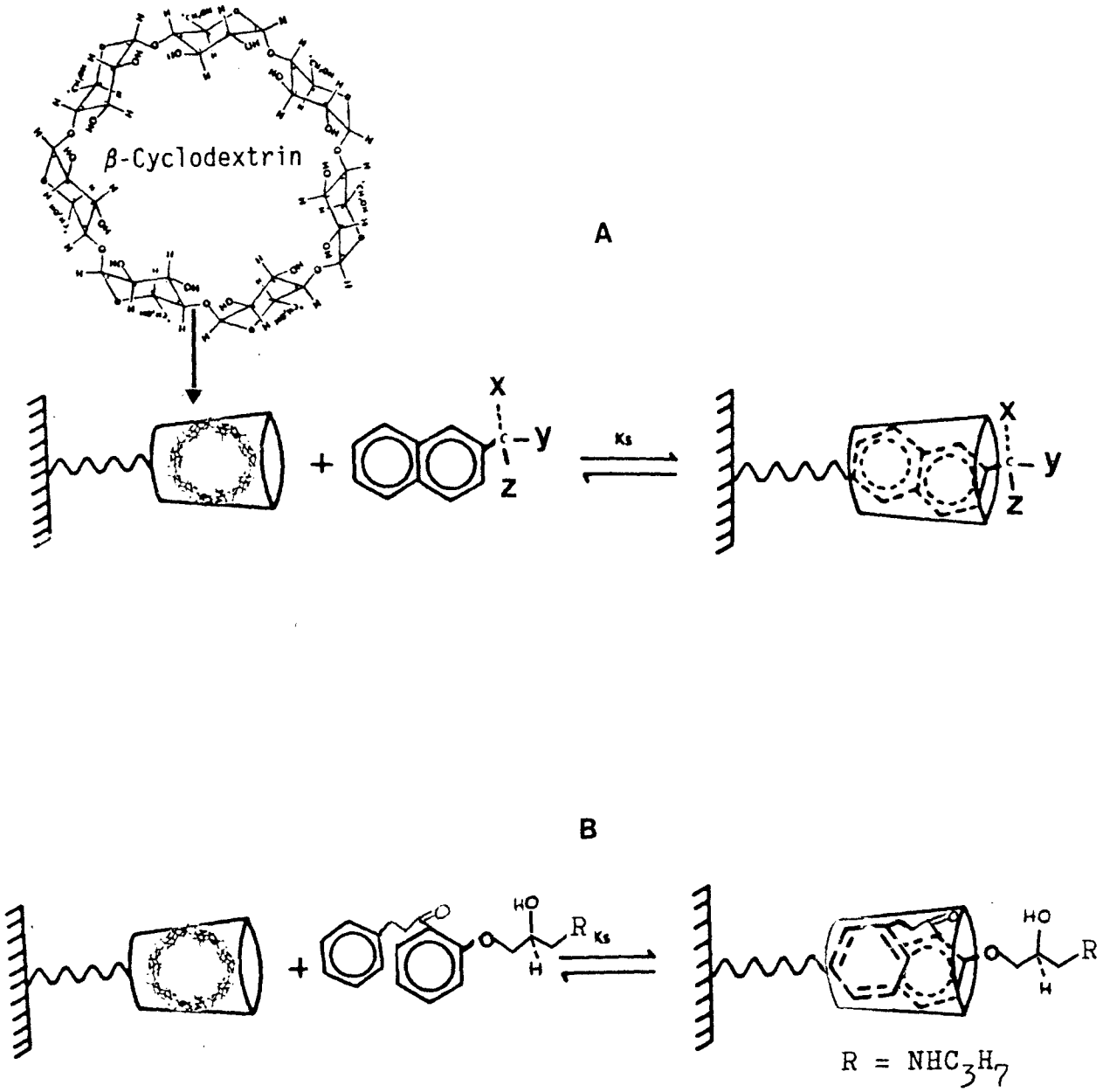


Figure 5. The General Mechanism of Inclusion-Complexing in β-Cyclodextrin Stationary Phase (A), and the Inclusion Complex Formation of Propafenone Enantiomers Within the β-Cyclodextrin Cavity (B). .

in Fig.5), leads to enantiomeric resolution. It was thus concluded that the essential interaction of the propafenone enantiomers were absent with the β-cyclodextrin phase.

4.2 High-Performance Liquid Chromatographic Resolution of the Propafenone Enantiomers

4.2.1 Resolution of R(+)-α-Methylbenzylisocyanate [R(+)MBIC] Derivatives of Propafenone Enantiomers

The first successful resolution of propafenone enantiomers was achieved by HPLC using commercially available R(+) α-methylbenzylisocyanate [R(+)MBIC] as the chiral derivatizing reagent. Both R,S propafenone and the internal standard, R,S Li-1115 reacted with R(+)MBIC at room temperature in 30 minutes. The resulting diastereomeric urea derivatives were resolved on a 5 u ODS column with a mobile phase of methanol/water (68:28) delivered at a flow rate of 0.8 mL/min. Resolution (R_S=1.25) of propafenone enantiomers was obtained in less than 60 minutes. The retention time could be decreased to 40 minutes (Fig. 6A) at the expense of resolution (R_S=1.15) using an increased proportion of methanol in the mobile phase of methanol/water (70:30). Addition of tetrahydrofuran and acetonitrile as organic modifiers to the methanol/water mobile phase abruptly decreased the resolution. Unfortunately, using this derivatizing reagent, the lower detection limit of each enantiomer was 500 ng/mL in plasma and the method was found not to be sufficiently sensitive (Table 1, page 61) for pharmacokinetic studies. Moreover, the reagent was extremely unstable and degradation of the reagent was observed during a few days of storage

Table 1. Data Representing the HPLC Separation of Enantiomers of Propafenone.

COLUMN USED	SOLVENT SYSTEM (1mL/min)	DETECTION METHOD	DERIVATIZING REAGENT	RETENTION TIME(MIN) R(-);S(+)	RESOLUTION FACTOR (R_s)	DETECTION LIMIT OF ENANTIOMER	SIG./NOISE RATIO
ODS	70:30 METHANOL/ WATER	254 nm	R(+)MBIC	40;42	1.15	500 ng	3:1
5μ (25 x 0.45cm i.d.)	75:25 METHANOL/ 0.2M PHOS-BUFF.pH2.8	250 nm	GITC	12;14.5	1.4	150 ng	4:1
	76:24 METHANOL/ WATER	230 nm	R(-)NEIC	19.5;21	1.25	100 ng	3:1
	75:25 METHANOL/ WATER	FLUORO. EX.220 nm EM.418 nm	DANSYL HYDRAZINE & R(-)NEIC	21.7; 23.4	1.35	2.5 ng	3:1

under nitrogen at 4°C. Similar information was reported by Gal et al., 1981.

4.2.2 Resolution of the 2,3,4,6 Tetra-O-Acetyl-β-D-Glucopyranosyl Isothiocyanate (GITC) Derivatives of Propafenone Enantiomers

Prior to conducting this experiment, the chiral derivatizing reagent GITC was synthesized from α-D-bromoglucose. The purity of the compound was checked by reverse phase HPLC and functional group characterization was studied by IR. A characteristic chromatographic peak and the C=N=S stretching frequency at 2100 cm^{-1} depicted in fig. 7 indicated that the GITC reagent thus synthesized was pure and no evidence of enantiomeric inversion was observed. The GITC synthesized was allowed to react with R,S propafenone at room temperature in 30 minutes and the resulting diastereomers were resolved on an ODS column (Fig.6B). The solvent system employed was methanol/phosphate buffer (0.2 M) at pH 2.8 (75:25) delivered at flow rate of 1 mL/min. Near baseline resolution (R_S=1.4) of the diastereomers was obtained within 15 minutes. The internal standard, desipramine as its thiourea derivative, eluted in 24 minutes. The thiourea derivatives, which have maximum molar extinction coefficients at 250 nm (Nimura et al., 1980) provided a minimum detection limit of 150 ng of each enantiomer of propafenone injected onto the column. In addition to effective resolution of the enantiomers, the GITC reagent could be easily handled and was found more stable than R(+)MBIC during storage.

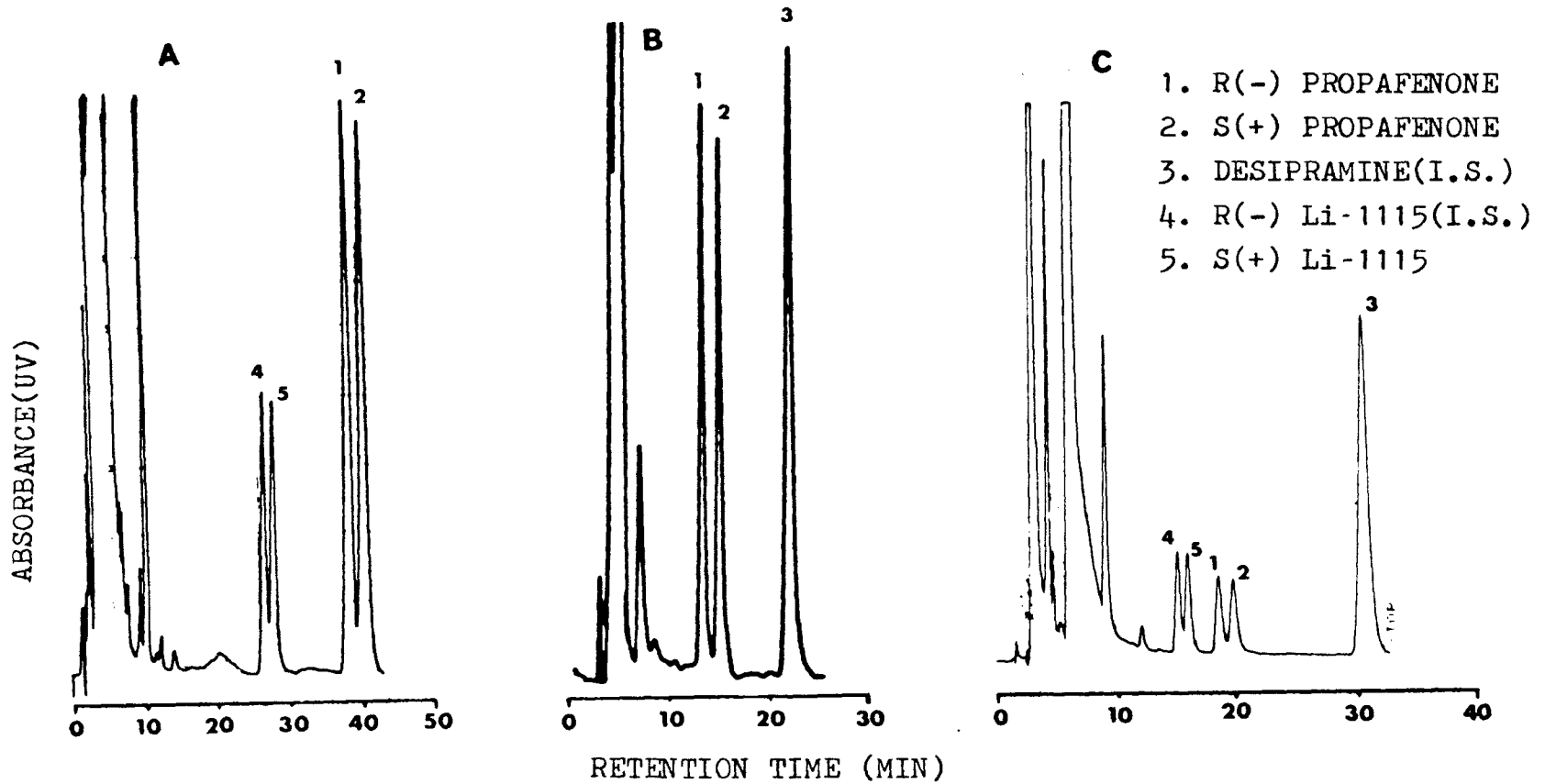


Figure 6. Reverse Phase HPLC Separation of Enantiomers of Propafenone as their Diastereomeric R(+)-α-Methylbenzyl Urea (A), β-D-Glucopyranosyl Thiourea (B) and R(-)-1-(Naphthyl)ethyl-Urea (C) Derivatives. Chromatographic Conditions are given in Table 1.

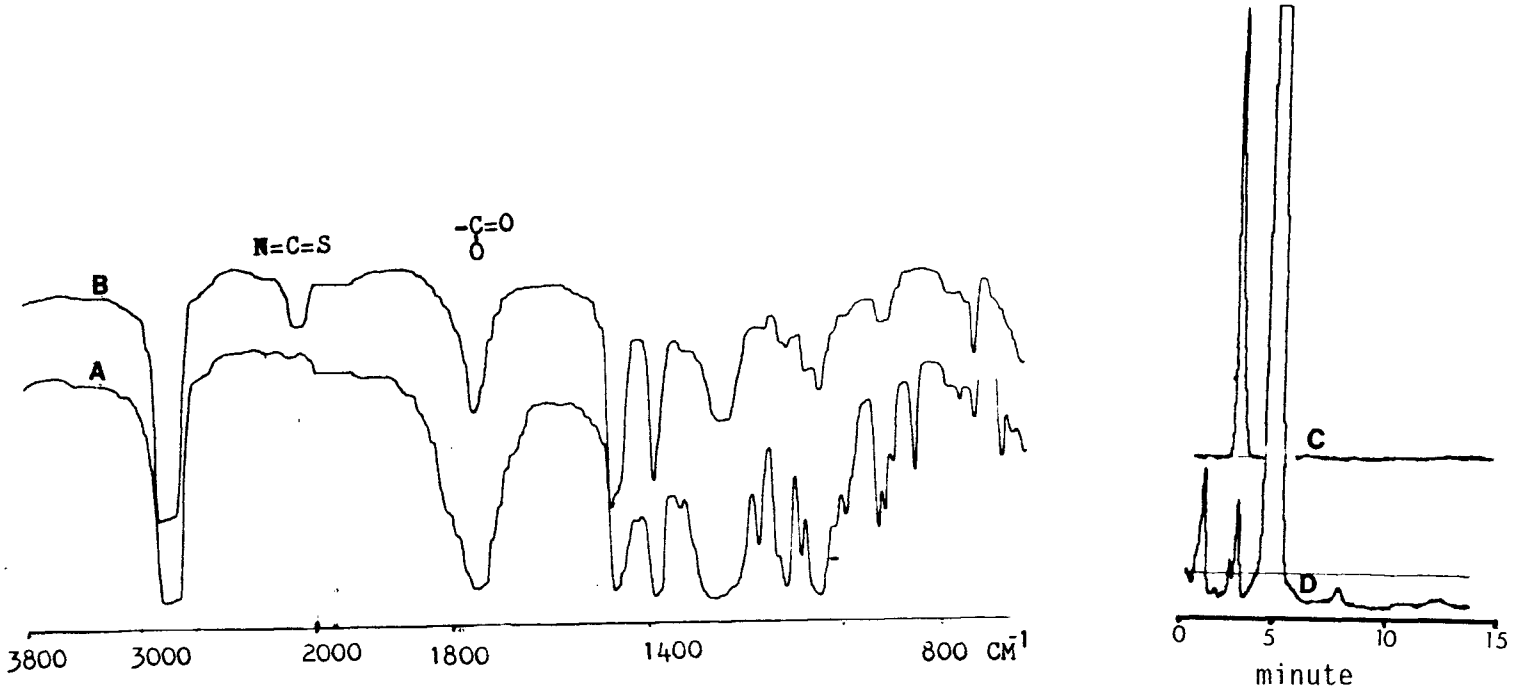


Figure 7. IR and HPLC Analyses of Acetylated α-D-Bromoglucose and GITC.

A) IR spectra of acetylated α-D-bromoglucose in nujol. Ester carbonyl stretching frequency at 1750 cm^{-1}.
B) IR spectra of acetylated GITC in nujol. Ester carbonyl stretching frequency at 1750 cm^{-1} and N=C=S stretching frequency at 2100 cm^{-1}.

C) Chromatogram of acetylated α-D-bromoglucose in HPLC. Column; ODS. Mobile phase; methanol/water (65:35) at 1.2 mL/min. Detection; 254 nm.
D) Chromatogram of GITC after third recrystallization.

4.2.3 Resolution of the R(-)-1-(Naphthyl)ethylisocyanate [R(-)NEIC] Derivatives of Propafenone

To take advantage of increased uv absorption characteristics of naphthyl over the benzyl moiety substituted on the chiral centre of the isocynate reagent, a third chiral reagent, R(-)-1-(Naphthyl)ethylisocyanate [R(-)NEIC] was chosen for the derivatization of R,S propafenone. This reagent also formed diastereomeric urea derivatives with propafenone. The resolution of the enantiomers (R_S 1.25) was obtained on a 5u ODS column within 24 minutes using methanol/water (76:24) as the mobile phase (Fig. 6C and Table 1). Detection at 230 nm was employed since the derivative provided maximum absorption at this wavelength (Fig. 8E). The minimum detectable quantity was 100 ng/mL in plasma for each enantiomer. When the pH of the mobile phase was adjusted below pH 7 using perchloric acid, the elution time of the diastereomeric derivatives from the ODS column was substantially increased, which was considered to be due to the reduced solvation effect of the acidic mobile phase with the naphthyl derivatives.

4.3 Elution Order of R and S Propafenone

In order to determine the elution order of the enantiomers of propafenone as their R(-)NEIC derivatives, the two diastereomeric fractions corresponding to the two peaks on the chromatogram (Fig. 6C) were separately collected in two flasks and the mobile phase was evaporated to dryness as described in section (3.9.9). The elution order of the peaks was determined to be R(-) propafenone, followed by S(+) propafenone. This was confirmed by polarimetric analysis of the

relative degree of rotation of each fraction of diastereomer. The rotation of two fractions containing the purified diastereomers was -22^{o} and -5^{o}, respectively. According to the 'rule of shift' reported by Freudenburg, 1933 and Finar, 1969, the more negative rotation was attributed to that from the R(-) propafenone, whereas the S(+) propafenone provided the least negative rotation due to the constant additive rotational contribution from the R(-)NEIC reagent. Similar elution order of R(+) propranolol and S(-) propranolol by reverse phase HPLC has been reported by Thompson et al., 1982 using S(-)MBIC as the derivatizing reagent.

4.4 Confirmation of Structures of R and S Propafenone Derivatives

The two HPLC fractions corresponding to the derivatives of R(-) and S(+) propafenone were individually collected and subjected to GC-MS analyses in the EI mode as described in section (3.9.10). Both fractions provided identical total mass-ion chromatograms (TIC) (Fig. 9A and 9B) and EI mass spectra from scan 160 (Fig. 10A and 10B). The fragmentation patterns of mass numbers of 77, 127, 155, 182 and 197 corresponded to the fractions of the derivatives, are depicted in fig 10. Underivatized propafenone apparently did not elute out from the GLC column under identical chromatographic conditions as evidenced by the lack of any peak in the TIC.

4.5 Sensitivity of Propafenone and its Derivatives

Unlike metoprolol, atenolol, or salbutamol, propafenone did not show any fluorescence despite the fact that a fluorogenic aryloxy moiety

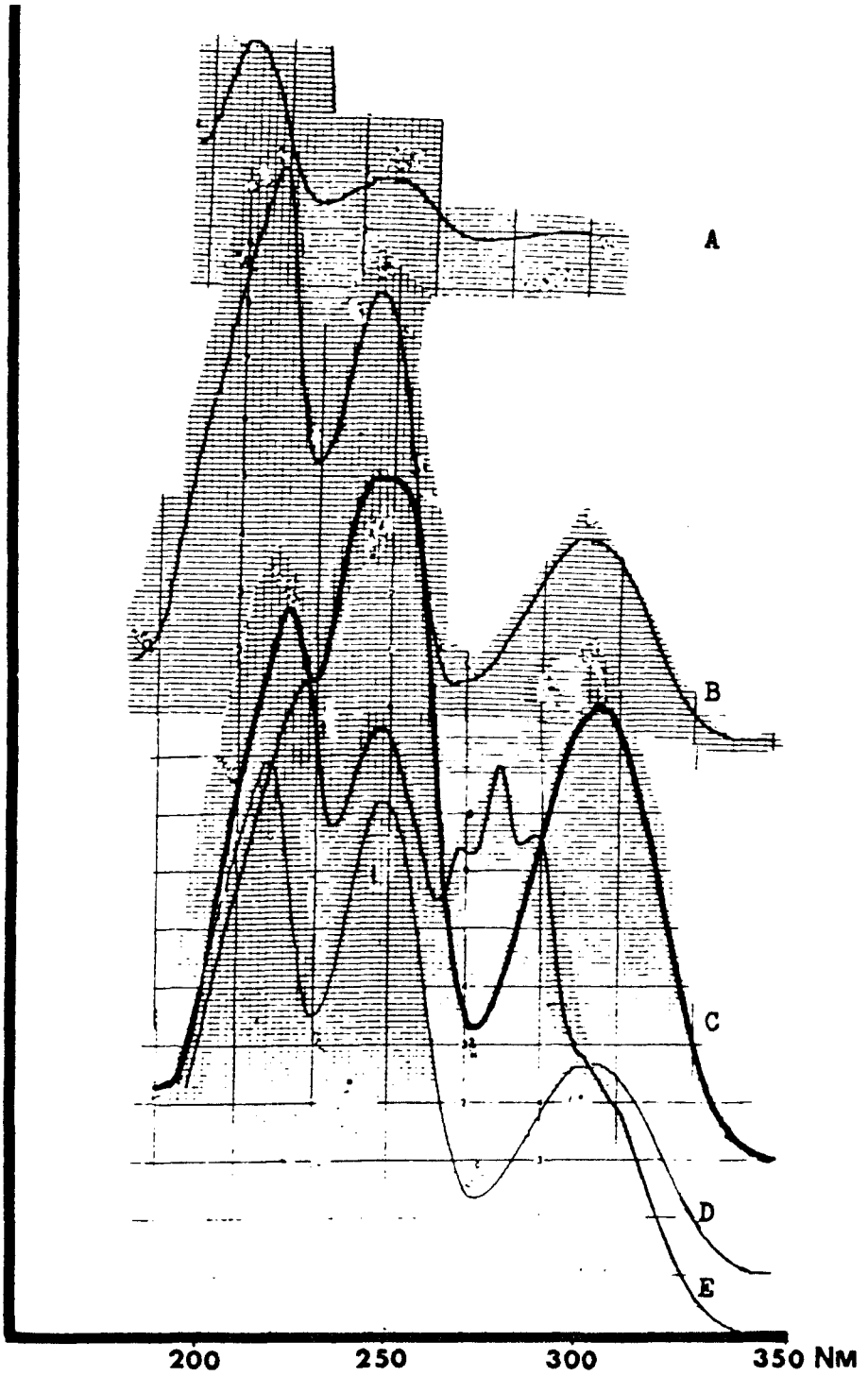


Figure 8.
Ultraviolet Absorption of Propafenone and its Derivative in Different Solvents.
Solvent systems: (A) Propafenone base in acetonitrile/ phosphate buffer (5 mM) of pH 2.8 (65:35). (B) Propafenone base in acetonitrile. (C) Propafenone base in methanol/phosphate buffer (0.2 M) of pH 2.8 (75:25). (D) Propafenone hydrochloride in water. (E) R(-)NEIC derivative of propafenone in methanol/water (75:25).

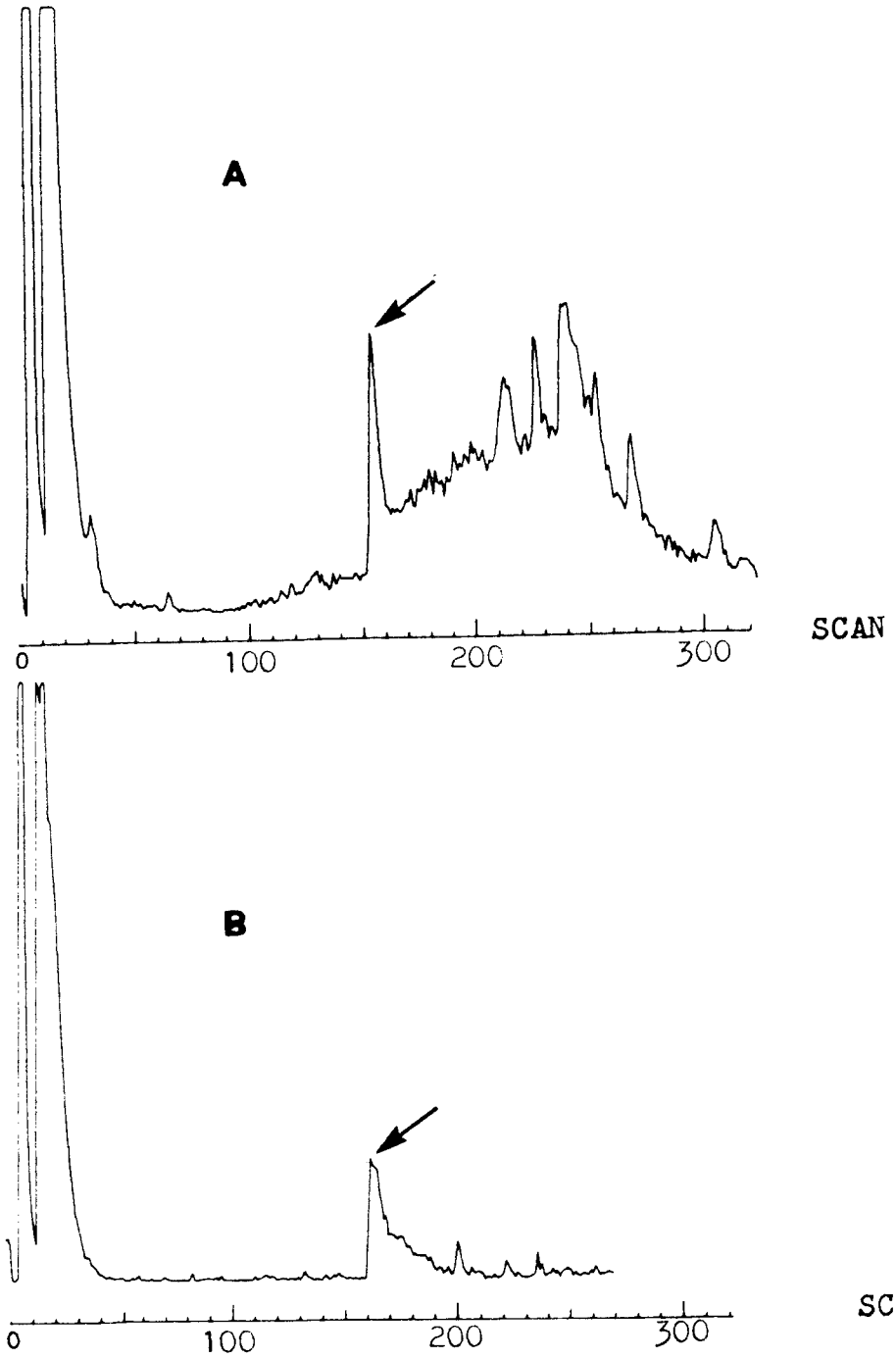


Figure 9.

Total-Ion-Mass Chromatograms of Derivatives of S(+)Propafenone (A) and R(-)Propafenone (B) with R(-)-1-(Naphthyl)ethylisocyanate. GC-MS conditions: Filament current,300 uA, Electron beam energy, 70 ev, Ion-source pressure, 8 X 10^{-6}torr, Injection port temperature, 250°C, Column (a 3 % silar 10 C on chromosorb W-HP, 2m X 2mm i.d.) temperature , 150° to 285°C at 8°C / min. Column helium flow rate, 20 mL/min.

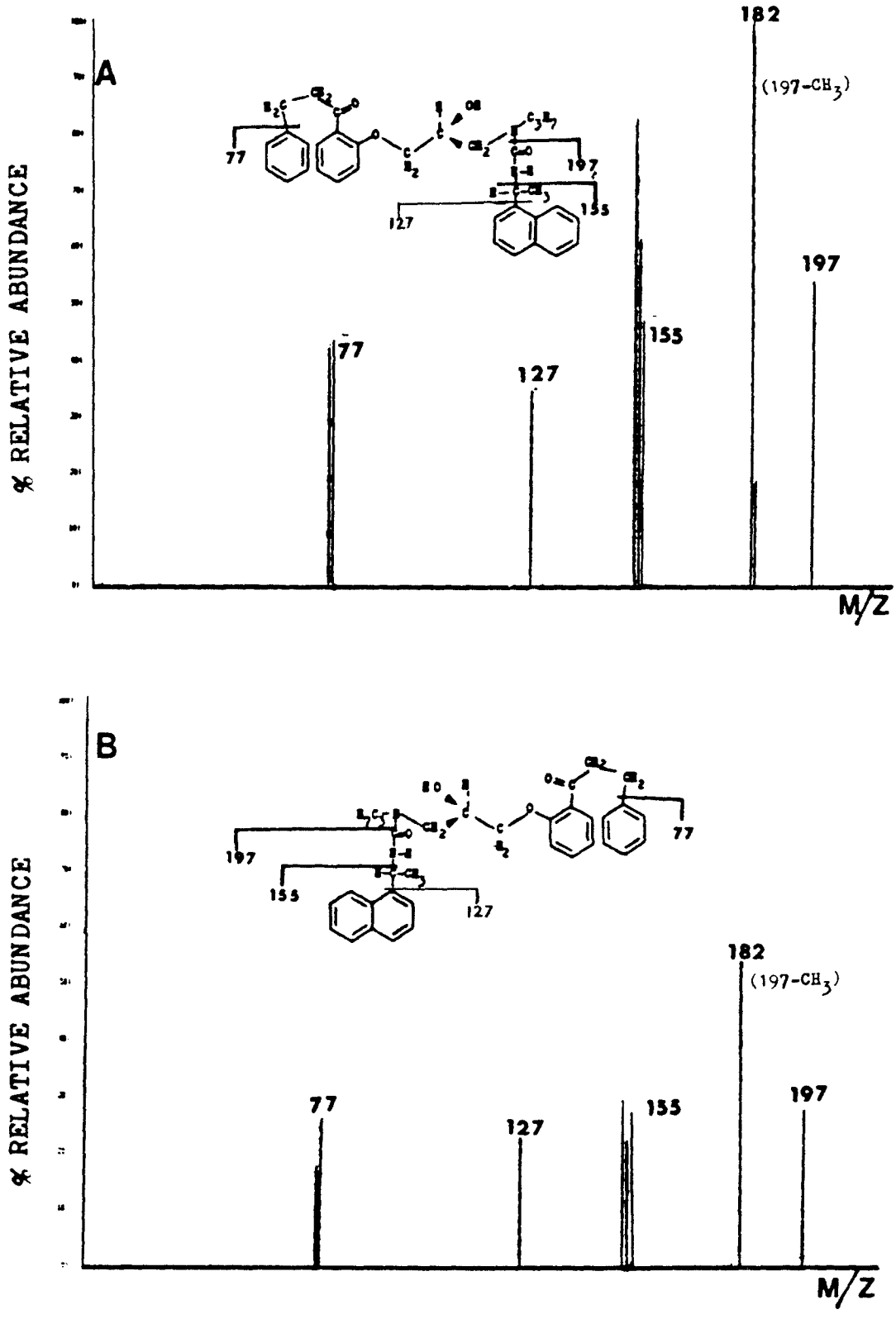


Figure 10 . EI Mass Spectra of S(+)Propafenone (A) and R(-)Propafenone (B) as their R(-)-1-(Naphthyl) ethylisocyanate Derivatives.

is common to all of these compounds. The absence of fluorescence of propafenone was ascertained on a Perkin-Elmer model 650-10S fluorescence spectrophotometer using a variety of solvents. The carbonyl group ortho to the aryloxy group was believed to provide quantum deactivation effects on propafenone thus eliminating any fluorogenic properties. The carbonyl group conjugated to the aryl moiety of propafenone structure did ,however, confer sensitivity for uv detection. A comparative study of the shift of the primary uv bands in different solvents is given in Fig. 8. Although the highest uv sensitivity for detection at 209 nm could be attained using acetonitrile/phosphate buffer at pH 2.8 (65:35), this did not provide enantiomeric resolution of propafenone derivatized with the chiral isocyanates. All solvent systems for enantiomeric resolutions required methanol in various proportions (Table 1) and hence it was necessary to work in the range of 248-254 nm. The R(-)NEIC derivative of propafenone provided the highest λ_{max} at 230 nm, using methanol/water (75:25) as mobile phase and a minimum detection limit of 100 ng (at the detector) for each enantiomer was observed (Table 1). The (+) naproxen derivative of propafenone, although assumed to provide better sensitivity due to its highly conjugated structure, the individual enantiomers could not be resolved by HPLC using methanol/water (76:24) as the mobile phase delivered at 1 mL/min. (figure not shown).

4.6 Mechanism of Diastereomeric Resolution of Propafenone

The mechanism of diastereomeric resolution of propafenone is depicted in figure 11. The essential criterion of resolution of the diastereomers was the formation of intramolecular hydrogen bonds between

the hydrogen of the secondary alcoholic group and the oxygen of the ureido carbonyl oxygen of the urea derivative formed, thus giving rise to maximum conformational dyssymmetry in two diastereomeric structures. These two structures however, have different partition coefficients and would enantiomerically separate by reverse phase HPLC. A similar approach has been reported by Thompson et al., 1982, for the separation of the enantiomers of propanolol as their S(-)MBIC derivatives by reverse phase HPLC. The same authors also emphasized the role of this hydrogen bonding by the fact that blocking the OH-group by conversion to its TMS ether, abolished the diastereomeric resolution.

From the structural consideration of the three kinds of chiral derivatizing reagents employed, which are shown in fig.12, the R(-)NEIC reagent imparted greater conformational restraint than R(+)MBIC and proved to be the better resolving reagent of the two. However, the most bulky acetylglycosyl residue of GITC reagent imparted the greatest conformational rigidity in the structure of the diastereomeric thiourea derivatives of propafenone and therefore provided the maximum resolution. According to Nimura et al., 1980, GITC derivatives of chiral amines confer both bulkiness and hydrophobicity, thus giving rise to an increased interaction with an ODS stationary phase and hence better resolution. In liquid chromatography, the differential solvation of the solutes by the eluting solvents also plays a major role on the mechanism of separation (Pirkle et al., 1981). The GITC residue of the derivative of propafenone was better solvated in the mobile phase employed and provided better resolution within a shorter time (15 minutes, Fig. 6B).

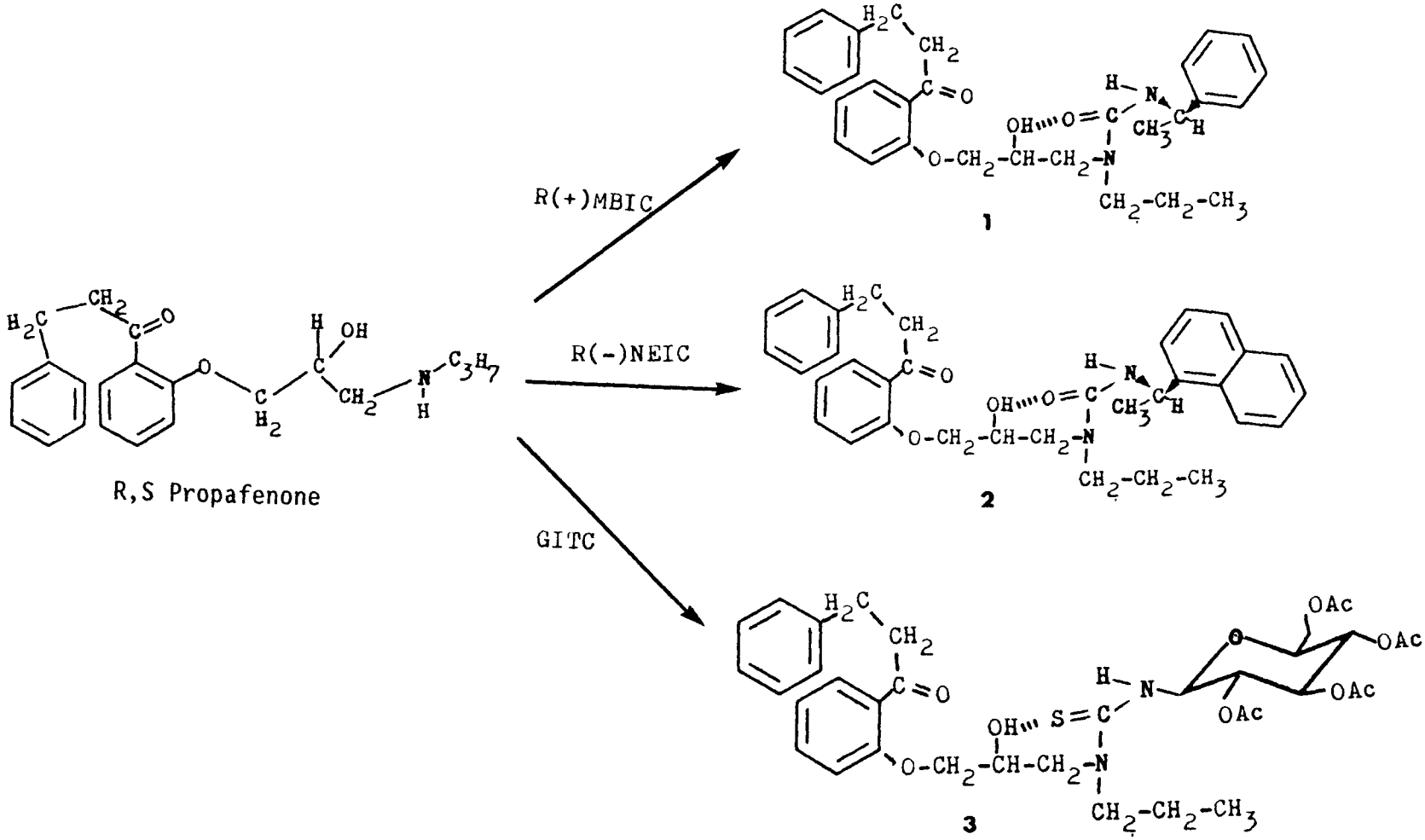
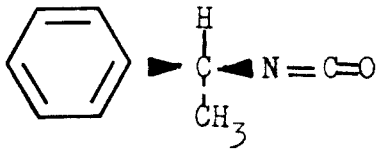
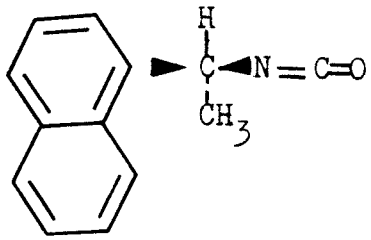


Figure 11. Mechanism of Diastereomeric Separation of Propafenone Urea and Thiourea Derivatives



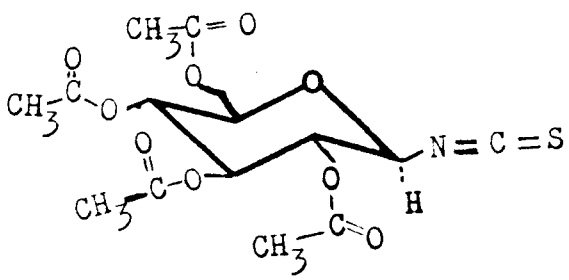
R(+)-α-Methylbenzylisocyanate

(R(+)MBIC)



R(-)-1-(Naphthyl)ethylisocyanate

(R(-)NEIC)



2,3,4,6 Tetra-O-Acetyl β-D-Glucopyranosylisothiocyanate

(GITC)

Figure 12. Structure of the Chiral Derivatizing Reagents.

4.7 Intramolecular Hydrogen Bonding and Conformation of Propafenone

4.7.1 HPLC Separation of the Conformers

Propafenone free base, when chromatographed on the ODS column, using a mobile phase of pH 5.5 containing methanol/phosphate buffer (0.2 M) (75:25), exhibited one major peak with a small shoulder (Fig. 13A). This was considered to be due to the existance of at least two conformational states with almost identical lipophilicity and solvation properties. Upon examining the molecular model of propafenone it was clear that the free base of propafenone could exist in two conformational states due to the formation of two alternate intramolecular hydrogen bonds. Due to the proximity of the aryl carbonyl group ortho to the side chain containing the secondary alcoholic and amino groups, two different conformations may result from intramolecular hydrogen bonding. This has been shown in scheme 1, where the conformers #2 and #3 are the propafenone free base in two different conformations, due to alternative intramolecular hydrogen bonds. The predominance of one, which corresponded to the major peak in the chromatogram of fig. 13A, was due to the greater flexibility of the cyclization. The ten membered cyclic form as shown in scheme 1, #3 would be more flexible than the nine membered form #2, thus giving rise to the existence of two conformational forms of propafenone. Further evidence of the formation of a hydrogen bond in propafenone was found when a low amplitude broad peak appeared in the range of 3275 to 3525 cm^{-1} in the IR spectrum (Fig.14). Similar phenomena of intramolecular hydrogen bonding and the predominance of one conformer over the other

has been reported by Kuhn et al., 1964 and Murthy et al., 1968, for 1,4-diols and other substituted aromatic alcohols and acids.

4.7.2 Reaction of Conformers with Chiral isocyanates and the HPLC Resolution of the Diastereomers

Of the two conformers of propafenone, the one with a free N-H group reacted with a chiral isocyanate to form diastereomeric ureide derivatives, where the two chiral centers in the diastereomer are separated by six atoms. The enantiomeric resolution of R(-) and S(+) propafenone as their ureide derivatives would happen when a stable conformational rigidity was attained by the formation of an intramolecular hydrogen bond between the alcoholic OH group and the ureido carbonyl group as discussed in section 4.6. However, an alternative intramolecular hydrogen bond, between the same OH group and the aryl carbonyl group of propafenone was also feasible. The later conformation would negate the diastereomeric resolution of propafenone enantiomers. This has been shown in scheme 1. The conformers #4 and #5 meet the essential criteria for resolution due to the formation of a very specific intramolecular hydrogen bond in each case. The conformer #6 however, is formed by an alternate hydrogen bond between the OH and the aryl carbonyl group and would not follow the criteria for resolution. From HPLC studies, both resolved and unresolved peaks at different retention times were found as shown in fig. 13B. In agreement with scheme 1, two pairs of resolved peaks #4 and #5 with R_t of 24 and 30 min. respectively and one unresolved peak with R_t of 35 min. were found in the HPLC chromatogram of fig. 13B.

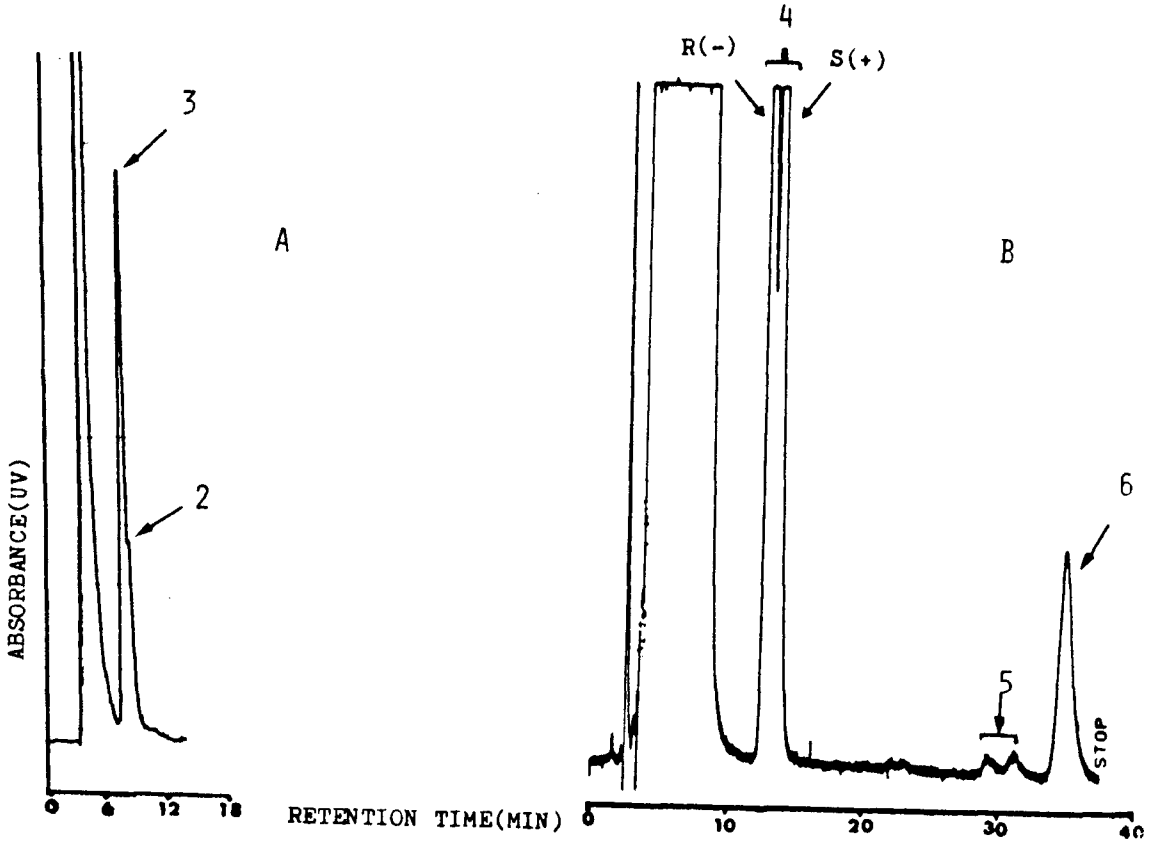


Figure 13.

Representative Chromatograms of Underivatized (A) and Derivatized (B) Propafenone in HPLC.
A) Propafenone free base elutes as one prominent peak (3) with a shoulder (2). HPLC conditions; mobile phase, 25% phosphate buffer (0 2 M) in methanol at pH 5.5, delivered at 1 mL/min. Column; 5u ODS, 25 x 0.45 cm i.d. Detection; 248nm.
B) Diastereomeric R(-) NEIC derivatives of propafenone appear as three conformational forms (4, 5, & 6) of which 4 and 5 resolve into R(-) and S(+) isomers. Mobile phase; 20% water in methanol delivered at 1 mL/min. Column; as above. Detection; 230 nm.

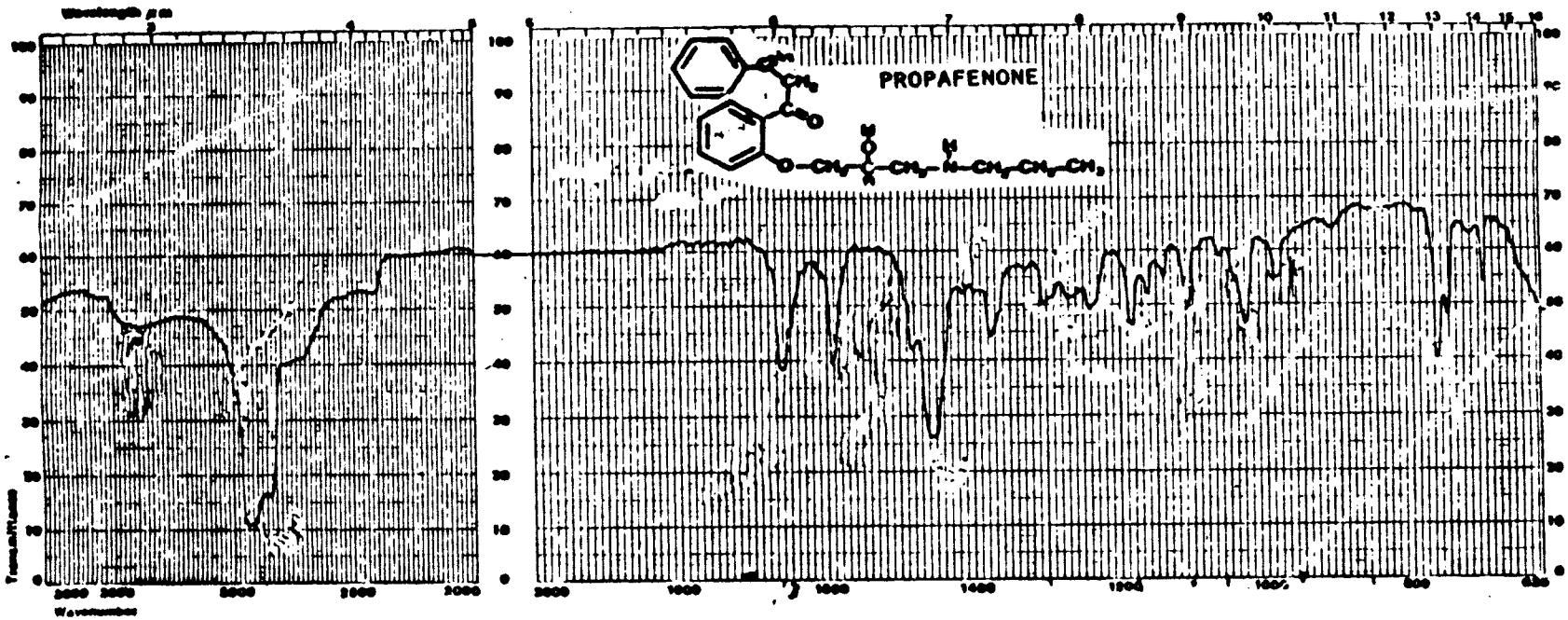
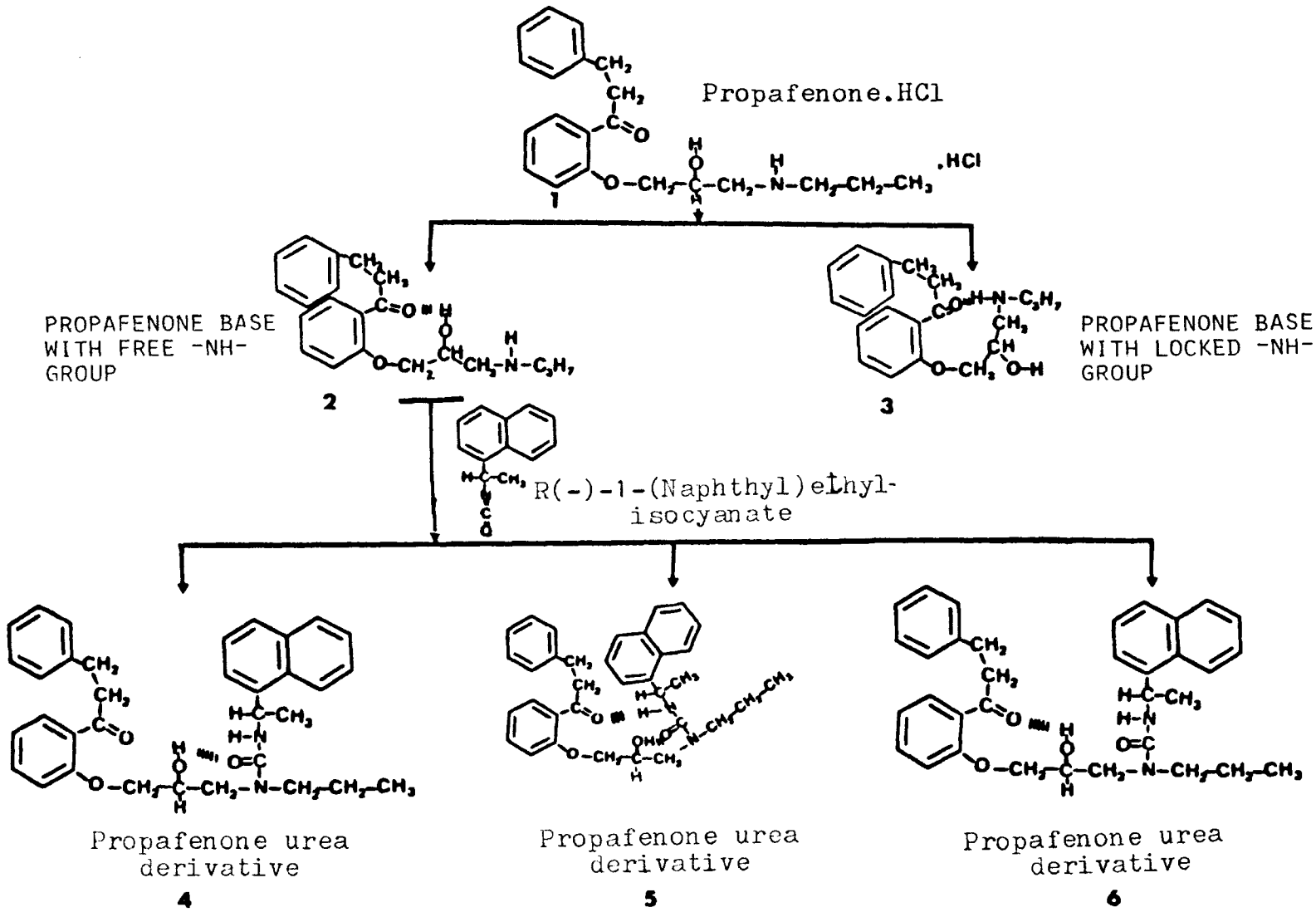


Figure 14. Infrared Spectrum of Propafenone in Nujol Mull.

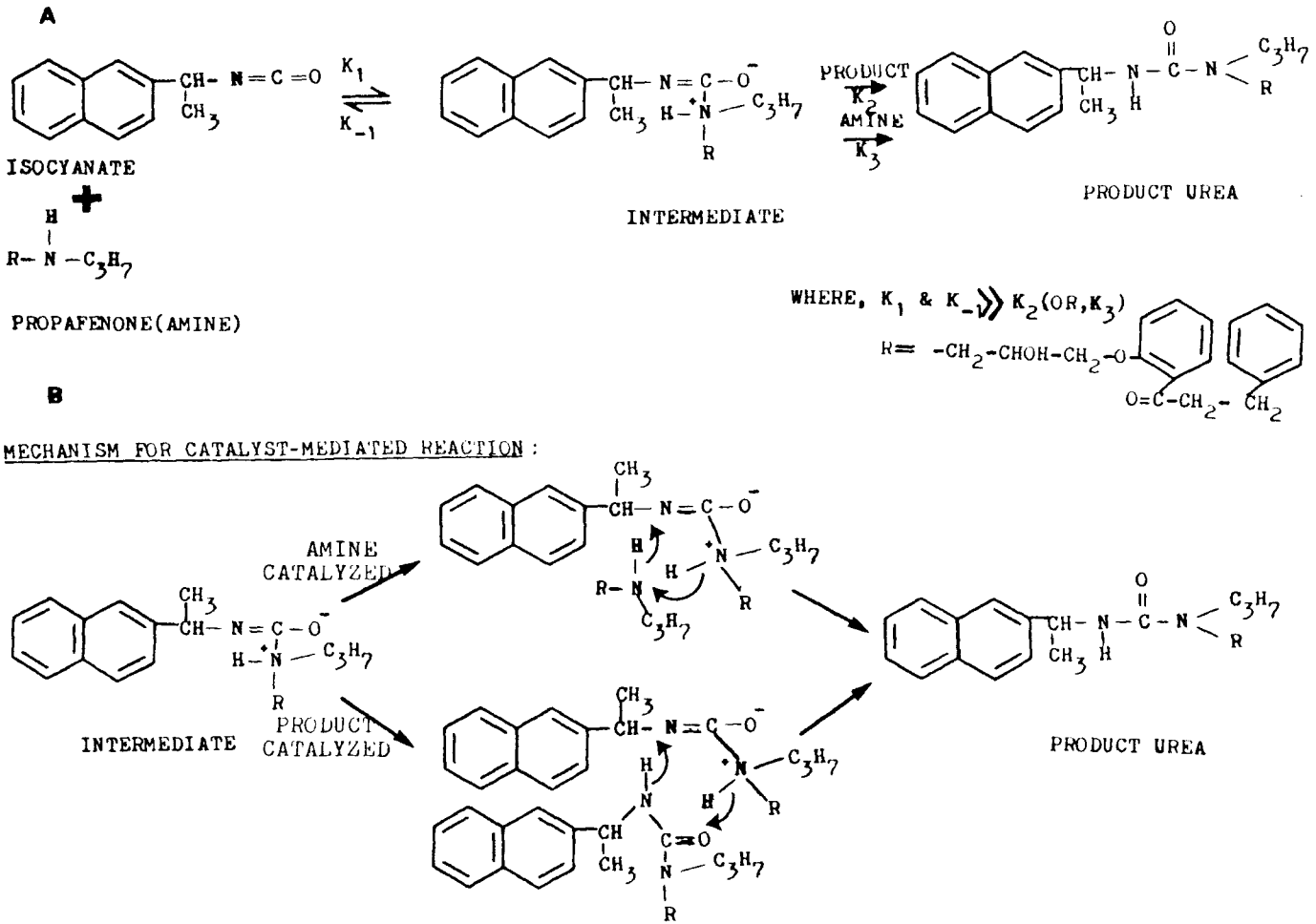


Scheme 1. Schematic Diagram Showing the Intramolecular Hydrogen Bonding and Conformational Isomers of Underivatized (2,3) and Derivatized (4,5 & 6) Propafenone.

4.8 Mechanism of Propafenone Isocyanate Reaction

The reaction of an amine with an isocyanate is governed primarily by the basicity or nucleophilicity of the N-H bond to form the substituted ureide. Isocyanate reactivity is decreased for substituents with increasing electron donating effect whereas the amine reactivity is increased for substituents with increasing electron donating effect of the substituents (Arnold et al., 1957). Briody and Narinesingh, 1971, determined that the reaction kinetics of amines with isocyanates strictly followed second order kinetics and that the rate limiting step was the slow proton transfer in the intermediate which was aided by a catalyst. The proton exchange mechanism between the catalyst and the amine-isocyanate intermediate has been confirmed by the primary hydrogen isotope effect, using a deuterated catalyst (Briody et al., 1971). Catalysis by the reacting amine, as well as by the product ureide, were also studied in such reactions (Satchell and Satchell, 1975). Bifunctional catalysts such as amides, ureides and carboxylic acids with very weak acidity were considered to be very effective. These catalysts form cyclic transition states in aprotic solvents which facilitate the slow proton transfer in the intermediate leading to completion of the reaction. In a few cases, triethylamine, as a weak basic catalyst, has been employed but the mechanism of catalysis is still uncertain (Briody et al., 1971, Satchell et al., 1975).

Propafenone is a secondary amine and was allowed to react with two different chiral isocyanates and a chiral isothiocyanate to form diastereomeric derivatives. A typical reaction scheme of propafenone and R(-)NEIC is depicted in scheme 2. The bipolar intermediate is autocatalysed by the reacting propafenone to the product ureide.

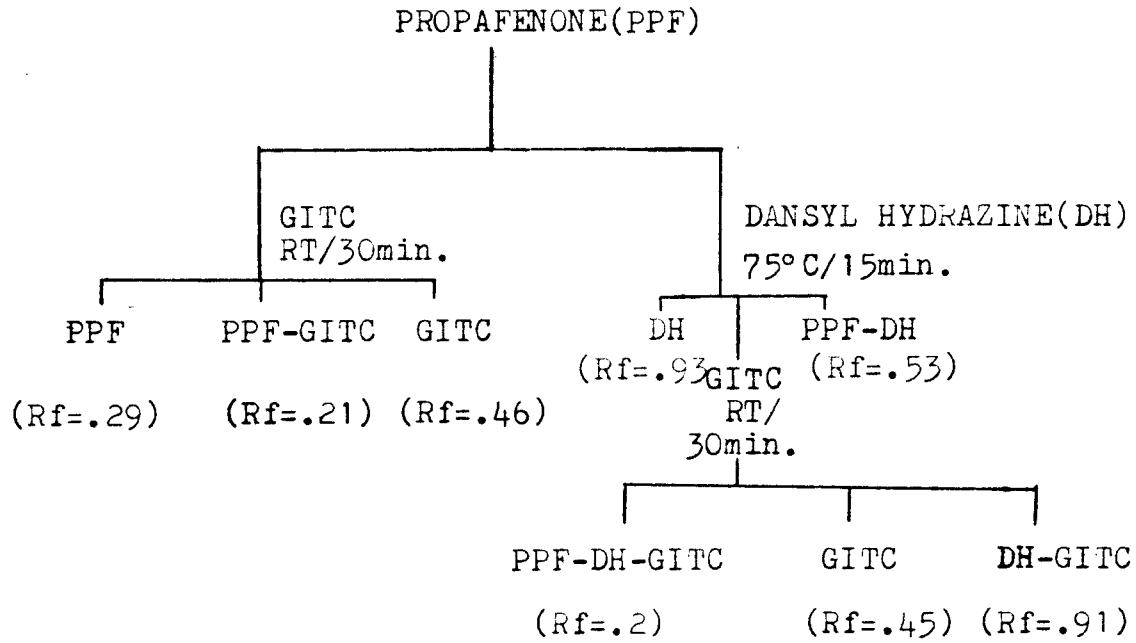


Scheme 2. Reaction Scheme for the Derivatization of Propafenone with R(-)-1-naphthyl ethylisocyanate.

However, the reaction of propafenone with isocyanate or isothiocyanate does not go to completion under the conditions employed and therefore a portion of residual propafenone is left unreacted. This was shown, with GITC as the derivatizing reagent, by evaluation of the reaction products using reverse phase TLC (Fig. 15). In the presence of excess reagent and at optimum reaction conditions a residual propafenone spot (R_f=0.29) was observed. Moreover, at very low concentrations of propafenone, below a reaction concentration of 1.67 ug/mL, the reaction between propafenone and isocyanate or isothiocyanate was found to be very slow and apparently stopped, since no increase in peak height of the urea derivative was observed between one to sixteen hours in HPLC.

The retarding effect of the reaction kinetics can be postulated to be due to two factors. At low concentrations of propafenone, the triggering auto-catalytic effect of the amine would be less prominent and the rate of the reaction would be retarded. However, the essential, and rate determining step, for the completion of the reaction is the proton exchange between the catalyst and the intermediate. Due to the possible intramolecular hydrogen bonding between the amino-hydrogen and the aryl carbonyl group, the exchangeable hydrogen is not readily available to perpetuate the catalysing activity. Moreover, the exchangeable proton in the intermediate is not sufficiently labile , since it is derived from propafenone itself. These two factors are believed to retard the isocyanate/ propafenone reaction and virtually impede the reaction when the propafenone level falls below a reaction concentration of 1.67 ug/mL.

Reverse Phase Thin-Layer Chromatography



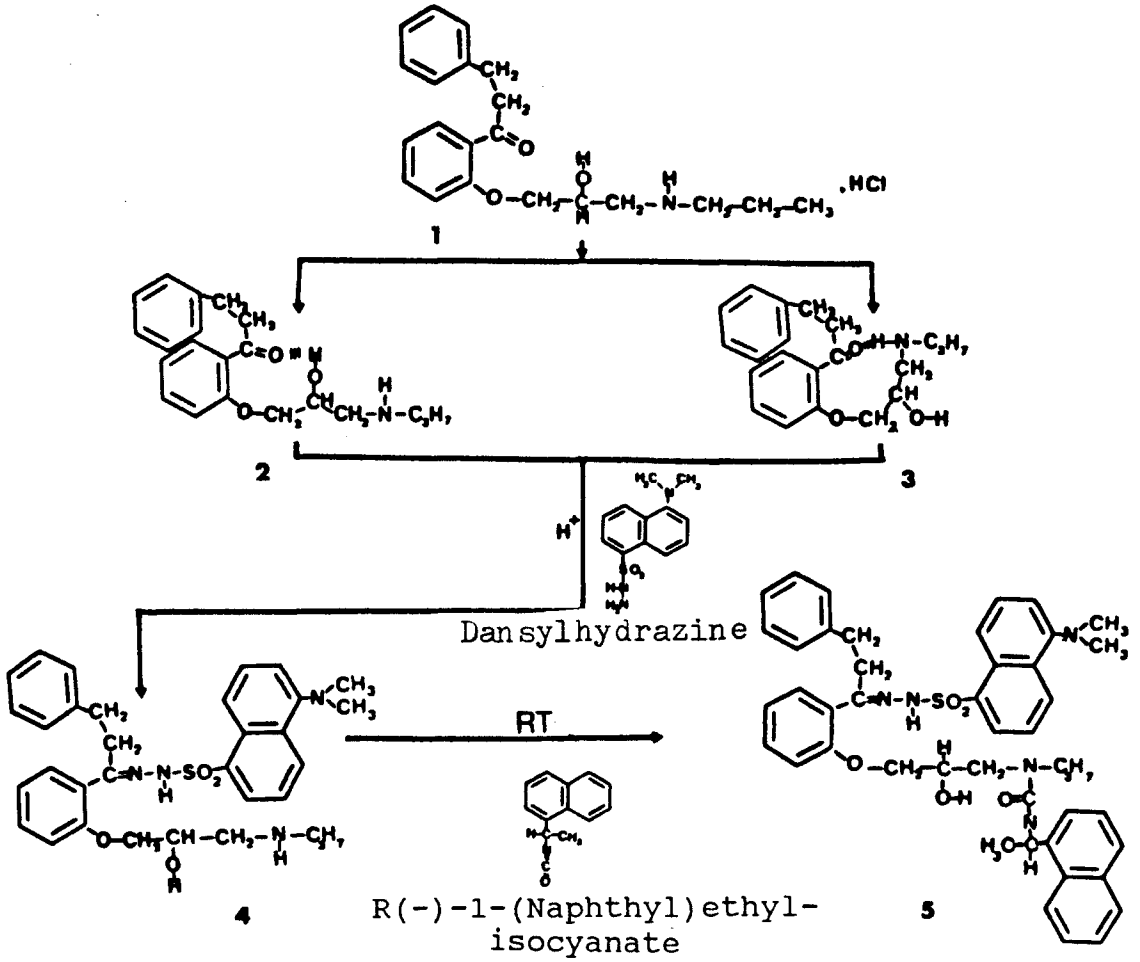
:: ELUTING SOLVENT: 76,24-METHANOL/PHOS.BUFFER(.2M),pH=2.8

Figure 15. Single vs. Dual Derivatization of Propafenone.

4.9 Dual Derivatization of Propafenone and HPLC Separation of Enantiomers

To overcome the effect of intramolecular hydrogen bonding between the aryl carbonyl group and the secondary amino hydrogen and to provide the necessary enantiomeric resolution, a dual derivatization technique was adopted (Scheme 3). The aryl carbonyl group was reacted with dansyl hydrazine in an acidic medium and the resulting dansyl hydrazone was further derivatized with a chiral isocyanate to form the diastereomeric derivatives.

From reverse phase thin-layer chromatographic experiments, it was observed that the derivatization of propafenone with GITC would go to completion if propafenone was pre-reacted with dansyl hydrazine (Fig. 15). Similarly R(-)-NEIC reacted quantitatively with the propafenone-dansylhydrazine derivative to yield the dual derivative which was enantiomerically separated on the ODS column (Fig. 16). The resolution factor (R_S=1.35) was slightly better than that of the propafenone-R(-)-naphthyl urea derivatives (R_S=1.25) presumably because of better solvation by the mobile phase of methanol/water (75:25). Using fluoroscence detection with excitation at 220 nm and emission at 418 nm, the detection limit for each enantiomer was 2.5 ng in plasma. However, there were limitations to this method. The excess fluorescent dansylhydrazine from the reaction mixture could not be efficiently removed using excess acetone to react with the residual dansyl hydrazine. As a result, the fluorescence detector photomultiplier tube was over excited with the excess derivatizing reagent. Moreover, the assay method could not be optimized to the desired precision level required for pharmacokinetic studies.



Scheme 3. Chemical Pathway for Dual Derivatization of Propafenone (2 & 3) from the Diastereomeric Urea Derivatives of Dansyl-Propafenone Hydrazone (5)

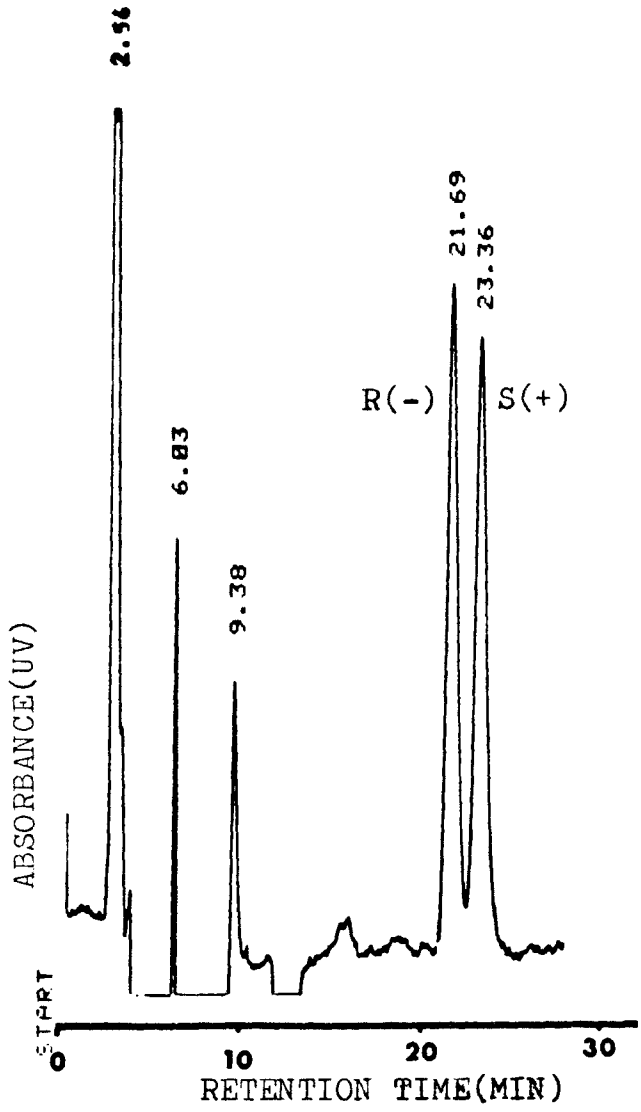


Figure 16. HPLC Separation of Enantiomers of Propafenone After Dual Derivatization. Chromatographic Conditions are given intable 1.

4.10 Kinetics of Derivatization and HPLC Analysis

The most effective derivatizing reagents that led to resolution of the enantiomers of propafenone were R(+)MBIC and GITC and R(-)NEIC Therefore, the optimal derivatization conditions for reaction of the reagents with propafenone were studied.

The peak-height ratio of R,S propafenone derivatized with R(+)MBIC did not significantly change between 30 to 60 minutes of derivatization at room temperature (Fig. 17A). The optimum time for the derivatization of R,S propafenone with GITC was found to be between 20 and 30 minutes (Fig. 17B). Therefore, the reactions were allowed to proceed for 30 minutes at room temperature in further studies.

The R(-)NEIC reagent provided constant peak-height ratios from 30 to 60 minutes at room temperature, but when the temperature was varied, keeping the time constant, a slight increase of peak-height ratio was observed between 60 to 75^{o}C (Fig. 17C and 17D).

Each of the three kinds of derivatives of propafenone formed with R(+)MBIC, GITC and R(-)NEIC were found to be stable for at least 72 hours at room temperature.

4.11 Stoichiometric Ratio of R(-)NEIC Reagent to Propafenone

In establishing the reaction kinetics of propafenone with R(+)MBIC and GITC a large excess of reagents were employed. The excess reagent, when destroyed with water resulted in smaller reagent peaks which did not interfere with the diastereomer peaks of the propafenone enantiomers. R(-)NEIC, on the contrary, being relatively more

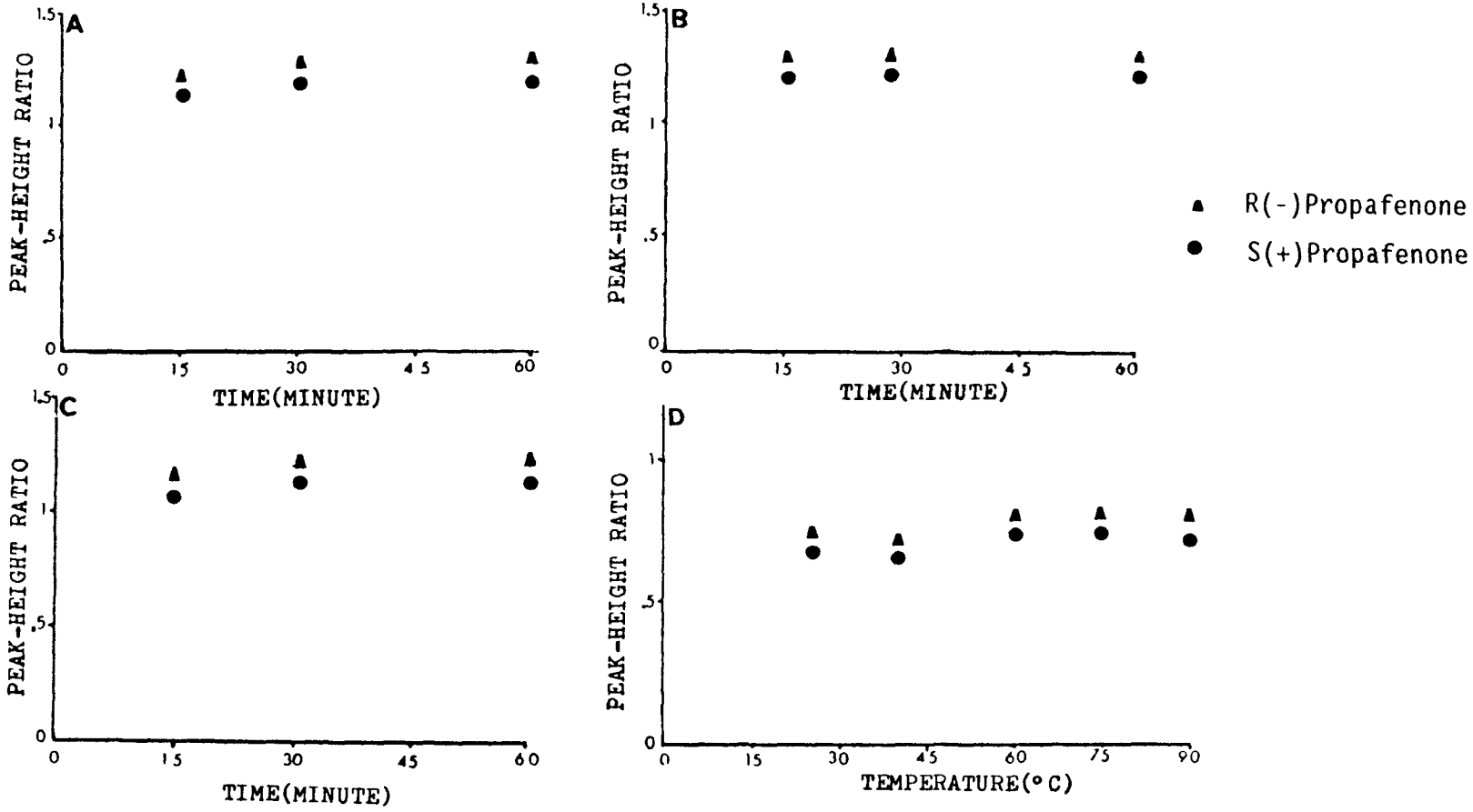


Figure 17. Time and Temperature Dependence for Derivative Formation.
A) At room temperature treated with R(+)MBIC.
B) At room temperature treated with GITC.
C) At room temperature treated with R(-)NEIC.
D) At varying temperature for 30 minutes treated with R(-)NEIC.

lipophilic exhibited a broad peak due to the excess reagent. In order to optimise the stoichiometric ratio of R(-)NEIC reagent and propafenone, the same amount of R,S propafenone was derivatized with 1.8, 4.8, 9, 45 and 90 molar excess of the reagent (Fig. 18). At concentrations of R(-)NEIC beyond a 45 molar excess, propafenone enantiomeric peaks were masked by the excess reagent peak. The peak height ratios did not significantly change above a 1.8 molar excess of reagent. For convenience of having a clean and uninterferred chromatogram of propafenone enantiomers, a 2 to 20 molar excess of reagents was used for the analytical assay.

4.12 Recovery of Propafenone Enantiomers from Plasma

Propafenone is highly protein bound. The initial recovery of total drug from alkalinized plasma (pH >12) was lower than compared to that of water. Plasma protein precipitation with 10% trichloroacetic acid prior to basification and extraction of the drug into benzene (6 mL) substantially increased the recovery of each enantiomer. The efficiency of recovery for each enantiomer as its R(-) NEIC derivative was calculated from the calibration curve of corresponding pure free base enantiomers derivatized similarly. For the measurement of peak-height ratio for each enantiomer α-bromonaphthalene was employed as an external standard. The recovery was 77.7% for R(-) propafenone and 78.2% for S(+) propafenone for concentrations of 500 ng and 1000 ng/mL (Table 2). Similar recovery values (78%) of racemic propafenone have been reported by Harapat et al., 1982, when the drug was extracted into 1% isoamyl alcohol in heptane. More than 90% recovery of racemic propafenone was reported by Chan et al., 1987, when the authors

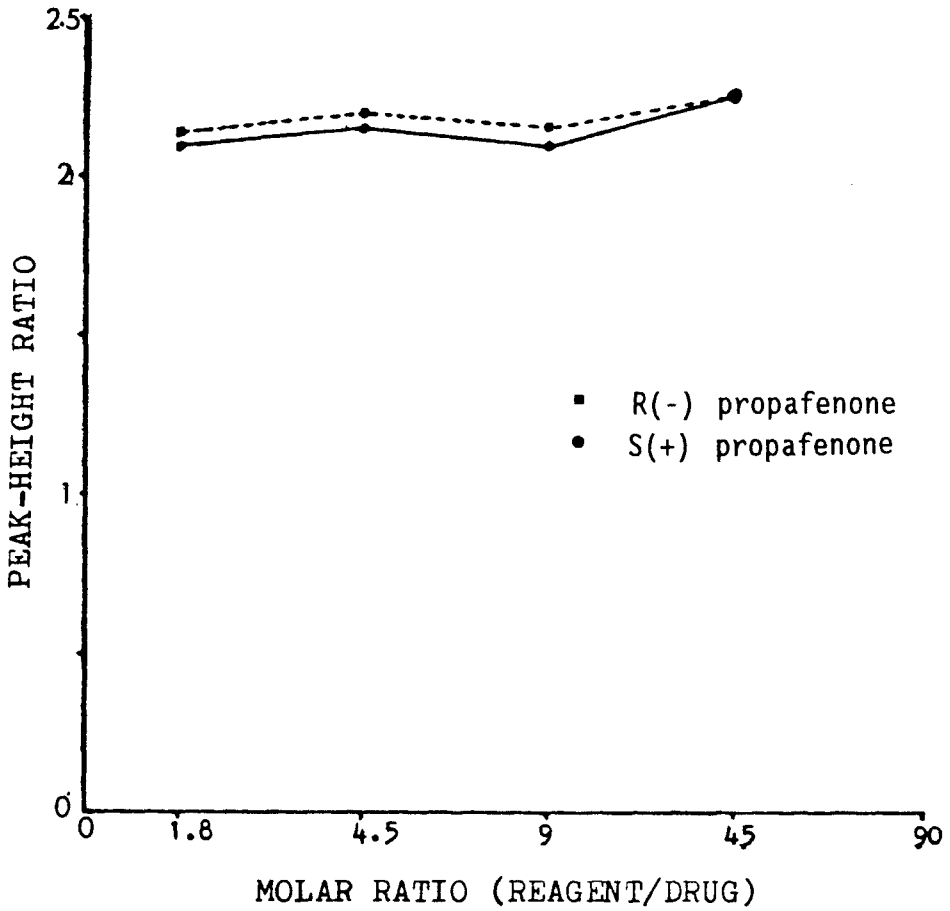


Figure 18. Stoichiometric Ratio of Reagent to Drug [R(-)-1-(Naphthyl)ethylisocyanate/Propafenone].

calculated the recovery of the racemic propafenone from the standard curve of propafenone hydrochloride reacted directly with heptafluorobutyric anhydride without extraction of the free base.

4.13 Linearity and Reproducibility of Assay

Among the stereoselective assay methods for propafenone developed, that using R(-)NEIC as the chiral derivatizing reagent (Scheme 4) was found to be the most sensitive. The linearity and precision of the assay were determined by analyzing plasma samples, which had been spiked with each enantiomer over the concentration range of 150 to 1000 ng/mL. The best fit through the data points for each enantiomer was obtained from linear regression analysis (Table 3). The coefficient of determinations, r^2, were 0.9988 and 0.9960 for all of the data points for the R(-) and S(+) propafenone respectively.

For inter-assay variability, the percent coefficient of variation, which is a measure of reproducibility in sample analysis, was within 5% above 250 ng of each enantiomer. Below this quantity the percent coefficient of variability increased to 8.6% for the R(-) isomer and 14.8% for the S(+) isomer of propafenone, indicating that at lower nanogram levels, the assay was not sufficiently reproducible. The intra-assay variability determined by triplicate injections of each of the samples was within 5% except for one which was 10.4% (Table 4).

ANALYTICAL PROCEDURE

PROPAFENONE.HCl (250-2000ng) IN PLASMA (1mL)

▼

ADD DESIPRAMINE.HCl/Li1115(I.S.), 10% TCA (0.4mL) SHAKEN
ADD NaOH TO pH 12 AND BENZENE (6 mL)

▼ Tumbled for 20 min. followed by centrifuge for 10 min. at 2500 r.p.m.

UPPER ORGANIC LAYER IS TRANSFERRED TO DRY TUBE AND
ADD 1gm OF ANHYDROUS SODIUM SULPHATE

▼ Tumbled for 5 min.; centrifuged and the liquid is transferred to another dry tube, and evaporated to dryness.

ADD 100uL OF 0.01% v/v R(-)NEIC IN DRY ACETONITRILE
PLUS 400uL OF DRY ACETONITRILE

▼

HEATED AT 75^{o}C FOR 30 MINUTES

▼

COOLED, ADD 1uL OF PIPERIDINE, HEATED FOR 20 min. AT 75^{o}C

▼

COOLED, EVAPORATED TO DRYNESS AND
RECONSTITUTED WITH 200uL OF IPA

▼

INJECTED ONTO THE HPLC COLUMN

Scheme 4. Assay Procedure for Propafenone Enantiomers by HPLC.

Table 2. Efficiency of Recovery of Propafenone Enantiomers from Plasma.

Quantity of enantiomer (ng)	Peak-Height Ratios▲ R(-) PPF unextracted	R(-) PPF extracted	Recovery (%)	Peak-Height Ratios▲ S(+) PPF unextracted	S(-) PPF△ extracted	Recovery (%)
500	0.436±0.04	0.337±0.03	77.13±1	0.426±0.34	0.334±0.29	77.34±0.5
1000	0.979±0.04	0.768±0.03	78.45±0.52	0.927±0.36	0.733±0.02	79.05±2.1

▲ mean ± standard deviation, n=3

△ PPF : Propafenone

Table 3. Calibration Curve Data and Inter-Assay Variability of Propafenone Enantiomers in Plasma.

Weight of each enantiomer(ng)	Peak-Height Ratio R(-) propafenone/ internal standard ▲	C.V. ▲▲ (%)	Peak-Height Ratio S(+) propafenone/ internal standard ▲	C.V. ▲▲ (%)
125	0.419±0.036	8.6	0.342±0.051	14.8
250	0.738±0.036	4.9	0.723±0.297	4.1
500	1.413±0.026	1.9	1.353± 0.049	3.6
1000	2.548±0.067	2.6	2.427± 0.051	2.1
Slope	1.2154		1.1775	
Intercept	0.1381		0.1076	
r^2	0.9988		0.9960	

▲ mean± standard deviation; ▲▲ coefficient of variability

Table 4. Intra-Assay Variability of Propafenone Enantiomers in Plasma

Weight of each enantiomer(ng)	Peak-Height Ratio R(-) propafenone/ internal standard▲	C.V.▲▲ (%)	Peak-Height Ratio S(+) propafenone/ internal standard▲	C.V.▲▲ (%)
125	0.412±0.021	5	0.280±0.029	10.4
250	0.789±0.038	4.8	0.763±0.030	3.9
500	1.401±0.031	2.2	1.360±0.049	3.5
1000	2.643±0.053	2.0	2.500±0.052	2.1

▲ mean± standard deviation; number of determinants,n=3

▲▲coefficient of variability

5. SUMMARY AND CONCLUSIONS

Stereoselective high-performance liquid chromatographic assay methods were developed for the resolution of the enantiomers of propafenone in plasma.

Reaction of the enantiomers contained in racemic propafenone with chiral derivatizing reagents to produce diastereomers lead to differing degrees of resolution of the derivatives and differing sensitivities for detection.

R(+)-α-methylbenzylisocyanate [R(+)MBIC] reacted with the enantiomers of propafenone and facilitated the resolution of the diastereomers on an ODS HPLC column (5 u, 25 x 4.5 cm i.d.) uing a mobile phase of methanol water (76:24) and ultraviolet detection. The resolution achieved for the diastereores was R_S=1.15 and the minimum detection limit was 500 ng of each enantiomer at the detector. The quantitative calibration curves were linear over the range 500 - 2000 ng at the detector, however, below this range the assay variation was unacceptably large.

The 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl-isothiocyanate [GITC] diastereomers formed with the propafenone enantiomers provided improved resolution (R_S=1.40) and improved sensitivity of detection down to 150 ng of each enantiomer at the detector. In a similar fashion to the quantitation of the diastereomers of propafenone formed with MBIC, quantitation was unacceptably variable below 250 ng at the detector.

Reaction of propafenone enantiomers with R(-)-1-(naphthyl)ethylisocyanate [R(-)NEIC] produced diastereomers which were effectively resolved on the ODS column with a resolution of R_S=1.25 and

improved sensitivity of detection down to 100 ng of each enantiomer at the detector. The calibration curve was determined in plasma over the concentration range 125-1000 ng/mL and was linear. The coefficient of variability was within 5% for the linear range of 250-1000 ng/mL, however, below 250 ng/mL the coefficient of variability increased to 8.6% and 14.8% for the R and S enantiomers, respectively.

Reaction of propafenone with dansyl hydrazine, followed by diastereomer formation with R(-)NEIC facilitated resolution of the diastereomers (R_S=1.35) and a minimum detectible limit of 2.5 ng/mL in plasma. While promising, this procedure was found to be unreliable below 100 ng/mL in plasma.

The presence of conformational isomers in propafenone was established to be responsible for the lack of reactivity of the parent drug and the chiral reagents. The intramolecular hydrogen bonding of the carbonyl group with either the secondary hydroxyl group or the secondary amino group was shown to be responsible for the presence, in solution, of two conformational arrangements. One of the conformers, however, reacted with the chiral reagents and led to the formation of three conformatoionally different diastereomeric pairs, of which one diastereomeric pair remained enantiomerically unresolved by reverse phase HPLC.

6. REFERENCES

Alebic-Kolbah, T., Rendic, S., Fuks, Z., Sunjic, V. and Kajfez, F., Enantioselectivity in the binding of drugs to serum proteins. Acta Pharm. Jugoslav., 29, 53-70, (1979)

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, 244-48, (1984).

Ames, M.A., Melmon, K.L., and Castagnoli, N. Jr., Stereochemical course in vivo of α-methyldopa decarboxylation in rat brains. Biochem Pharmacol., 26, 1757-62, (1977)

Ariens, E.J., Soudijin,W. and Timmermans,P.B.(Eds): Stereochemistry and biological activities of drugs. Backwell Sci. Publication, Oxford, London (1983).

Ariens, E.J., Chirality in bioactive agents & its pitfalls. TIPS. May 200-205, (1986)

Arnold, R.G., Nelson, J.A., and Verbane, J.J., Recent advances in isocyanate chemistry. Chem. Rev. 57, 49-50, (1957)

Armstrong, D.W., Chiral stationery phases for HPLC separation of enantiomers - a mini review. J. Liq. Chromatogr., 7(S-2) 353-370, (1984)

Asshauer, F. and Vorpahl, U. Results of a double-blind comparison of propafenone with a standard (Ger) Drug Dev. Eval. 1, 135-39, (1977)

Axelson, J.E., Chan, G.L.Y., Kristen, E.B., Mason, W.D., Lanman, R.C., and Kerr, C.R. Food increases the bioavailability of propafenone. Br. J. Clin. Pharmacol., 23, 735-41, (1987)

Bachour, G. & Hochrein, H. Haemodynamic responses to propafenone (Ger). Drug Dev. Eval. 1, 39-44 (1977)

Beck, O.A. & Holchrein, H., Combined use of propafenone and Lidocaine in chronic atrial fibrillation and flutter. Dtsch. Med. Wochenschr., 105, 1243-46, (1980)

Beck, O.A., Caren, J., Hochrein, H., Propafenone and propranolol in the management of cardiac extrasystoles (Ger). Dtsch. Med. Wschr., 107, 579-83, (1982)

Belanger, O.G., Turgeon, J., Gilbert, M., Stereoselective disposition of mexiletine in man. Br. J. Clin. Pharmacol. 21, 481-487, (1986)

Belanger, O.G., Turgeon, J., HPLC assay for mexiletine enantiomers in human plasma. J. Chromatogr., 337, 172-177, (1985)

Belz, C.G., Matthews, J., Sci, M., Doering, M.D. & Belz, G. Digoxin-antiarrhythmias, pharmacodynamics & pharmacokinetic studies with quinidine, propafenone and verapamil. Clin. Pharm. Ther., 31, 202-3, (1982)

Blaschke, G. and Walther, B. Racemattrennung von. propafenon and diprafenon, konfiguration der propafenon-enantiomeren. Leibigs Ann. Chem., 561-563, (1987)

Breithardt, G., Jochum, E., Kuhn, H., Seipel, L. The effect of some antiarrhythmic drugs on systolic time intervals in normal subjects (Ger). Z. Kardiol., 67:680-87, (1978)

Borggree, M., Wiebringhaus, E. and Seipel, L. Effect of propafenone in the wolf-parkinson-white syndrome. Electrophysiologic findings and longterm follow up. Am. J. Cardiol., 54, 29D-49D, (1984)

Briody, J.M., Narinesingh, D. Bifunctional catalysis by amides and ureas in the reaction of amines with phenylisocyanate. Tetrahedron letters., 40, 4143-46, (1971)

Brode, E., Kripp, U. and Hollmann, M. Simultaneous determination of Propafenone and 5-hydroxy propafenone in plasma by means of HPLC. Drug Res., 34(11), 1455-60, (1984)

Brode, V.E., Sache, R and Hoffmann, H.D. Studies on the analysis of propafenone by means of internal analog standardization. Drug Res., 32(1), (1982)

Connolly, S.J., Kates, R.E., Lebsack, C.S., Harrison, D.C. and Winkle, R.A. Clinical pharmacology of propafenone. Circulation, 68, no. 3, 589-96, (1983)

Connolly, S., Lebsack, C., Winkle, R.A., Harrison, D.C. and Kates, R.E. Propafenone disposition kinetics in cardiac arrhythmia. Clin. Pharm. Ther., 163-168, (1984)

Corbin, J.A. and Rogers, L.B. Improved gas chromatographic separation of enantiomeric secondary amine derivatives. Anal. Chem. 42, 974-79, (1970)

Coumel, P., Leclerca, J.F., Assayag, P. European experience with the antiarrhythmic efficacy of propafenone for supraventricular and ventricular arrhythmias. Am. J. Cardiol., 54, 60D-66D, (1984)

Dabrowiak, J.C., & Cook, D.W. GLC of the optical isomers of threonine and allothreonine. Anal. Chem., 43, 791, (1971)

Dalgliesh, C.E. The optical resolution of aromatic amino-acids on paper chromatography. J. Am. Chem. Soc., 3940-42, June (1952)

Davenkov, W., Bockov, A.S., Kuraganov, A.A., Unger, K.K. and Roumeliotis, P. Advances in chromatography. 18, 139 (1980) And the references therein.

Dayer, P., Balant, L., Kupfer, A., Striberni, R. and Leeman, T. Effect of oxidative polymorphism (debrisoquine/sparteine type) on hepatic first-pass metabolism of bufuralol. Eur. J. Clin. Pharmacol. 28, 317-320, (1985)

De Aguirre, I. and Jungers, J.C. No 191 Chimie de l'uree. Bull. Soc. Chim. France, 1316-27, (1965)

De Brito,F.S.,Pereira,M.H., Castro,I., Mala,I.G. Propafenone in ventricular arrhythmia caused by ischemic cardiopathy. Quantitative Study using the holter system. Arq. Bras. Cardiol. 33, 303-10, (1979).

Durante, G., Del Forno, P., Vergara, G., Frisanco, L., Furlanello, F. Utilization of propafenone for the prophylaxis of ventricular hyperkinetic arrhythmias in mitral valve prolapse syndrome. Fifth international seminar on cardiology, Abstract, 98-99, (1980)

Eichelbaum, M., Mikus, G. and Vogelgesany, B. Pharmacokinetics of (+), (-) and (+) verapamil after i.v. administration. British J. of Clin. Pharmacol. 17, 453-58, (1984)

Feibush, B. Interaction between asymmetric solutes and solvents. L-lauroyl-L-valyl-t-butylamide as stationary phase in gas-liquid partition chromatography. Chem. Commun., 544, (1971)

Fill, W.D., Rulhn, P., Probst, P. and Zilcher, H. Use of propafenone in man: Clinical results on the effect on electrophysiological and hemodynamic parameters. Drug Dev. Eval., 1:71-99, (1977)

Finar, I.L., Organic chemistry (volume two) ELBS ed.,pp 8-9 and 609, (1969)

Findlay, J.W.A., Warren, J.T., Hill, J.A. and Welch, R.M. Stereospecific radio-immunoassays for d-pseudoephedrine in human plasma and their application to bioequivalency studies. J. Pharm. Sci., 70, 624-30 (1981)

Frank, H., Nicholson, G.J. and Bayer, E. Spectrometric analysis of optically active metabolites and drugs on a novel chiral stationary phase. J. Chromatogr., 146, 197-206, (1978)

Freudenberg (1933) quoted from Finar, I.L., Organic chemistry 9volume two) ELBS ed. pp9 and 609, (1969).

Gal, J., Vito, D. and Harper, T.W. Gas Chromatographic resolution of enantiomeric secondary alcohol. Drug Metabol. Dispos., 9(6),557,(1981)

George, C.F., Fenjvesi, T., Connolly, M.E. and Dollery, C.T. Pharmacokinetics of dextro, levo and racemic propranolol in man. Eur. J. Clin. Pharmacol., 4,76-6, (1972)

Gil-Av, E., Feibush, B. and Sigler, R.C. Separation of enantiomer by GLC with an optically active stationary phase. Tetrahedron letters., 1009 (1966). And references therein

Gillis, A.M. and Kates, R.E. Influence of protein-binding on the myocardial uptake and pharmacodynamics of propafenone. J. Cardiovasc. pharmacol., 8,1163-67, (1986)

Gillis, A.M., Yee, Y.G. and Kates, R.E. Binding of antiarrhythmic drugs to purified human α_1-glycoprotein. Biomed. Pharmacol., 34,4279-82, (1985)

Gordis, E. Gas chromatographic resolution of optical isomers in microgram samples of amphetamine. Biochem., Pharmacol. 15, 2124, (1966)

Gray, J.A., Lullmama, H., Mitchelson, F. and Rert, G.H. Stereoselective binding in cardiac tissue of the enantiomers of benzetimide, an antiarrhythmic drug. Br. J. Pharmacol., 56, 486-90, (1976)

Gross, S.J. & Soares, J.R. Immunoassays for drugs subject to abuse. S.J. Mule, I. Sunshine Eds. CRC Press. Cleveland, Ohio, 3-11, (1974)

Gubitz, G. and Mihellyes, S. Optical resolution of β-blocking agents by thin-layer chromatography and high-performance liquid chromatography as diastereomeric R(-)-1-(1-naphthyl)ethylureas. J. Chromatogr. 314, 462-66, (1984)

Harapat, S.R., Kates, R.E. High-performance liquid chromatographic analysis of propafenone in human plasma samples. J. Chromatogr., 230, 448-53, (1982)

Hege, H.G., Hollmann, M., Kaumeier, S. and Lietz, H. The metabolic fate of ^{2}H-labelled propafenone in man. Europ. J. Drug Metabol. & Pharm., 9(1) 41-55, (1984)

Hege, H.G., Hietz, H. and Weymann, J. Studies on the metabolism of propafenone (2nd common.), Drug. Res., 34(II), 972-979, (1984)

Heger, J.J., Hubbard, J., Zipes, D.P., Miles, W.M. & Prystowsky, E.N. Propafenone treatment of recurrent ventricular tachycardia: Comparison of continuous electricardiographic recording and electrophysiologic study in predicting drug efficacy. Am. J. Cardiol., 54, 40D-44D, (1984)

Helmchen, G. and Stubent, W. Determination of optical purity by high-performance liquid chromatography. Chromatographia, 7(12), 713-15, (1974)

Hendel, J. and Brodthagen, H. Enterohepatic cycling of methotrexate estimated by the use of D-isomer as a reference marker. Eur. J. Clin. Pharmacol., 26:103-7, (1984)

Hermansson, J. Determination of R and S alprenolol and (R) and S metoprolol as their diastereomeric derivatives in human plasma by reversed phase liquid chromatography. J. Chromatogr., 227,113-127, (1982)

Higuchi, S., Urano, C. & Kawamura, S. Determination of plasma protein-binding of propafenone in rats, dogs and humans by high sensitive GCMS. J. Chromatogr., 341, 305-311, (1985)

Hodges, M., Salerno, D., Granrud, G. Double blind Placebo-Controlled evaluation of propafenone in suppressing ventricular ectopic activity. Am. J. Cardiol., 54, 45D-50D, (1984)

Hollmann, M., Brode, E., Holtz, D., Kaumeier, S. and Kehrhahn, O.H. Investigations on the pharmacokinetics of propafenone in man. Drug Res., 33(1):763-770, (1983)

Hollmann, M., Hege, H.G., Brode, E., Buhler, V., Hotz, D., Kaumeier, S., Kehrhahn, O.H., Lietz, H., Schwary, J.A., Steieren, B. & Weymann, J. Pharmacokinetics and metabolic studies of propafenone in volunteers. Schlepper, M., and Olson, B. eds. Cardiac Arrhythmia. Proceedings of the first international rytmonorm congress. N.Y., Springer-Verlag, 125-132, (1982)

Kaiser, D.G., Vangiessen, G.J., Reischer, R.J. and Wechter, W.J. Isomeric inversion of ibuprofen R enantiomer in humans. F. Pharm. Sci., 65, 269-73, (1976)

Kates, R.E., Yee, Y.G. & Winkle, R.A. Metabolic cumulation during chronic propafenone dosing in arrhythmia. Clin. Pharmacol. Ther., 37, 610-614, (1985)

Kawashima, K., Levi, A. & Spector, S. Stereospecific radioimmunoassay for propranolol isomers. J. Pharmacology and Experimental Ther., 196, 517-523, (1976)

Kawashima, K. & Ishikawa, H. Pharmacokinetics of propranolol isomers and their relationships with beta adrenoceptor blocking activity in rabbits administered with dl Propranolol. J. Pharmacol. Exp. Ther., 213, 628-31, (1980)

Kaye, C.M. A simple spectrofluorometric method for measurement of I.C.I. 66082 in plasma and urine. Br. J. Clin. Pharmacol. 1, 84-85, (1974)

Keberle, H., Hoffman, K. & Bernhardt, K. The metabolism of glutethimide. Experimentia, 18, 105-11, (1962)

Keller, K., Meyer-Estorff, G., Beck, O.A., Hochrein, H. Correlation between serum concentration and pharmacological effect on atrioventricular conduction time of antiarrhythmic drug propafenone. Eur. J. Clin. Pharmacol., 13,17-20, (1978)

Kennedy, K.A. and Fischer, L.J. Quantitative and stereochemical aspects of glutethimide metabolism in humans. Drug Metabol. Dispos., 7, 319-24, (1979)

Klempt, H.W., Nayebagha, A. and Fabry, E. Antiarrhythmic effect of mexiletine, propafenone, flecainide with ventricular extra systoles. Zeit fur Kardiol, 71, 340-49, (1982)

Kohlhardt, M. Der EinfluB von propafenon auf der transmembranen Na+ and Ca++ strom der warmblutler blyokardfaser membrana. In: Hochrein, H., Kapke, H.J. and Beck, O.A. (eds.). Fortschritte in der pharmakotherapic Von Herzrhythmusstorunger. Fischer, Stuttgart, 35-38, (1977)

Kohlhardt, M. Block of sodium current by antiarrhythmic agents: Analysis of the electrophysiological effects of propafenone in heart muscle. Am. J. Cardiol., 54, 13D-19D, (1984)

Konig, W.A., Stoelting, K. and Kruse, K. GC separation of optically active compounds in glass capillaries. Chromatographia., 10, 444, (1977)

Konig, W.A., Frank, W. and Benecke, I. Gas chromatographic separation of enantiomers of chiral alcohols. J. Chromatogr., 239,227, (1982)

Koreeda, M., Weiss, G. and Nakanishi, K. Absolute configuration of natural (+) abscisic acid. J. Am. Chem. Soc., 95, 239-40, (1973)

Kotake, M., Sakan, J., Nakamura, N. and Senoh, S. Resolution into optical isomers of some amino acids by paper chromatography. J. Am. Chem. Soc., 73, 2973, (1951)

Kretzschman, R., Gries, J., Hoffmann, H.P., Muller, D., Von-Philipsborn, G., Raschack, M. and Teschendorf, H.J. Pharmacological studies with 5-OH-propafenone on active metabolite of propafenone. Archives Pharmacol., 324, R38, (1983)

Kretzschman, R., Hoffmann, H.P., Von Philipsborn, G., Teschendorf, H.J. II. World conference of clinical pharmacology and therapeutics. Washington, D.C. (July 31-Aug. 9), (1983a)

Kripalani,K.J., Addin,Z.E., Dean,A.V. and Shreiber,E.C. Stereospecific inversion of 1-α-methylfluorine-2-acetic acid to its d-enantiomer in the dog. Xenobiotica, 6, 159-63, (1976)

Kuhn, L.P., Schleyer, P.R., Bactinger, W.F., Eberson, L. Conformational effects and hydrogen bonding in 1,4-diols. Am. Chem. Soc., 86, 650-58, (1964)

Kupfer, A., Bircher, J. Stereoselectivity of differential routes of drug metabolism: The fate of enantiomers of 14C mephenytoin in dog. J. Pharmacol. Exp. Ther., 209, 190-5, (1979)

Latimer, W.M. and Rodebush, W.H. A definitive discussion of the H-bond. J. Am. Chem. Soc. 42, 1419-33, (1920)

Ledain, L., Sourdille, N., Colle, J.P., Ohayon, J. and Besse, P. Treatment of severe ventricular arrhythmias with propafenone. First rytmonorm Congress, 231-236, Springer-Verlag eds., (1982)

Ledda, F., Mantelli, L., Manzini, S., Amerini, S. and Mugelli, A. Electrophysiological and antiarrhythmic properties of propafenone in isolated cardiac preparation. J. Cardiovasc. Pharmacol., 3, 1162-73, (1981)

Lemmes, L. and Bathe, K. Stereospecific and circadian-phase-dependent kinetic behavior of d,l-, l- and d-propranolol in plasma, heart and brain of light-dark synchronized rats. J. Cardiovasc. Pharmacol., 4, 635-44, (1982)

Lennard, M.S., Tucker, G.T., Silas, J.H., Freestone, S., Ramsey, L.E. and Woods, H.F. Differential stereoselective metabolism of metoprolol in extensive and poor debrisoquine metabolizers. Clin. Pharmacol. Ther., 34, 732-37, (1983)

Lewis, R.J., Trager, W.F., Chan, K.K., Breckenridge, A., L'orma, M., Rowland, M. and Schary, W. Warfarin: Stereochemical aspects of its metabolism and the interaction with phenylbutazone. J. Clin. Invest., 53, 1607-17, (1974)

Lima, J.L., Jungbluth, G.L., Devine, T. and Robertson, L.W. Stereoselective binding of disopyramide to human plasma protein. Life Sci. 35, 835-38, (1984)

Linder, W. and Leitner, C. Liquid chromatographic separation of enantiomeric alkanolamines via diastereomeric tartaric acid monoesters. J. Chromatogr., 316, 605-616, (1984)

Liu, J.H., Ku, W.W., Tsay, J.T., Fitzgerald, H.P. and Kim. S. Approaches to drug sample differentiation. III. A comparative study of the use of chiral and achiral capillary column GCMS for the determination of Methamphetamine enantiomers and possible impurities. J. Forensic Sci., 27, 39, (1982)

Liu, J.H. and Ku, W.W. Chiral stationary phases for the GLC separation of enantiomers. J. Chromatogr., 271, 309-323, (1983)

Lochmuller, C.H., Souter, R.W. Direct GC resolution of enantiomers on optically active mesophases (II). J. Chromatogr., 88, 41-54, (1974)

Marchensini, B. and Boschi, S. Identification of metabolite of propafenone in human urine by means of HPLC and GC-MS. J. Chromatogr., 278, 173-178, (1983)

McErlane, K.M. and Pillai, G.K. Gas liquid chromatographic resolution and assay of tocainide enantiomers using chiral capillary column and study of their selective disposition in man. J. Chromatogr., 274, 129-138, (1983)

McErlane, K.M., Igwemezie, L. Stereoselctive analysis of the enantiomers of mexiletine by HPLC using fluorescent detection and study of their stereoselctive disposition in man. J. Chromatogr., 415, 335-48, (1987)

McMenamy, R.H. and Oncley, J.L. The specific binding of L-tryptophan to serum albumin. J. Biol. Chem., 233, 1436-47, (1958)

Meyer-Estorff, G., Keller, K., Beck, O.A., Cochrein, H. Antiarrhythmic effect of propafenone in relation to serum concentration and conduction time (Ger). Z. Kardiol., 67, 352-56, (1978)

Midha, K.K., Hubbard, J.W., Cooper, J.K. and Mackonka, C. Stereospecific radioimmunoassays for l-ephedrine and d-ephedrine in human plasma. J. Pharm. Sci., 72, 736-39, (1983)

Miller, L.G. and Greenblatt, D.J. Determination of albuterol in human plasma by HPLC with fluorescence detection. J. Chromatogr., 381, 205-208, (1986)

Mieller-Peltzer, H., Greger, G., Neugebauer, N., and Hollmann, M. Beta-blocking and electrophysiological effects of propafenone in volunteers. Eur. J. Clin. Pharmacol., 25, 831-33, (1983)

Muller, W.E. and Wollert, U. High stereospecificity of the benzodiazepine binding sites on human serum albumin. Mol. Pharmacol. 11, 52-57, (1975)

Murphy, P.J., Nickandes, R.C., Bellamy, G.M. and Kurtz, W.L. Effects of l-propoxyphene on plasma levels and analgesic activity of d-propoxyphene in the rat. J. Pharmacol. Exp. Ther., 199, 415-22, (1976)

Murthy, A.S.N. and Rao, C.N.R. Spectroscopic studies of the hydrogen bond. Applied Spectr. Rev., 2(1), 69-191, (1968)

Neumann, B. Distribution: Plasma-proteins binding of propafenone in man. BASF Pharm. Div. Report no. VP/FBB-78041, March, (1978)

Ibid., Distribution: Plasma protein binding of propafenone in dog and rat. BASF Pharm. Div. Report no. 20. VP/FBB-7911, June, (1979)

Nimura, N., Ogura, H., Kinoshita, T. Reverse phase liquid chromatographic resolution of amino acid enantiomers by derivatization with 2,3,4,6 tetra-O-acetyl-β-D-glucopyranosylisothiocyanate. J. Chromatogr., 202, 375-379, (1980)

Parr, W. and Howard, P.Y. Molecular interactions in a unique solvent-solute system. J. Chromatogr., 71, 193-201, (1971)

Palma, J.L., Bayes de Luna, A., Fiol, M., Yuste, P., Aza, V., Sadwine, J., Y Cajal, C.E.R., Dureta, S.R. and Rallo, H.S. Antiarrhythmic effect and study of segmentary contractility following the administration of propafenone in coronary patients with ventricular arrhythmias. Proceedings of the first rytmonorm congress, Springer-Verlag, N.Y., 199-210, (1982)

Patil, P.N., Patel, D.G. and Drell, R.D. Steric aspects of adrenergic drugs. XV. Use of isomeric activity ratio as a criterion to differentiate adrenergic receptors. J. Pharmacol. Exp. Ther., 176, 622, (1971)

Patil, N., LaPidus, J.B. and tye,A. Steric aspects of adrenergic drugs. J. Pharm. Sci., 59:1025-30, (1970)

Pettersson, C. and Schill, G. Separation of enantiomeric amines by ion-pair chromatography. J. Chromatogr., 204, 179-183, (1981)

Pettersson, C. Chromatographic separation of enantiomers of acids with quinine as chiral counter ion. J. Chromatogr., 316, 553-65, (1984)

Pimentel, G.C. and McClellan, A.L. The hydrogen bond. W.H. Freeman and Co. San Fransisco and London, (1960)

Pirkle, W.H. and House, D.W. Chiral HPLC stationary phases. 1. Separation of the enantiomers of sulfoxides, amines, amino acids, alcohols, hydroxyacids, lactones and mercaptans. J. Org. Chem., 44, 1957-79, (1979)

Podrid, P.J., Cytryn, R., Lown, B. Propafenone: noninvasive evaluation of efficacy. Am. J. Cardiol., 54, 53D-59D, (1984)

Powel, M.L., Wagoner, R.R., Chen, C.H., Nelson, W.L. Aromatic hydroxylation of propranolol. Stereochemical aspects of the formation of 4′-hydroxy propranolol in the rat. Res. Commun. Chem. Path. Pharmacol., 30, 387-400, (1980)

Prachar, H., Jobst, C.H., Kiss, E., Nobis, H., Pollak, H., Spiel, R., Swoboda, O. and Erenkel, W. Propafenone HCl: results of a double-blind study against a reference preparation. Drug Dev. Eval. 1, 140-147, (1977)

Rose, H.C., Stern, R.L. and Karger, L.B. Studies on the mechanism of separation of diastereomeric esters by GLC - Effect of bulk dissymmetry and distance between optical centres. Anal. Chem., 38, 469-72, (1966)

Salerno, D.M., Granrud, G., Sharkey, P., Asinger, R. and Hodges, M. A controlled trial of propafenone for treatment of frequent and repetitive ventricular premature complexes. Am. J. Cardiol., 53, 77-83, (1984)

Satchell, D.P.N. and Satchell, R.S. Acylation by ketens and isocyanates. A mechanistic comparison. Chem. Soc. Rev., 4, 231-50, (1975)

Schmidt, W. and Jahnchen, E. Stereoselective drug distribution and anticoagulant potency of the enantiomers of phenprocumon in rats. J. Pharm. Pharmacol., 29, 266-71, (1977)

Scholz, H. Pharmacology of propafenone. Proceedings of first rytmonorm Congress. Springer-Verlag eds. N.Y. 103-111, (1982)

Sedman, A.J. and Gal, J. Resolution of enantiomers of propranolol and other beta-adrenergic antagonists by high performance liquid chromatography. J. Chromatogr., 278, 199-203, (1983)

Sedman, A.J., Gal, J., Both, A., Wiebringhaus, E. Effects of propafenone on the sinus nodes and intracardiac conduction in man (Ger.). Drug Dev. Eval., 1, 45-53, (1977)

Siepel, L. and Breithardt, G. Propafenone: a new antiarrhythmic drug. Eur. Heart Journal, 1, 309-13, (1980)

Sellers, E.M. and Koch-Weser, J. Interaction of warfarin stereoisomer with human albumin. Pharmacol. Res. Commun., 7:331-335, (1975)

Shindo, H., Komai, T. and Kawai, K. Studies on the metabolism of D- and L-isomers of 3,4-DOPA. Mechanism of intestinal absorption of D- and L-DOPA in rats. Chem. Pharm. Bull., 21(9), 2031-38, (1973)

Siddoway, L.A., Thompson, K.A., McAllister, B., Wang, T., Wilkinson, G.R., Roden, D.M. and Woosley, R.L. Polymorphism of propafenone metabolism and disposition in man. Clinical and pharmacokinetic consequences. Circulation, 75(4), 785-791, (1987)

Siddoway, L.A., Roden, D.M., Raymond, L.W. Clinical pharmacology of propafenone, pharmacokinetics, metabolism and concentration-response relations. Am. J. Cardiol., 54, 9D-12D, (1984)

Siddoway, L.A., McAllister, C.B., Wang, T., Bergstrand, R.H., Roden, D.M., Wilkinson, G.R., Woosley, R.L. Polymorphic oxidative metabolism of propafenone in man. Circulation, 68(111) 64, (1983b)

Silber, B. and Riegelman, S. Stereospecific assay for (-) and (+) propranolol in humans and dog plasma. J. Pharmacol. Exp. Ther., 215, 643, (1980)

Silber, B., Holford, N.H.G. and Riegelman, S. Stereoselective disposition of glucuronidation of propranolol in humans. J. Pharm. Sci., 71(6), 669-703, (1982)

Simonyi, M. On chiral drug action. Med. Res. Rev. 4, 359, (1984)

Smith, D.F.(Ed) CRC handbook of Stereoisomers: Drugs in Psychopharmacology. CRC Press Inc. Boca Raton, Florida , (1984)

Spector, R. and Lorenzo, A.V. Specificity of ascorbic acid transport system of the central nervous system. Am. J. Physiol., 226, 1468-73, (1974)

Steinbach, K., Frohner, K., Meisl, F. and Unger, G. Interaction between propafenone and other drugs. In: Schlepper, M. and Olson, B. eds. Cardiac Arrhythmias, N.Y. Springer Verlag, 141-7, (1983)

Tamura, S., Kuzuna, S., Kawai, K.and Kishimoto, S. Optical isomerization of R(-) clidanac to the biologically active S(+) isomer in guinea pigs. F. Pharm. Pharmacol., 33, 701-6, (1981)

Tocco, D.J., Hooke, K.F., Deluna, F.A. and Ducen, A.E.W. Stereospecific binding of timolol, a beta-adrenergic blocking agent. Drug Metabol. Dispos., 4, 323-29, (1976)

Thompson, J.A., Hull, J.E. and Norris, K.J. 4-hydroxypropranolol. Glucuronidation of propranolol and substrate specificity and stereoselectivity of rat liver microsomal glucuronyltransferases. Drug Metabol. Dispos., 9, 466, (1981)

Thompson, J.A., Holtzman, J.L., Tsuru, M., Lerman, C. and Holtzman, J. Procedure for the chiral derivatizations and chromatographic resolution of R(+) and S(-) propranolol. J. Chromatogr., 238, 470-75, (1982)

Von Bahr, C. Hermansson, J. and Tawara, K. Plasma levels of (+) and (-) propranolol and 4-hydoxy propranolol after administration of racemic (+) propranolol in man. Br. J. Clin. Pharmacol. 14, 79-83, (1982)

Von-Philipsborn, G., Gries, J., Hoffmann, H.P., Kreiskott, H., Kretzschman, R., Muller, C.D., Raschack, M. and Jeschendorf, H.J. Pharmacological studies on propafenone and its main metabolite, 5-hydroxy propafenone. Drug Res., 34(11), 1487-97, (1984)

Wade, D.N., Mearrick, P.T. and Morris, J.L. Active transport of L-dopa in the intestine. Nature, 242, 463-65, (1973)

Waleffe, A., Mary-Rabine, L., de Rijbel, R., Sojeur, D., Legrand, V. and Kulbertus, H.E. Electrophysiological effects of propafenone studied with programmed electrical stimulation of the heart in patients with recurrent PSVT. Europ. Heart J., 2:345-352, (1981)

Walle, T. and Walle, U.K. Stereoselective oral bioavailability of racemic propranolol in the dogs: A GCMS study using stable isotope techniques. Res. Commun. Chem. Pharmacol., 23, 453, (1979)

Weddle, O.H., Amick, E.N. and Mason, W.D. Rapid determination of atenolol in human plasma and urine by high performance liquid chromatography. J. Pharm. Sci., 67, 1033-35, (1978)

Wedlung, P.J., Aslanian, W.S.,Jacqz, E., McAllister, C.B., Branch, R.A. and Wilkinson, G.R. Phenotypic differences in mephenytoin pharmacokinetics in normal subjects. J. Pharmacol. Exp. Ther., 234(3), 662-9, (1985)

Weiss, Y.A., Safar, M.E., Lebner, J.P., Levenson, J.A., Simson, A. and Alexandre, J.M. (±) propranolol clearance, an estimation of hepatic blood flow in man. Br. J. Clin. Pharmacol., 5, 457-60, (1978)

Wester, H.A. and Mooselimis, N. The effect of antiarrhythmic drugs on myocardial function (Ger). Dtsch. Med. wschr. 107, 1262-66, (1982)

Westly, J., Halpern, B. and Krger, B.L. Effect of solute structure on separation of diastereomeric esters and amides by GLC. Anal. Chem., 40, 2046, (1968)

Williams, K. and Lee, E. Importance of drug enantiomers in clinical pharmacology. Drugs, 30, 333-36, (1985)

Wilson, J.E. and Vigneaud, V. Inhibition of the growth of the rat by L penicillamine and its prevention by aminoethanol and related compounds. J. Biol. Chem., 184, 63-70, (1950)

Wright, J., Cho, A.K. and Gal, J. The metabolism of amphetamine in vitro by rabbit liver preparations: A comparison of R(-) and S(+) enantiomer. Xenobiotica, 7, 257-66, (1977)