Comparative metabolism of warfarin and acenocoumarol: stereoselectivity in oxidative and reductive biotransformation routes

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**COMPARATIVE METABOLISM OF WARFARIN AND
ACENOCOUMAROL:
STEREOSELECTIVITY IN OXIDATIVE AND REDUCTIVE
BIOTRANSFORMATION ROUTES**

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Comparative metabolism of warfarin and acenocoumarol: stereoselectivity in oxidative and reductive biotransformation routes

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CHAPTER 1:

GENERAL INTRODUCTION

1.1 STEREOCHEMICAL TERMS:

Three-dimensional structures, such as molecules, can exist as each others mirror images without being superimposable on each other. The property of an object or a molecule to have a non-identical mirror image is termed **chirality** (IUPAC, 1970). The term chirality is similar to 'handedness' and is derived from the Greek $\chi\epsilon\iota\rho$. The phenomenon chirality is evident if two hands are considered: although the left and right hand appear identical, and can be considered each others mirror images, they are not superimposable. This has great implications since other chiral objects such as gloves may fit to one hand but not to the other.

Molecules that are each others mirror image but that are not superimposable are called **enantiomers** (enantios morphe: opposite shape) (IUPAC 1970, Waine 1988). Enantiomeric compounds mostly possess a chiral centre, but sometimes a chiral axis or plane. In most cases, chiral centres are tetravalent carbon atoms (figure 1.1) but amongst others, may also be nitrogen, phosphorus or sulphur. Enantiomers display virtually similar physico-chemical properties such as solubilities, boiling- and melting points. Compounds containing more than one chiral element are called **diastereomers**. Since diastereomers are not necessarily each others mirror image, they may possess quite different physico-chemical properties.

Pure enantiomers or chiral diastereomers possess **optical activity**, i.e. when a beam of plane-polarized light is transmitted through (a solution of) a chiral compound, the plane of polarization will be rotated. Enantiomers will rotate the polarization plane in the opposite direction than their enantiomeric counterparts. Optical activity is annotated (+) or d (dextrorotatory) when the polarization plane of the light is rotated to the right (clockwise) and (-) or l (levorotatory) when the polarization plane is rotated to the left (counterclockwise). 1/1 mixtures of the corresponding (+)- and (-)- enantiomers are **racemates** and do not possess optical activity. Optical activity of natural tartrate was first discovered by Jean-Baptiste Biot in 1815 (reviewed by Drayer 1988). However, it was Louis Pasteur in 1848, who related this phenomenon with molecular asymmetry, by comparing crystals of natural and synthetic tartrate (which is not optically active). Finally, Van 't Hoff and Le Bell postulated that this asymmetry was due to the presence of an **asymmetrical carbon atom** (i.e. a carbon atom to which four different groups are attached).

Optical rotation by a compound is not only dependent on its structure (although there is no clear structure-optical activity relationship) but also depends on its concentration, the solvent in which the compound is dissolved and the wavelength of the light with which optical activity is determined (Roberts and Caserio, 1977). Therefore, optical activity gives virtually no information on the three dimensional structure of the enantiomers. In order to obtain insight in the three dimensional structure of enantiomers, and to introduce a clear nomenclature, chiral molecules were originally chemically transformed to an arbitrarily chosen standard (+)-glyceraldehyde, which was assigned the D configuration by Fisher in 1919.

Chapter 5

The in vitro and in vivo pharmacokinetics of drugs are determined by their physicochemical properties. The in vitro pharmacokinetics of drugs are determined by their physicochemical properties. The in vivo pharmacokinetics of drugs are determined by their physicochemical properties.

Three-dimensional structures, such as molecules, can be represented in two-dimensional images. The in vitro pharmacokinetics of drugs are determined by their physicochemical properties. The in vivo pharmacokinetics of drugs are determined by their physicochemical properties. The in vitro pharmacokinetics of drugs are determined by their physicochemical properties. The in vivo pharmacokinetics of drugs are determined by their physicochemical properties.

Figure 1.1: Representation of a chiral molecule. Groups A,B,C, and D represent four different atoms (or isotopes) or groups of atoms. The (carbon) atom to which these groups are attached is called an asymmetric (carbon) atom.

TABLE 1.1: Stereochemical terms in pharmacology.

Enantiomer:	a compound with a non-superimposable mirror image.
Chirality:	the property of objects or molecules to be non-superimposable on their mirror image.
Diastereomer:	compounds containing more than one chiral element.
Eutomer:	enantiomer with higher biological activity or affinity towards biomolecules than its counterpart.
Distomer:	enantiomer with lower biological activity or affinity towards biomolecules than its counterpart.
Eudismic ratio:	quotient of the affinity/activity of the eutomer and the distomer.
Eudismic index:	logarithm of the eudismic ratio.

The opposite enantiomer was assigned the L configuration (not to be confused with the d and l prefixes of optical activity). This **Fisher Convention** is still often used for naming (derivatives of) enantiomeric biological molecules such as sugars or amino acids. Since other techniques for determining the three-dimensional structure of molecules became available and because uniform conversion to (+)- or (-)-glyceraldehyde often is difficult, the **RS Convention** of Cahn, Ingold, and Prelog was introduced (Cahn et al, 1956).

This RS Convention implies that enantiomers are named on the basis of the rank order of the atoms or groups that are attached to a chiral centre. Rank orders are determined by sequence rules (atomic number, mass number in the case of isotopes, number of carbon atoms in alkanes, etc) as described by Cahn et al (1956) and the IUPAC (1970). The rank order numbers of the attached groups increase with decreasing priorities of the groups. If the group with the highest rank order number is positioned behind the plane of projection and the (other) rank order numbers increase clockwise, the enantiomers will have the R- (*rectus*; right) configuration. If the rank orders increase counterclockwise, the enantiomers will be assigned the S- (*sinister*; left) configuration. This nomenclature allows us to derive the three dimensional structure of enantiomers or diastereomers directly from their name and *vice versa*.

To date, the above discussed nomenclatures are all in use but the application of one single nomenclature for optical activity and one single nomenclature for absolute configurations would be beneficial as has been discussed by Simonyi et al (1989).

1.2 CHIRALITY IN CHEMICAL-BIOLOGICAL INTERACTIONS:

As has been stated above, apart from optical activity, enantiomers possess virtually identical physico-chemical properties. Nevertheless, large differences between enantiomers in their interaction with biological systems are often observed (reviews on this subject are given by Simonyi, 1984; Lam, 1988; Smith, 1989; and Maher and Johnson 1991). This is not surprising if it is realized that biological molecules are generally asymmetric. Firstly, the building blocks of macromolecules (amino acids and sugars) are chiral. Secondly, the three-dimensional structure of macromolecules only allows the binding and interaction of ligands if specific spatial criteria are met. In fact, many hormones (for instance peptide hormones and steroids), neurotransmitters (such as (nor)adrenalin) and other regulating endogenous substances (e.g. prostaglandins) are chiral. However, for regulating biological compounds, chirality is not a rule: for instance, acetylcholine, dopamine, gamma-aminobutyric acid, serotonin, and histamine all are achiral. Nevertheless, their receptors display marked stereoselectivity towards chiral drugs (Simonyi, 1984).

It has been hypothesized (Mason, 1984; Mason, 1986; Waechtershauser, 1991) that homochirality of biological molecules in the prebiotic era arose from chemical synthesis on asymmetric mineral catalytic templates such as pyrite or quartz to form asymmetric amino acids and sugars. The amino acids then polymerized to form peptides that served as new, and more efficient chiral catalysts.

Chirality of the resulting biomolecules can be considered the basis of the observed differences between enantiomers in their interaction with biological systems.

Already in 1933, Easson and Stedman proposed a model to explain the large differences in the biological activities of (+)- and (-)-adrenaline and in the mitotic activities of some asymmetric urethanes (Easson and Stedman, 1933).

Figure 1.2: The three point attachment model proposed by Easson and Stedman (1933). An optimal fit of the ligands groups with the receptor is achieved when the ligand groups a, b, and c are able to bind to receptor groups A, B, and C respectively. For a chiral drug this is possible for only one enantiomer (left figure). Of the other enantiomer only two ligand groups can be attached to their corresponding receptor site (right figure).

Their three point attachment model (figure 1.2) implies that in an optimal receptor ligand interaction, three ligand groups are important. In asymmetric molecules, only one of the enantiomers (the most active) will have the configuration that allows all three groups to interact with the receptor. In the case of the less active enantiomer however, maximally two

groups interact with the receptor and thus the ligand-receptor interaction will be less effective.

From a pharmacological point of view, Rodrigues de Miranda (1975) and Lehman (1976, 1986) introduced the term **eutomer** for the most active enantiomer and the term **distomer** for the less active. The **eudismic ratio** is defined as the quotient of the affinity or activity of the eutomer and that of the distomer. The **eudismic index** is the logarithm of the eudismic ratio. The eudismic ratio will increase with the affinity of the eutomer. This relation was first given by Pfeiffer (1956) and demonstrates that the higher the affinity of the eutomer is, the more complementary the ligand-receptor interaction will be.

The fact that enantiomers have different biological properties has lead Ariëns to the statement that racemic drugs are actually a mixture of two completely different drugs, with different pharmacological and toxicological properties, and that one of the enantiomers can be considered as isomeric ballast (Ariëns, 1984). An example, which is often cited to illustrate the importance of recognizing differences between enantiomers in their interaction with biological systems is thalidomide (Simonyi, 1984; Mason, 1984; de Camp, 1989; Williams, 1990; Lennard, 1991). This compound was initially used as a sedative; later it was shown to be teratogenic in humans. This prompted several investigators to study the teratogenicity of the thalidomide enantiomers. Fabro et al (1967) did not find any difference between the enantiomers and the racemate in teratogenic potency. However, closer inspection of these data by Simonyi revealed that the racemate was in fact twice as potent as teratogen as the individual enantiomers (Simonyi, 1984). Other studies reported that the teratogenicity of thalidomide may predominantly reside in the S(-)-enantiomer or metabolites thereof (Blaschke et al, 1979). As has been discussed by de Camp (1989), species and strain differences may play a role in the teratogenicity of thalidomide and its enantiomers. This example shows the complexity of enantiomeric interactions with biological systems.

In paragraphs 1.3 and 1.4, some examples of the differences between enantiomers on the pharmacodynamic (paragraph 1.3) as well as on the pharmacokinetic level (paragraph 1.4) will be given. The examples will demonstrate that enantiomers have different effects not only because of their distinct three dimensional structure, but also because of the large diversity of biomolecules with which they can or cannot interact.

1.3 PHARMACODYNAMIC DIFFERENCES BETWEEN ENANTIOMERS:

Pharmacodynamics describes the effects of a compound on a biological system. Compounds elicit their effect by interaction with effector molecules (such as receptors, ion channels, and enzymes).

Differences between enantiomers (or stereoisomers) in their interaction with receptors are well documented (Ariëns et al, 1983; Jamali et al, 1989). These differences are the result of the different three-dimensional structures of the enantiomers in combination with the stereochemical requirements of the receptors with which the enantiomers interact (Ariëns et

al, 1983). Different interactions at the receptor level can occur because: 1) enantiomers have different affinities towards receptors 2) enantiomers have different intrinsic activities (maximal responses) or 3) enantiomers interact to various extents with different (sub)types of receptors.

Differences in affinities between enantiomers towards receptors are observed for fenoldopam, a dopamine agonist. The EC₅₀ value of the R-enantiomer to relax norepinephrine contracted rabbit arterial segments is about 10 times lower than that of the S-enantiomer (Ohlstein et al, 1984).

Another example of chiral ligands of which the enantiomers have different affinities towards receptors will be discussed for β -adrenoceptor ligands. Morris and Kaumann (1984) determined the affinities of the enantiomers of chiral β -adrenoceptor agonists (isoprenaline, adrenaline, and noradrenaline) partial agonists (prenalterol, practolol, alprenolol, and pindolol) and antagonists (atenolol, bupranolol, and propranolol) towards β_1 -receptors in calf heart and β_2 -receptors in calf lung. In all cases, the affinity of the (-)-enantiomers was higher than the affinity of the (+)-enantiomers. However, the eudismic ratios observed for β_1 -adrenoceptors were higher than those observed for the β_2 -adrenoceptors. For instance, of the antagonists tested eudismic ratios for β_1 -receptors varied from 12 (atenolol) to 1450 (pindolol), whereas for β_2 -receptors eudismic ratios varied from 6 (atenolol) to 200 (pindolol). These differences between β_1 - and β_2 -receptors could be partially attributed to the fact that β_2 -receptors are more dependent on the lipophilicity of the ligands, so that the contribution to the binding to these receptors by factors different from the complementary 3 dimensional orientation of the ligand is relatively higher (El Tayar et al, 1988).

Different stereochemical demands between receptor subtypes are also shown for α_1 - and α_2 -adrenergic receptors (reviewed by Ruffolo, 1983). It appears that α_2 -receptors are more sensitive for the three-dimensional structure of their ligands than α_1 -receptors. For instance, the (-)/(+) enantiomeric activity ratio of noradrenaline is 107 for α_1 -receptors and 479 for α_2 -receptors, whereas the RS/SR ratio of the diastereomeric compound α -methylnoradrenaline is 60-fold for α_1 - and 550-fold for α_2 -receptors (Ruffolo, 1983).

The different pharmacological effects of enantiomers are not only due to the different affinities that the enantiomers may have towards a variety of receptors, but may also be due to disparities in the intrinsic activities of the enantiomers when bound to a certain type of receptor. An example of this is 3-(3-hydroxyphenyl)-N-n-propylpiperidine (Hjorth et al, 1983). Both enantiomers are agonists for presynaptic dopaminergic receptors but at postsynaptic dopamine receptors, the S-enantiomer is an agonist, whereas the R-enantiomer is an antagonist. Different intrinsic activities are also observed for the enantiomers of dobutamine, which possesses both α - and β -adrenergic activity (Ruffolo, 1983). Both enantiomers possess β -agonist activity, the (+)-enantiomer being the most potent. However, only the (-)-enantiomer has significant α -agonist activity which can be antagonized by the (+)-enantiomer, possessing only very weak partial agonist activity.

As has been stated above, in some cases enantiomers or stereoisomers interact with different receptor (sub)types, resulting in differential effects. An example thereof is provided by labetalol, which actually is a diastereomeric compound. The mixture of the four diastereomers has α - and β -adrenoceptor blocking activities. Brittain et al (1982) showed that in anaesthetized dogs and in isolated tissues, only the RR-diastereomer has significant β_1 - and β_2 -blocking activity; of the other diastereomers 10-40 times higher doses are required. In contrast, the α_1 -blocking potency of labetalol resides in the SR-diastereomer, the other diastereomers being 5-20 times less potent. Of all diastereomers, only the RR-diastereomer is equally effective as antihypertensive as the mixture of the four stereoisomers (Gold et al, 1982).

All of the above considerations concerning enantiomer-receptor interactions (i.e. enantiomeric differences in affinity, intrinsic activity and in the selectivity towards specific biomolecules) also hold for chiral compounds that elicit an effect by enzyme inhibition or by modulation of ion channels. For example, the S-enantiomers of NSAIDs of the arylpropionic acid type display a higher affinity towards cyclooxygenases *in vitro* and are therefore effective as inhibitors of the prostaglandin synthesis at lower concentrations than their enantiomeric counterparts (Caldwell et al, 1988). Inhibition of different enzymes by enantiomers is for instance observed for the competitive monoamine oxidase inhibitor N[3-(2,4-dichlorophenoxy)propyl]-N-methyl-3-butyn-2-amine (a clorgyline derivative). In rat liver mitochondria, the R-enantiomer has a higher affinity towards monoamine oxidase A, whereas the S-enantiomer has a higher affinity towards monoamine oxidase B (Dostert et al, 1992). Finally, the occurrence of differences in the intrinsic activities of enantiomers is exemplified by the dihydropyridine BAY K 8644. In rabbit heart and aorta, the R(+)-enantiomer of BAY K 8644 is a calcium entry blocker; in contrast, the S(-)-enantiomer activates calcium channels (Franckowiak et al, 1985).

1.4 PHARMACOKINETIC DIFFERENCES BETWEEN ENANTIOMERS:

Pharmacokinetics describes the fate of a compound in the body. This involves uptake, distribution (plasma protein binding and tissue uptake) and elimination (renal and biliary clearance and metabolism) processes. For all these processes, with the possible exception of biliary clearance, differences between enantiomers have been described (reviewed by Simonyi, 1984; Williams and Lee, 1985; Drayer et al, 1986; Ariëns and Wuis 1987; Lam, 1988; Tucker and Lennard, 1990; Campbell, 1990). The net effect of compounds is determined by their pharmacodynamic properties as well as the concentration at which the compounds are present at their effector site. Therefore, stereoselectivity in pharmacokinetics is an important factor in determining the differential effects of enantiomers in biological systems.

In the next sections some examples of enantiomeric differences in the above described pharmacokinetic processes will be given.

1.4.1 Stereoselective uptake of drugs through the gut wall:

Differences between enantiomers in their uptake after oral administration are relatively rare since most drugs enter cells by passive diffusion, which is dependent on the physico-chemical properties (showing no differences between enantiomers) of the drug (Van Ginneken et al, 1983). Differences may, however, occur if facilitated or active transport is at stake. Thus, stereoselective resorption after oral intake is observed for dopa. Its L-enantiomer, which is used as antiparkinson drug, is taken up by active transport (probably by intestinal amino acid transporters) as well as by passive diffusion, whereas D-dopa is taken up by passive diffusion only (Wade et al, 1973). As a result, L-Dopa is absorbed more readily from the intestine than D-dopa. Another, more extreme difference in the intestinal uptake of enantiomers is observed for methotrexate. This chemotherapeutic drug is marketed as the L-enantiomer. L-Methotrexate is extensively resorbed from the gut if applied orally; in contrast, resorption of the D-enantiomer is less than 3% of the applied dose (Hendel and Brodthagen, 1984). This indicates a highly stereoselective transport of the L-enantiomer through the gut wall.

1.4.2. Stereoselective tissue uptake:

Stereoselectivity in tissue uptake is relatively seldom observed since tissue uptake is often attributable to physico-chemical properties of the compound. There are however some examples of differences between enantiomers in tissue distribution.

The β -adrenoceptor blocking agent atenolol is shown to be taken up by neurotransmitter vesicles by an active transport mechanism which can be inhibited by reserpine and (-)-norepinephrine (Walle et al, 1988). This transport system is shown to be stereoselective since the uptake of (-)-atenolol is 5 fold greater than that of (+)-atenolol. A similar mechanism has been proposed for the enantiomers of propranolol (Walle et al, 1988); uptake of these β -adrenoceptor blocking agents in nerve endings may be of importance since it may account for a prolongation of their action after a single dose.

An example of stereoselective drug disposition, which is not dependent on stereoselective transport, is the NSAID ibuprofen. When racemic ibuprofen is given to Wistar rats for 7 days, the R-enantiomer of ibuprofen is found to be stored in fat tissue as the coenzyme A thioester; only trace amounts of the S-enantiomer are detected in fat tissue (Williams et al, 1986). Whether or not this has toxicological consequences is currently still under debate (Caldwell et al, 1988; Evans, 1992).

1.4.3 Stereoselective plasma protein binding:

Plasma protein binding is an important factor in pharmacokinetics since it has an impact on the (volume of) distribution and the clearance of drugs. For the binding of drugs to plasma

proteins, differences between enantiomers are generally modest (Mueller, 1988; Lam, 1988; Campbell, 1990).

Drugs may bind to albumin (mainly acidic drugs), α_1 -acid glycoprotein (mainly basic drugs) or to both. An example of a basic drug that binds stereoselectively to plasma proteins is verapamil. The free fraction of its (+)-enantiomer is 6.5 %, whereas that of the (-)-enantiomer is 11 % (Eichelbaum et al, 1984). The stereoselectivity of the plasma protein binding of verapamil offers an explanation for the higher systemic clearance of the (-)-enantiomer (table 1.2) of this high clearance drug. The uricosoric agent indacinone offers an example of an acidic drug that binds stereoselectivity to plasma proteins. The free fraction of (+)-enantiomer is 0.9%, whereas that of the (-)-enantiomer is only 0.3 % (Drayer, 1988). Propranolol binds to both plasma proteins (although mainly to α_1 -acid glycoprotein) but with different stereoselectivity. In human plasma, the unbound fraction of (+)-propranolol is higher than that of (-)-propranolol (Walle et al, 1983). Using isolated α_1 -acid glycoprotein, the unbound fraction of the (+)-enantiomer of propranolol is higher than that of the (-)-enantiomer. The inverse is observed for the binding of propranolol to isolated human albumin, for which the unbound fraction of the (-)- enantiomer is higher (Walle et al, 1983).

Albumin possesses two main non-overlapping binding sites (Sudlow et al, 1975). It is interesting to note that ligands bound to one of these two binding sites may influence the stereoselectivity of the other binding site and *vice versa* by allosteric interactions. Fitos et al (1990) showed that the binding of the enantiomers of a benzodiazepine derivative to immobilized human albumin is non-stereoselective. However, S-warfarin and the S-enantiomer of the benzodiazepine mutually increased the binding of each other, whereas bilirubin enhanced the binding of the R-enantiomer of the benzodiazepine.

In plasma protein binding, considerable species differences are observed (Mueller, 1988). For example, the free fraction of the R-enantiomer of the anticoagulant drug phenprocoumon is higher than that of the S-enantiomer in human, monkey, cat, dog, pig and horse. However, in the rat, mouse, guinea pig and the rabbit, the opposite is observed (Schmidt and Jaenchen, 1978).

1.4.4 Stereoselective renal clearance:

Stereoselective renal clearance is described for only a limited number of drugs. Often, stereoselectivity is attributable to enantiomeric differences in plasma protein binding (Tucker and Lennard, 1989). Stereoselectivity in the intrinsic renal clearance of drugs is expected only for compounds that are actively secreted in the kidney. This is indeed observed for the tubular secretion of some cationic compounds.

The antiarrhythmic drug disopyramide is subjected to active tubular secretion (Lima et al, 1985). The unbound renal clearance of the S-enantiomer is shown to be twice as high as that of the R-enantiomer (Lima et al, 1985). Stereoselective renal clearance, in combination with stereoselective plasma protein binding and stereoselective metabolism are the basis of

TABLE 1.2: Examples of stereoselective clearances in humans.

Drug	admin. route	Enantiomer		reference
		(+)	(-)	
Nitrendipine	p.o.	24 l/min	13 l/min	Soons et al, 1990
	i.v.	1.6 l/min	1.5 l/min	Soons et al, 1990
Nimodipine	i.v.	2.3 l/min	2.2 l/min	Sporckmann and Eichelbaum, 1991
Verapamil	p.o.	1.7 l/min	7.5 l/min	Vogelgesang et al, 1984
	i.v.	0.8 l/min	1.4 l/min	Eichelbaum et al, 1984
Propranolol	p.o.	2.8 l/min	2.0 l/min	Ward et al, 1986
	i.v.	1.2 l/min	1.0 l/min	Paxton and Norris, 1984
Propafenone (EM)	p.o.	5.8 l/min	11 l/min	Gross and Korner, 1989
(PM)*	p.o.	0.3 l/min	0.6 l/min	Gross and Korner, 1989
Mephénytoin (EM)	p.o.	4.7 l/min	0.03 l/min	Wedlund et al, 1985
(PM)*	p.o.	0.03 l/min	0.02 l/min	Wedlund et al, 1985
Phenprocoumon	p.o.	18 ml/kgd	14 ml/kgd	Jaenchen et al, 1976
Indoprofen	p.o.	32 ml/min	49 ml/min	Björkman, 1985
Pentobarbitone	p.o.	40 ml/min	32 ml/min	Cook et al, 1987

EM: extensive metabolizers (of sparteine/debrisoquine in the case of propafenone).

PM: poor metabolizers

the different clearances of the enantiomers of disopyramide (Lima et al, 1985; table 1.2).

In man, the renal clearance of the S(-)-enantiomer of pindolol is about 1.3 times higher than that of the R(+)-enantiomer (Campbell, 1990; Somogyi et al, 1992), despite the fact that pindolol enantiomers are bound to plasma proteins to equal extents (Campbell, 1990). Coadministration of cimetidine, an inhibitor of tubular organic cation transporters, decreases the renal clearance of pindolol stereoselectively: the renal clearance is reduced by 34% for R-pindolol *versus* 26% for the S-enantiomer, indicating stereoselectivity of the organic cation transporters in the tubule (Somogyi et al, 1992).

Stereoselective renal clearance is also observed for the antimalarial drug chloroquine, of which the (+)-enantiomer has a 1.6 times higher renal clearance, despite its stronger binding to plasma protein (Ofori-Adjei et al, 1986).

1.4.5 Stereoselective metabolism:

In drug metabolism three types of stereoselectivity are observed: 1) substrate stereoselectivity i.e. the preferential metabolism of one of the enantiomers of a chiral substrate, 2) product stereoselectivity i.e. formation of a chiral product in enantiomeric excess and 3) chiral inversion i.e. the conversion from one enantiomer to the other.

All three types of stereoselective metabolism and some of their implications will be discussed below. Another complicating aspect which is of importance in the stereoselectivity of (drug) metabolism is the existence of genetic polymorphisms. Some implications of genetic polymorphisms in substrate- and product stereoselectivity will be put forward. Finally, stereoselectivity in first-pass metabolism will be discussed since first pass-metabolism is an important factor in determining the bioavailability of high clearance drugs when applied orally.

1.4.5a Substrate stereoselectivity.

Substrate stereoselectivity may occur because enantiomers have different affinities towards the metabolizing enzymes or because the catalytic activity of the metabolizing enzymes is higher for one of the enantiomers (Testa, 1988).

The racemic calcium antagonist felodipine is an example of a compound with stereoselective metabolism due to different affinities of the metabolizing enzyme(s) towards the enantiomers. Its enantiomers are oxidized by cytochrome P450 to their common pyridine metabolite. In human liver microsomes, the formation of this metabolite for both enantiomers has similar V_{max} values (i.e. maximal reaction velocities, reflecting catalytic activity). However, the affinity of the R-enantiomer towards the metabolizing enzyme(s) is twice as high as that of the S-enantiomer. As a result, the intrinsic clearance, due to formation of this metabolite, is two times higher for the R-enantiomer (Eriksson et al, 1991). Since felodipine is a high clearance drug, which implies that its rate of elimination is determined by blood flow, this stereoselective metabolism has no effect on its systemic clearance. However, when felodipine is applied orally the bioavailability of the S-enantiomer will be higher than that of the R-enantiomer, due to the more rapid first-pass metabolism (i.e. the metabolism of a compound during its passage from the gastrointestinal tract to the circulation) of this latter enantiomer. Felodipine may also serve as a clear example of species differences that may occur in stereoselective metabolism. The liver microsomal metabolism of felodipine is stereoselective for the R-enantiomer in man (as stated above), non-stereoselective in rat, whereas in dog liver microsomes stereoselectivity for the S-enantiomer is observed (Eriksson et al, 1991).

Different catalytic activities of the metabolizing enzymes towards enantiomers may also be a cause of stereoselective metabolism. In man, the enantiomers of 4-hydroxypropranolol (an active metabolite of propranolol) are metabolized to their sulfate conjugates by

cytosolic enzymes. In platelets both 4-hydroxypropranolol enantiomers are sulfated with similar K_m values (i.e. dissociation constants of the enzyme-substrate complex), but the V_{max} of the (+)-enantiomer is 5 times higher than that of the (-)-enantiomer (Walle and Walle, 1991). A similar phenomenon is observed for the glucuronidation of the enantiomers of the antibiotic ofloxacin in rat liver microsomes. This reaction appears to be carried out by the same isozyme but the V_{max} value of the reaction for the S-enantiomer is 8 times higher than that of the R-enantiomer, whereas the affinities of the enantiomers are similar (Okazaki et al, 1991).

Sometimes, metabolizing enzymes display a catalytic activity to only one enantiomer, despite the fact that both enantiomers are able to bind to the enzyme. In that case one enantiomer is a substrate of the enzyme, whereas its enantiomeric counterpart is an inhibitor. In this respect, nicotine is a clear example. The natural S(-)-enantiomer of nicotine has been shown (Cundy et al, 1985) to be a competitive inhibitor (K_i about 60 μM) of an aromatic N-methyltransferase whereas the R(+)-enantiomer is a substrate of this enzyme (K_m about 10 μM).

In the above examples, both enantiomers follow similar metabolic routes that account for a similar percentage of their total metabolic clearance. However, from enantiomers, different metabolites may be formed. Also, the metabolites that are formed, may contribute differently to the total metabolic clearance of the enantiomers. In addition, enantiomers may follow similar metabolic routes but the reactions are carried out by different isozymes with different enantioselectivities. Propranolol exemplifies a chiral drug of which the enantiomers follow metabolic routes to different extents. In man, both enantiomers undergo side chain hydroxylation, glucuronidation, as well as aromatic hydroxylation to the 4-hydroxy metabolite (Walle et al, 1984). However, of the metabolites, about 60 % of the (+)-enantiomer *versus* 30 % of the (-)-enantiomer is recovered as 4-hydroxypropranolol, demonstrating that 4-hydroxylation is more important for the (+)- than for the (-)-enantiomer. This explains why quinidine, which is an inhibitor of the formation of 4-hydroxypropranolol, reduces the clearance of (+)-propranolol more than that of (-)-propranolol in man (Zhou et al, 1990). The above demonstrates that enantiomers may interact differently when other drugs are coadministered. This is because enantiomers may be metabolized by different combinations of enzymes or because enantiomers have different affinities to the enzymes. In the latter case, the enantiomer with the lowest affinity will be most sensitive for drug interactions at the metabolic level.

A clear example of different isozymes catalysing a similar reaction but with different enantioselectivities is observed in the metabolism of bufuralol. The main metabolite of this β -adrenoceptor blocking agent, 1'-hydroxybufuralol, is formed by two isozymes of the cytochrome P450 family: P450buf1 and P450buf2 (Gut et al, 1986). The R/S ratio of the intrinsic formation clearance of 1'-hydroxybufuralol by P450buf1 is about 5. P450buf2 shows an R/S ratio of only 1.3. As a result, the degree of stereoselectivity of 1'-hydroxylation will be determined by the ratio at which these two isozymes (in their active form) are present.

Since P450b_uf1 is presumably related to P450 2D6 (Gut et al, 1986; Beaune and Guengerich, 1988) which is responsible for the sparteine/debrisoquine polymorphism, the activity ratios of these isozymes, and as a result the stereoselectivity of bufuralol 1'-hydroxylation, may vary considerably among subjects.

The anticonvulsant drug mephenytoin is an additional example of a compound of which the enantiomers are metabolized by different enzymes. This example clearly illustrates which implications this can have on the stereoselectivity of (metabolic) clearances. The metabolism (more specifically the 4'-hydroxylation) of mephenytoin is known to be controlled by a genetic polymorphism which is different from the sparteine/debrisoquine polymorphism (Guengerich, 1989). In extensive metabolizers (EM), a clear stereoselectivity (150 fold) for the clearance of the (+)-enantiomer is observed (table 1.2; Wedlund et al, 1989). In fact in EM, the (+)-enantiomer is a high clearance drug, whereas (-)-mephenytoin is a low clearance drug. In poor metabolizers (PM), the stereoselectivity in the clearance of mephenytoin is completely lost due to a marked reduction of the clearance of the (+)-enantiomer (table 1.2; Wedlund et al, 1989). This shows that if chiral drugs are metabolized by genetically polymorphic enzymes, the stereoselectivity of their metabolism may show large interindividual differences. Nevertheless, genetic polymorphisms do not always have an impact on the stereoselectivity of metabolic drug clearances. If both the polymorphic and the constitutive enzymes display similar enantioselectivities, no differences in the stereoselectivity of drug metabolism between EM and PM will be observed. This is for instance the case for the antiarrhythmic drug propafenone. The metabolism of this drug is controlled by the sparteine/debrisoquine polymorphism (Siddoway et al, 1987; Kroemer et al, 1989). In EM, the clearance of propafenone is clearly stereoselective for the (-)-enantiomer (table 1.2, Gross and Komer, 1989). In PM however, this stereoselectivity is not altered since the clearance of both enantiomers is equally reduced.

The above examples demonstrate that the stereoselectivity of drug metabolism is a combination of the stereoselectivity of the metabolizing enzymes and the amount at which these enzymes are present (which may show large interindividual differences).

1.4.5b Product stereoselectivity.

Product stereoselectivity occurs if by metabolic conversion a chiral product is formed from an achiral substrate or if by metabolism an additional chiral centre is introduced in a chiral substrate. In the latter case a combination of product- and substrate stereoselectivity is possible.

An interesting example of product stereoselectivity is the 4-hydroxylation of debrisoquine. For the 4-hydroxylation of debrisoquine a genetic polymorphism is observed. In extensive metabolizers, the product formed is almost exclusively (>99%) in the S-configuration (Meese and Eichelbaum, 1986; Lennard et al, 1988). In contrast, in poor metabolizers, up to 20 % of the 4-hydroxydebrisoquine formed, is in the R-configuration (Meese and Eichelbaum 1986).

To illustrate various aspects of product- and substrate stereoselectivity in drug metabolism and its complexity due to the involvement of multiple enzymes, one of the metabolic routes of the hypnotic agent hexobarbitone will be discussed in greater detail. The enantiomers of hexobarbitone are known to be eliminated stereoselectively in man due to stereoselective metabolism (Breimer et al, 1973). One of its metabolic pathways, 3'-hydroxylation (Vermeylen et al, 1983) introduces a second chiral centre. This reaction is under polymorphic control (Knodell et al, 1988). In microsomes from extensive metabolizers (EM) S-hexobarbitone is converted more rapidly to its 3'-hydroxy metabolite than R-hexobarbitone (Yasumori et al, 1990). In microsomes from poor metabolizers (PM), however, substrate stereoselectivity is lost due to a more drastic reduction of the 3'-hydroxylation of the S- than of the R-enantiomer. In EM microsomes, from S-hexobarbitone, the 3' β -hydroxy metabolite is formed in 8 fold excess over the 3' α -hydroxy metabolite; this product stereoselectivity is completely absent in PM microsomes. From R-hexobarbitone, the 3' α metabolite is formed in 1.5 fold excess in EM microsomes, whereas in PM microsomes an inversion of product stereoselectivity is seen: 2.5 times more 3' β - than 3' α -hydroxyhexobarbitone are formed (Yasumori et al, 1990). Once again, this demonstrates the role of the multiplicity of enzymes involved in the metabolism of a chiral substance in determining substrate- and product stereoselectivity.

1.4.5c Chiral inversion:

A specific type of stereoselective metabolism is chiral inversion, i.e. the conversion from one enantiomer to the other. Well known examples of drugs for which chiral inversion plays an important role are derivatives of 2-arylpropionic acid. 2-Arylpropionic acids are an important group of NSAIDs. These compounds contain a chiral centre and from *in vitro* studies it is clear that the S(+)-enantiomers are far more potent as inhibitors of the prostaglandin synthesis than the R(-)-enantiomers (Caldwell et al, 1988; Evans, 1992). However, when tested *in vivo* the stereoselectivity of their action is very markedly reduced. For instance, *in vitro*, S-ibuprofen is 165 times more effective than the R-enantiomer as inhibitor of the prostaglandin synthesis. In contrast, both enantiomers are approximately equipotent as analgetics (Adams et al, 1976). This apparent discrepancy is largely explained by the chiral inversion that is observed for 2-arylpropionic acids, which is highly selective for the transformation of R(-) in S(+)-enantiomers (Caldwell et al, 1988 and Evans, 1992). Inversion may be very rapid, as is for instance observed for fenoprofen in man (Rubin et al, 1985) and flunoxaprofen in rats (Iwakawa et al, 1991). On the other hand, some arylpropionic acids show very few inversion as is the case for indoprofen in man (Tamassia et al, 1984) and naproxen and carprofen in the rat (Iwakawa et al, 1991). Although the mechanism of this inversion is still not unambiguously determined, it may involve stereoselective conversion of the enantiomers to their acyl CoA esters and subsequent enzymatic epimerization (Caldwell et al, 1988; Evans, 1992; Tracy and Hall, 1992).

1.4.5d Stereoselective first-pass metabolism:

First-pass metabolism is the metabolism of a compound during its passage from the gastro-intestinal tract to the circulation. First-pass metabolism has no effect on systemic parameters as $t_{1/2}$ or the systemic clearance of enantiomers but determines their bioavailability when applied orally and thus the initial ratio at which the enantiomers will be present in plasma. This is most important for high clearance drugs.

Stereoselective first-pass metabolism of the calcium channel blocking agent verapamil has been shown to be the cause for the marked differences in plasma-concentration (of the racemate) effect relations after oral or iv administration (Eichelbaum, 1988). Plasma concentrations after oral administration must be 3 times higher than after iv administration to elicit the same effect (Eichelbaum et al, 1980). (-)-Verapamil is about 8-10 times more potent than (+)-verapamil with regard to its negative dromotropic effect. On the other hand the systemic and oral clearance of (-)-verapamil is substantially higher (table 1.2; Vogelgesang et al 1984). Because of stereoselective first-pass metabolism the (+)/(-) ratio of plasma verapamil concentrations is 5 after oral but only 2 after iv administration, explaining the observed discrepancies between plasma concentration effect relationships (Echizen et al, 1985). The reason of the stereoselectivity of the first-pass metabolism of verapamil probably is the higher affinity of the (-)-enantiomer towards the verapamil metabolizing enzymes (Kroemer et al, 1991).

Stereoselective first-pass metabolism is also observed for the dihydropyridine calcium antagonists nitrendipine (Soons et al, 1990) and nimodipine (Sporckmann and Eichelbaum, 1991). The systemic clearance of the latter compound is non-stereoselective (table 1.2), but because of first-pass metabolism, the bioavailability of the R-enantiomer after oral administration is 22% whereas that of the more potent S-enantiomer is only 3.5%.

1.5 STEREOCHEMICAL ASPECTS IN THE PHARMACOLOGY OF WARFARIN AND ACENOCOUMAROL:

From the previous paragraphs it will be clear that differences between enantiomers in pharmacology are commonly observed and that enantiomeric differences occur at many levels in the pharmacodynamics and pharmacokinetics of chiral compounds.

Warfarin and some of its phenyl ring substituted analogues have been used as model compounds in this thesis to study the stereochemical aspects of their pharmacokinetics and biotransformation. Therefore, the second part of this introduction section will specifically deal with the stereochemical aspects of the pharmacology of warfarin and its 4'-nitro analogue acenocoumarol, which like warfarin is in use as oral anticoagulant. In paragraph 1.5.1 some general information of these drugs will be given. Their mode of action, pharmacokinetics and interactions with other drugs will be described in paragraphs 1.5.2 to 1.5.5. Finally, in paragraphs 1.6 and 1.7, the aim and outline of this thesis will be discussed.

1.5.1 Warfarin and acenocoumarol: general information.

4-Hydroxycoumarins (figure 1.3) are widely used as oral anticoagulants in the treatment of thrombo-embolic disease and post-operative prevention of blood clotting. Furthermore, treatment with 4-hydroxycoumarins is proposed for reinfarction prevention after myocardial infarction (Jafri et al, 1992). In addition, 4-hydroxycoumarins are used as rodenticides.

In 1922, in North America, extensive bleeding of cattle that had consumed spoiled clover occurred (Schofield, 1922). Later, in 1931, this was attributed to a depressed prothrombin level (Roderick, L.M., 1931). Campbell and Link (1941) isolated the active compound dicoumarol from sweet clover as the first coumarin anticoagulant.

Figure 1.3: Structural formulas of 4-hydroxycoumarin (A), warfarin (B), acenocoumarol (C), and phenprocoumon (D)

Among the 4-hydroxycoumarins used as anticoagulants are warfarin, which is world wide the most extensively used, its 4'-nitro analogue acenocoumarol, and phenprocoumon (figure 1.3). These coumarins possess a chiral centre, which implies that two optically active enantiomers exist. West et al (1961) isolated the enantiomers of warfarin by cristallization of their quinidine and quinine salts and demonstrated that (-)-warfarin is related to S(-)- β -caproic acid and therefore assigned the S-configuration to the (-)-enantiomer. In a later study by Wheeler and Trager (1977), the absolute configuration of the enantiomers of warfarin was confirmed by X-ray crystallography. The same authors showed that (-)-acenocoumarol also has the S-configuration. Warfarin and acenocoumarol are weak acids due to the acidic 4-hydroxy group of the coumarin moiety (pKa values of about 4.8) that are protolysed at physiological pH. In aqueous solution, warfarin exists as an open ring form. In organic solution however warfarin can also be present as two cyclic diastereomeric hemiketals (Heimark and Trager, 1984). In addition, keto-enol tautomerism of the coumarin ring is possible (Vanhaelen, 1978).

1.5.2. The mechanism of action of 4-hydroxycoumarin anticoagulants:

The action of 4-hydroxycoumarins on blood coagulation is indirect and is due to their ability to decrease the regeneration of vitamin K. The metabolism of vitamin K and the function of the vitamin have been reviewed by Olsen (1984) and Suttie (1987).

The hydroquinone of vitamin K, next to molecular oxygen and carbon dioxide, serves as an obligatory cofactor for a microsomal carboxylase. This enzyme posttranslationally converts glutamyl residues of a number of protein precursors to gamma-carboxyglutamyl residues. Thereby, these proteins will become more negatively charged and will therefore be able to form complexes with calcium. The introduction of gamma-carboxyglutamyl residues is, among others, important for the activation of the plasma clotting factors II, VII, IX, and X and of the plasma proteins C, and S that are involved in fibrinolysis and anticoagulation (Olsen, 1984; Suttie 1987). During the carboxylation reaction, the hydroquinone of vitamin K (figure 1.4) is converted to vitamin K 2,3-epoxide (Larson et al, 1981). This epoxide is subsequently converted to vitamin K by a dithiol-dependent reductase, which is located in the endoplasmatic reticulum (Zimmerman and Matschiner, 1974). Finally, vitamin K is reduced to its active form, the hydroquinone, by a dithiol dependent microsomal enzyme (Fasco and Principe, 1980 and 1982) and by NAD(P)H dependent enzymes, although the latter enzymes probably play only a minor role in the formation of vitamin K hydroquinone under physiological conditions (Wallin and Martin, 1985).

The microsomal dithiol dependent vitamin K and vitamin K epoxide reductase are possibly one and the same enzyme (Preusch and Suttie, 1984; Gardill and Suttie, 1990). The nature of the endogenous reductant of these reductase activities is still not unambiguously

determined but reduced lipoic acid (Whitlon et al, 1978; Lee and Fasco, 1984) or thioredoxin (van Haarlem et al, 1987; Silverman and Nandi, 1988) are able to serve as reductant.

4-Hydroxycoumarins inhibit the reduction of vitamin K-epoxide to vitamin K as well as the reduction of vitamin K to its hydroquinone. This has been demonstrated both *in vitro*, where 4-hydroxycoumarins are potent inhibitors of the microsomal dithiol-dependent reductases (Fasco et al, 1982) and *in vivo*, where accumulation of vitamin K epoxide is observed after application of a pharmacological dose of vitamin K to warfarin treated patients or volunteers (Choonara et al, 1985 and 1986b). Because the formation of the hydroquinone of vitamin K is inhibited, the synthesis of blood clotting factors will be hampered, and as a result, anticoagulation occurs.

It has been proposed (Silverman 1980, Fasco et al, 1983) that the dithiol-dependent enzyme(s) responsible for the reduction of vitamin K and its epoxide contain(s) two sulphhydryl groups that are oxidized to form a disulfide during reduction of the substrate. Later, the vitamin K 2,3 epoxide reductase was indeed shown to follow Ping-Pong kinetics and to shuttle between a reduced and an oxidized state (Hildebrandt et al, 1984). At high levels of dithiothreitol the extent of inhibition of the vitamin K epoxide reductase by warfarin is diminished (Zimmerman and Matschiner, 1974; Fasco et al, 1983). In the absence of reductant, the binding of warfarin to the dithiol dependent vitamin K and vitamin K 2,3-

Figure 1.4: The vitamin K cycle.

epoxide reductases is essentially irreversible, since washing of microsomes treated with warfarin does not diminish the inhibiting effect of warfarin on the reductases (Fasco and Principe, 1982). Therefore, a model has been proposed in which 4-hydroxycoumarins bind to the vitamin K epoxide reductase in the oxidized state (Fasco et al, 1983), whereas vitamin K epoxide binds to the enzyme in the reduced state. This model has been affirmed by Thijssen and Baars (1989), who showed the binding of ^{14}C -warfarin to be shifted to the right by dithiothreitol. Nevertheless, the exact mechanism of the inhibition of the reduction of vitamin K and its epoxide by 4-hydroxycoumarins at the enzyme level has still not yet been revealed. However, Silverman et al (1988), without presenting data, stated the inhibition by warfarin to be competitive for the reductant dithiothreitol.

1.5.3 Clinical pharmacology of warfarin and acenocoumarol.

4-Hydroxycoumarins have a narrow therapeutic range and large interindividual differences in the required dose are observed (Holford, 1986; Shetty et al, 1989). A clear relationship between the dose and the anticoagulant effects is still not known. Racemic acenocoumarol is more potent as anticoagulant than racemic warfarin. To obtain therapeutic anticoagulation (i.e. prothrombin complex activities of about 10 % control values) total plasma concentrations of warfarin range from 1 to 10 μM (Holford, 1986), whereas those of acenocoumarol range from 60 to 600 nM (Thijssen et al, 1988b). Plasma-concentration anticoagulant effect curves for warfarin are steep (Holford, 1986). One of the reasons for this is that (at least in the rat) the dithiol dependent vitamin K epoxide reductase appears to have an overcapacity. At least 70-75% of its hepatic activity has to be eliminated before any clinical effect on clotting factor synthesis is observed (Mosterd and Thijssen, 1991 and 1992). Thus, a no-effect level for 4-hydroxycoumarins exists, which must be exceeded to have an anticoagulant effect.

In man and in the rat, the S-enantiomer of warfarin is about 2-5 times more potent as anticoagulant, than the R-enantiomer (Yacobi and Levi, 1974; Wingard et al, 1978; Fasco and Principe 1982; Choonara et al, 1986b). Conversely, R-acenocoumarol is more potent than S-acenocoumarol in man (Meinertz et al, 1978) and in the rat (Thijssen et al, 1985). However, if stereoselective clearance is dealt with, S-acenocoumarol is more potent than the R-enantiomer (Thijssen et al 1985). Thijssen et al (1988) showed that the higher anticoagulant potency of the S-enantiomers in the rat is due to the slower release of the S-enantiomers, when bound to the vitamin K-epoxide reductase.

1.5.4 Pharmacokinetics of the enantiomers of warfarin and acenocoumarol:

When applied orally, warfarin and acenocoumarol are well and readily absorbed from the gastro-intestinal tract (Breckenridge and Orme 1973, Dieterle et al 1977, Thijssen et al 1983, Gill et al 1989). Plasma peak concentrations are observed after about 1 to 4 hours. The absorption does not appear to be stereoselective.

TABLE 1.3: Reported pharmacokinetic parameters of the enantiomers of warfarin and acenocoumarol in man.

Warfarin	R-	S-	R/S	References
t1/2 (h)	46	28	1.6	Lewis et al 1974
	46	32	1.4	O'Reilly 1976
	46	30	1.5	Kelly and O'Malley 1979
	33	22	1.5	Banfield et al 1983
	48	38	1.3	Choonara et al 1986a
	44	33	1.3	O'Reilly et al 1987
	34	29	1.2	Abernethy et al 1991
Cl (ml/min)	3.1	4.2	0.7	Choonara et al 1986a
	2.2	3.3	0.7	Choonara et al 1986b
	2.5	3.6	0.7	O'Reilly et al 1987
	3.5	4.2	0.8	Abernethy et al 1991
Vd (l)	9.5	9.8	1.0	Kelly and O'Malley 1979
	11.2	11.2	1.0	Banfield et al 1983
	12.8	12.0	1.1	Choonara et al 1986a
	9.3	8.4	1.1	O'Reilly et al 1987
	9.8	9.8	1.0	Abernethy et al 1991
f* (%)	1.2	0.90	1.4	Yacobi and Levi, 1977
	0.49	0.44	1.1	Banfield et al 1983
	0.85	0.50	1.7	Toon and Trager 1984
	0.86	0.73	1.1	Abernethy et al 1991
Acenocoumarol	R-	S-	R/S	References
t1/2 (h)	11.5	1.1	10	Thijssen et al, 1986
	6.8	0.9	7.6	Gill et al, 1988
	7.1	1.6	4.4	Gill et al, 1989
Cl (ml/min)	35	496	0.1	Thijssen et al, 1986
	24	317	0.1	Gill et al, 1988
	24	225	0.1	Gill et al, 1989
Vd (l)	32	38	0.8	Thijssen et al, 1986
	16	23	0.6	Gill et al, 1988
	15	31	0.5	Gill et al, 1989
f* (%)	1.8	2.0	0.9	Godbillon et al, 1981
	0.7	1.0	0.7	Gill et al, 1988

* f is the fraction of drug in the plasma which is not bound to plasma proteins.

Warfarin and acenocoumarol are extensively bound to plasma protein. In humans S-warfarin is bound more tightly to plasma proteins than R-warfarin and as a result, the free fraction of the R-enantiomer is 1.1 to 1.7 times higher than that of the S-enantiomer (table 1.3). The contrary is observed in the case of acenocoumarol for which the R-enantiomer is more tightly bound; the free fraction of R-acenocoumarol is about 0.7 to 0.9 times that of S-acenocoumarol. In rats, however S-warfarin and S-acenocoumarol are more tightly bound to plasma proteins than the R-enantiomers (table 1.4). In rats, the free fraction of R-warfarin is 1.6 times higher than that of S-warfarin, whereas the free fraction of R-acenocoumarol is 1.1 times higher than that of S-acenocoumarol.

Both warfarin and acenocoumarol are eliminated stereoselectively. There are however, considerable species differences in the stereochemical course and speed of elimination. In man, S-warfarin is eliminated faster than R-warfarin. The half-life of R-warfarin in man is about 1.2 to 1.6 times higher than that of S-warfarin, and its clearance is about 0.7 to 0.8 times that of the S-enantiomer (table 1.3). In man, a qualitatively similar, but quantitatively more extreme stereoselectivity is also observed for the elimination of acenocoumarol. Half-lives are reported to be 4.4 to 10 times higher for R-acenocoumarol, whereas the clearance of R-acenocoumarol is about 10 times lower than that of S-acenocoumarol (table 1.3).

Contrary to what is observed in man, R-warfarin is eliminated faster than S-warfarin in the rat. Half-lives of R- and S-warfarin are about 7.3 and 14 hours, whereas the clearance of the R-enantiomer is two times higher than that of S-warfarin. This inversion of stereoselectivity is not observed for acenocoumarol. In the rat, S-acenocoumarol is eliminated faster than R-acenocoumarol but the difference between the enantiomers is less pronounced than in man. As in man, acenocoumarol enantiomers are eliminated substantially faster than the enantiomers of warfarin: clearances of R- and S-acenocoumarol are about 10 and 90 times higher in man and about 10 and 70 times higher in rats (tables 1.3 and 1.4).

TABLE 1.4 Reported pharmacokinetic parameters of the enantiomers of warfarin and acenocoumarol in rats.

Warfarin	R	S	R/S	References
t1/2 (h)	7	14	0.5	Yacobi and Levy, 1974
Cl (ml/h)	6.7	3.6	1.9	" " "
Vd (ml)	64	63	1.0	" " "
f (%)	1.31	0.84	1.6	Yacobi and Levy, 1977
Acenocoumarol	R	S	R/S	References
t1/2 (h)	1.6	0.4	4.0	Thijssen et al, 1985
Cl (ml/h)	68	257	0.3	" " "
Vd (ml)	167	185	0.9	" " "
f (%)	1.6	1.4	1.1	" " "

1.5.5 Biotransformation of the enantiomers of warfarin and acenocoumarol:

In both man and rat warfarin and acenocoumarol have been shown to be eliminated mainly by biotransformation (Lewis et al, 1974, Toon et al 1986, Thijssen et al, 1986, Dieterle et al 1977, Thijssen and Baars 1987, Pohl et al 1976). Warfarin metabolites have been shown to have virtually no anticoagulant activity (Lewis et al 1973, Obaseki and Coker, 1987).

Various metabolic routes of warfarin have been described (figure 1.5). In man the main metabolites of R-warfarin are the RS alcohol (formed by reduction of the acetyl side chain), 6-hydroxywarfarin and to a lesser extent 7- and 8-hydroxywarfarin. In contrast, the main metabolite of S-warfarin in man is the 7-hydroxy metabolite next to the 6-hydroxy metabolite, which is found in 2-4 times smaller quantities. (Lewis et al 1974; Banfield et al, 1983; Toon et al 1986; Niopas et al 1991; Abernethy et al, 1990). In the rat however, the main metabolites of both R- and S-warfarin are the 4'-, 6- and 7-hydroxy metabolites (Pohl et al 1976). In addition to the above described metabolites, small quantities of dehydrowarfarin, 9- and 10-hydroxywarfarin (figure 1.5) and the SS, SR, and RR alcohols have been found in humans and rats (Lewis et al, 1974; Pohl et al, 1975; Abernethy et al, 1991; Pohl et al, 1976).

The enantiomers of acenocoumarol are mainly hydroxylated to their 7- and 6-hydroxy metabolites in man and in rat (Dieterle et al, 1977; Thijssen et al, 1986; Thijssen and Baars, 1987). In addition, the diastereomeric alcohols of acenocoumarol have been found in man, but information on the configurations of the alcohols was not given (Dieterle, 1977).

Figure 1.5: The biotransformation of warfarin and acenocoumarol.

A) Arrows indicate hydroxylation sites of warfarin and acenocoumarol by cytochrome P450.

Dehydrowarfarin possesses a double bond between C9 and C10

B) The diastereomeric alcohols, produced by reduction of the acetyl side chain

*) Stereocentre

1.5.6. Stereoselective drug-interactions with warfarin and acenocoumarol.

In man various interactions of drugs with warfarin have been described. These interactions occur both at the pharmacodynamic and the pharmacokinetic level. Stereoselective interactions with the S-enantiomer, the R-enantiomer, as well as non-stereoselective interactions are found. For acenocoumarol, up till now, fewer interactions have been described.

In the next section, some drug-interactions with warfarin and acenocoumarol will be briefly discussed.

1.5.6a Stereoselective interactions with the S-enantiomer:

The anti-inflammatory drug phenylbutazone potentiates the anticoagulant activity of warfarin, without changing the clearance or elimination half life of the racemate. However, in a study in which the separated enantiomers of warfarin were given it was revealed that the clearance of the more potent S-enantiomer was markedly decreased, whereas that of the R-enantiomer was increased by phenylbutazone (Lewis et al, 1974; Banfield et al 1983). This has been ascribed to the displacement of warfarin from albumin in combination with a decrease of the intrinsic clearance of S-warfarin. Because of the higher levels of unbound R-warfarin, the clearance of this enantiomer will be enhanced. On the other hand, the formation of the 6- and 7-hydroxy metabolites of S-warfarin has been shown to be markedly decreased upon coadministration of phenylbutazone, which explains the decreased intrinsic clearance of this enantiomer. The interaction of warfarin with phenylbutazone proves that it is necessary to evaluate the fate of the single enantiomers if racemic drugs are applied, since data on the racemate only may lead to substantial misinterpretation.

A similar stereoselective interaction with warfarin as that of phenylbutazone, is observed for the uricosuric agent sulfinpyrazone (O'Reilly, 1982). This drug also enhances the clearance of the R-enantiomer by displacement of the plasma protein binding sites (Toon and Trager 1984) but decreases the clearance of the S-enantiomer by inhibition of its oxidative metabolism (Toon et al, 1986). Sulfinpyrazone has also been reported to potentiate the anticoagulant effect of acenocoumarol (Michot et al, 1981).

Other compounds that potentiate the hypothermic response to racemic warfarin by inhibition of the metabolic clearance of the S-enantiomer are the antibiotics metronidazole (O'Reilly 1976) and cotrimoxazole (O'Reilly 1980).

Pharmacodynamic interactions with S-warfarin have been described for disulfiram (O'Reilly, 1981) and the blood lipid lowering compound clofibrate (Bjornsson et al, 1977). These compounds are without effect on the pharmacokinetics of S-warfarin but potentiated its anticoagulant effect.

1.5.6b Stereoselective interactions with the R-enantiomer:

The histamine H₂ antagonist cimetidine is known to decrease the clearance of R- but not S-warfarin (Choonara et al 1986) by inhibition of the formation of the 6- and 7-hydroxy

metabolites (Niopas et al, 1991). Cimetidine also decreases the clearance of R-acenocoumarol albeit only at relatively high doses (Thijssen et al, 1986; Gill et al, 1989). These interactions have been shown to increase the hypothermibrinaemic response in some patients.

The calcium antagonist diltiazem, has been shown to decrease the clearance of R-warfarin in man (Abemethy et al, 1991) by a decrease of the formation of the 6- and 8-hydroxy metabolites of R-warfarin.

Other drugs that decrease the clearance of R-warfarin are the antibiotic enoxacin, which inhibits the formation of the R-6-hydroxy metabolite (Toon et al, 1987), and the gastric hydrogen-potassium pump inhibitor omeprazole (Sutfin et al, 1989).

1.5.6c Non stereoselective interactions:

A non-stereoselective decrease of the clearance of warfarin has been described for the antiarrhythmic amiodarone (O'Reilly et al 1987); this has been attributed to a non-stereoselective inhibition of the metabolism of warfarin (Heimark et al, 1992).

Amiodarone is also shown to enhance the anticoagulant activity of acenocoumarol but information on the mechanism and on stereochemical aspects is lacking. (Pini at la, 1985; Fondevilla, 1988).

1.5.6d Other interactions:

The antiarrhythmic agent propafenone is known to potentiate the anticoagulant effect of warfarin by a decrease of its clearance but the stereochemistry has not been studied (Kates et al, 1987). Potentiation of the anticoagulant effect of warfarin, without information on the mechanism and stereoselectivity has been described for the antimalarial drug proguanil (Armstrong et al 1991) and the blood sugar lowering drug glibenclamide (Jassal 1991).

Finally, warfarin clearance is shown to be enhanced, probably by enzyme induction, by barbiturates, phenytoin, glutethimide, rifampicin and griseofulvin (reviewed by Serlin and Breckenridge 1983). Some of these inductions involve one enantiomer mainly, thus rifampicin induces particularly the metabolic clearance of R-warfarin (Heimark et al, 1987).

Enhancement of the clearance of racemic acenocoumarol in man has been described for pentobarbitone (Kroon et al, 1990).

1.6 AIM OF THIS THESIS:

The above paragraphs show that the enantiomers of warfarin and its 4'-nitro analogue acenocoumarol display clear differences in their pharmacokinetics and the stereoselectivity thereof. In addition, differences between species are observed (human and rat). Substitution of the 4'-hydrogen of warfarin by an amino or acetamido group was also shown to influence the rate and stereoselectivity of the elimination of the 4-hydroxycoumarin (Thijssen and Baars, 1983; Thijssen et al, 1985). This may point to an important role of the phenyl ring of

warfarin in determining the stereochemical course of its pharmacokinetics.

To study whether this is indeed the case, the effect of phenyl ring substitution of warfarin on the stereochemical course of its pharmacokinetics has been investigated. Since no studies on the *in vitro* biotransformation of acenocoumarol have been conducted before, we compared the *in vitro* biotransformation of warfarin and acenocoumarol, in an attempt to explain the pharmacokinetic differences between these 4-hydroxycoumarins. Both the oxidative and reductive metabolism of these compounds have been investigated. The role of the multiplicity of enzymes in the differences in the (stereochemical course of the) metabolism of warfarin and acenocoumarol has been evaluated.

1.7 OUTLINE OF THIS THESIS

In chapter 2, the pharmacokinetics of various phenyl ring substituted warfarins in the rat will be described. In chapter 3 the stereochemical aspects of the rat liver microsomal cytochrome P450 mediated metabolism of acenocoumarol and warfarin will be compared and the effect of enzyme inducers, the cytochrome P450 inhibitor and antibodies raised to cytochromes P450 of the 3A and 2C subfamily on the metabolism of both compounds will be discussed. Chapter 4 deals with the (stereochemical aspects of) human liver microsomal cytochrome P450 mediated metabolism of warfarin and acenocoumarol. In addition, the effect of some selected cytochrome P450 inhibiting drugs on their metabolism will be described.

In chapter 5, stereochemical aspects of another metabolic route, reduction of the ketone group of warfarin and analogues and species differences in this metabolic route will be considered. Chapter 6 describes characteristics of two warfarin ketone reductases that have been partially purified from rabbit liver cytosol. Finally, in chapter 7 the experimental data of this thesis will be discussed and some considerations on the use of chiral drugs in pharmacology will be put forward.

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CHAPTER 2:

ENANTIOSELECTIVE STRUCTURE-PHARMACOKINETIC RELATIONSHIPS OF RING SUBSTITUTED WARFARIN ANALOGUES IN THE RAT.

2.1 ABSTRACT:

The enantiomer specific pharmacokinetics of ring substituted warfarin analogues have been studied in the rat after the administration of 2 mg/kg of the racemates. The stereoselective differences observed are due to stereoselective plasma binding and stereoselective intrinsic hepatic clearance. Greater binding is observed for the S-enantiomers, except for the 2'-substituted analogues, where the R-enantiomers are more tightly bound. The stereoselectivity of plasma protein binding ranges up to a factor of about 4.

All substituted warfarins show a higher intrinsic clearance than warfarin. Enantiomer selectivity of the intrinsic clearance depends on the position of the substituent; warfarin and 3'-analogues show R>S; 4'- and 2'-substituted analogues show S>R. Exceptions to this generality are observed for 4'-methoxy and 4'-methylwarfarin for which there are no differences between the enantiomers in the intrinsic clearances.

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2.2 INTRODUCTION:

Stereoselective differences in drug disposition and drug activity have received increasing attention as awareness has grown that pharmacokinetic and pharmacodynamic descriptions of a drug applied as a racemic mixture are meaningless unless the fate of the individual enantiomers is established (Ariëns, 1984; for reviews, see Walle and Walle, 1986; Evans et al, 1988; Tucker and Lennard, 1990). The closely related 4-hydroxycoumarin anticoagulants warfarin and acenocoumarol (4'-nitrowarfarin) are in use as racemic mixtures. The stereoselectivity of the pharmacokinetics and the pharmacodynamics of their enantiomers in man and rat has been widely studied (Breckenridge et al, 1973; Yacobi and Levy, 1974; Toon et al, 1986; Godbillon et al, 1981; Thijssen et al, 1985, 1986). In man the S-enantiomers of both compounds are eliminated more rapidly than the R-enantiomers; the differences are modest for the warfarin enantiomers but 10-20 fold for the enantiomers of acenocoumarol. The body clearance of the acenocoumarol enantiomers is at least 100 times that of R/S-warfarin (Godbillon et al, 1981; Thijssen et al, 1986). Those differences between warfarin and acenocoumarol are also observed in the rat. However, in the rat, R-warfarin is eliminated faster than S-warfarin. The stereoselectivity of acenocoumarol elimination in the rat is comparable to that of man, i.e. S-acenocoumarol is cleared 3-4 times faster (Thijssen et al, 1985). The effects of the introduction of the 4'-nitro substituent on clearance and stereoselectivity prompted us to investigate the disposition of a series of phenyl-substituted warfarin (figure 2.1) analogues in the rat. The compounds were administered as their racemates and

the individual enantiomers were analyzed by a stereoselective HPLC procedure. Stereoselectivity in plasma protein binding was accounted for in the pharmacokinetic evaluation.

Figure 2.1: The structure of warfarin (R=H) and the ring substituted analogues. The chiral center is indicated by *.

2.3 MATERIALS AND METHODS:

Synthesis of warfarin analogues:

Racemic warfarin was obtained from Sigma Chemicals (St. Louis, USA); racemic 4'-chlorowarfarin was from Aldrich (Brussels, Belgium); racemic 4'-nitrowarfarin was a gift from Ciba-Geigy (Amhem, The Netherlands). Optically pure isomers of warfarin, 4'-chlorowarfarin and 4'-nitrowarfarin were prepared by the method of West et al (1961). The purity of the enantiomers was tested by the formation of their t-BOC-L-proline diastereomers and subsequent analysis by HPLC (Lichrosorb Si60; n-hexane/ethylacetate/methanol 75/25/0.2) as described by Banfield and Rowland (1984) and proved to be higher than 97%.

Phenyl ring substituted warfarin analogues were synthesized in our laboratory according to standard procedures. Briefly, substituted benzalacetones were prepared from the respective benzaldehydes by condensation with acetone under alkaline conditions in an ethanol-water

mixture. The benzalacetones were purified by crystallization (TLC was used to monitor purity) and identified by mass spectrometry (GC-MS, Hewlett Packard 5995). Conjugation of the benzalacetone with 4-hydroxycoumarin via Michael addition was by refluxing an equimolar mixture of the reactants in methanol as described by Bush and Trager (1983). The compounds were purified by crystallization (TLC and HPLC were used for analysis). GC-MS identification of the warfarins was performed after derivatization with diazomethane. The acidity (pKa) of the warfarins was estimated from the pH-dependent change in UV absorption (at 272 nm) of 10 µg/ml solutions in buffers (pH range 2-11, ionic strength 0.5 M).

Drug analysis:

Total drug concentrations, i.e. the R- and S-enantiomer were assayed by reversed phase HPLC based on techniques described by Thijssen et al (1985). The eluent consisted of a mixture of acetonitrile and 0.1 % v/v/ acetic acid in water, brought to a pH of 5.0 to 5.2 with ammonia. For each compound the eluent was adapted for optimal resolution and sensitivity. The stationary phase consisted of a Chromspher C18 column 3x100 mm (Chrompack, Middelburg, the Netherlands). To 80 µl plasma, 0.5 ml of 0.1 M sodium acetate buffer (pH=4.4) was added. The drugs were extracted by shaking the acidified plasma samples with 4 ml of a 1/1 (v/v) mixture of methylene chloride and petroleum ether (boiling range 40-60 °C). Before extraction, one of the enantiomers of either warfarin, 4'-chlorowarfarin, or 4'-nitrowarfarin was added as internal standard. The choice depended on the chromatographic behaviour in relation to the compound to be assayed. Following evaporation of the organic phase, the residue was dissolved in 50 µl of acetonitrile; 10 µl was injected on HPLC as described above. Total concentration was estimated from peak area ratios. The detection limit, using 80 µl plasma was 30-50 ng/ml.

The ratio of the R- and S-enantiomers was estimated by the procedure of Banfield and Rowland (1983). Briefly, 20 µl of a solution of dicyclohexane-carbodiimide (10 mg/ml in acetonitrile) and 20 µl of a solution of N-carbobenzyloxy-L-proline (10 mg/ml in acetonitrile) were added to the remaining 40 µl of extract.

The reaction was completed overnight at 40°C. The diastereomeric esters were separated by straight phase chromatography using a Chromspher Si column (3x100 mm) and mixtures of hexane-THF and acetic acid as eluting solvent. The esters of the R- and S-enantiomers were completely separated, with the esters of the S-enantiomers eluting first. The concentrations of the individual enantiomers in a sample were calculated from the R/S ratio and the total concentration.

Partition coefficients:

The partition coefficients of the warfarin analogues for the n-octanol water system at pH 7.4 was estimated as follows. To 1 ml 0.1 M phosphate buffer, pH 7.4, saturated with N-octanol, was added 20 µg of the compound (20 µl of a 1 mg ml⁻¹ pH 8-10 aqueous stock

solution). Three 0.3 ml samples were equilibrated with 0.3 ml n-octanol saturated with buffer by shaking for 30 min. The concentrations in the aqueous phase were assayed by HPLC.

Plasma protein binding:

Plasma protein binding was determined by equilibrium dialysis. Rat plasma pooled from six rats was used. Dialysis bags (diameter 6.4 mm) were filled with 1 ml plasma containing 15 µg of the racemate. Dialysis was performed against 4 ml of 0.066 M phosphate buffer, pH 7.4, containing 0.1% sodium azide in screw stoppered 5 ml flasks. The flasks were gently tumbled overnight at room temperature (22°C). Assay of the enantiomers in the buffer compartment and the plasma compartment were performed as described above.

Animal experiments:

Male Wistar rats, 250-300 g, were used and had free access to labfood and water. Under light ether anesthesia, the rats were fitted with a catheter (PE-10) in the left femoral artery. The catheter exteriorized in the neck. The animals were allowed to recover at least two days before use. The warfarin analogues, as racemates (1 mg/ml saline, pH 7-8), were administered subcutaneously in 2 mg/kg amounts. Blood samples, 200 µl, were taken at regular times and collected on 22 µl 0.1 M trisodium citrate in Eppendorf cuvettes. Plasma was prepared immediately and stored at -20°C until analyzed.

Determination of blood clotting activity:

Blood clotting activity was measured by the thrombotest assay (Nycomed, Oslo, Norway) and was expressed as % of control prothrombin complex activity (%PCA). Briefly, 50 µl citrated plasma was diluted with 50 µl Michaelis buffer, then 100 µl of a prewarmed solution of Thrombotest reagent was added (Nycomed, Oslo, Norway). After preincubation at 37°C for 1 minute the clotting process was started by the addition of 100 µl of a 0.033 M calcium dichloride solution. Clotting time was assayed automatically with a LC-78 coagulometer (Lode Instruments, Groningen, the Netherlands). Clotting time was converted to % PCA from a calibration curve obtained from various dilutions of pooled plasma from control rats.

Pharmacokinetic analyses:

The plasma half-life $t_{1/2}$ was estimated from the terminal elimination phase by linear regression. The area under the plasma concentration-time curve was calculated by the trapezoidal rule with the addition of the extrapolated part; $AUC_t + C_t \cdot t_{1/2} / \ln 2$, where AUC_t is the area from $t=0$ to the last concentration C_t estimated. Total plasma clearance Cl, assuming complete absorption of the dose D, was calculated by D/AUC , the apparent volume of distribution Varea by $Cl \cdot t_{1/2} / \ln 2$. The pharmacokinetic constants for the unbound fraction were obtained by correcting for the protein binding; e.g. $Cl_i = Cl/f_u$ is the clearance of the unbound fraction (intrinsic clearance).

Statistical evaluation:

Enantiomeric differences were tested for their significance by two-tailed paired t-tests.

TABLE 2.1: pKa and log P constants of ring-substituted warfarins

Compound	pKa	log P	log Px-logPh	Π_X
Warfarin	5.0	1.20	0.00	0.00
4'-nitro	5.0	0.98	-0.22	-0.28
3'-nitro	4.9	0.92	-0.28	-0.28
4'-cyano	n.d.	0.64	-0.56	-0.57
4'-chloro	4.9	2.03	0.83	0.71
3'-chloro	5.0	2.00	0.80	0.71
2'-chloro	5.1	2.02	0.82	0.71
4'-bromo	5.0	2.24	1.04	0.86
3'-bromo	n.d.	2.24	1.04	0.86
4'-methyl	4.9	1.71	0.51	0.56
3'-methyl	n.d.	1.73	0.53	0.56
2'-methyl	n.d.	1.92	0.72	0.56
4'-methoxy	5.1	1.25	0.05	-0.02
3'-methoxy	5.0	1.20	0.00	-0.02
2'-methoxy	5.2	1.59	0.39	-0.02

pKa and log P values were determined as described in the method section. n.d., not determined

Figure 2.2: Plasma concentration-time curves of the R- (open circles) and S-enantiomers (closed circles) of warfarin and some of its phenyl ring substituted analogues. Data are presented as the mean $\pm$ SD of 3-4 rats.

TABLE 2.2: Pharmacokinetic constants of ring substituted R/S warfarins in the rat.

Analogue		t _{1/2} (h)	AUC (µg.h/ml)	Cl (ml/h.kg)	Varea (ml/kg)
H	R	4.75 ± 0.29**	32.0 ± 4.0**	32.3 ± 4.2**	215 ± 14.7*
	S	8.75 ± 0.43	92.2 ± 4.3	10.9 ± 0.5	135 ± 0.6
4'-NO ₂	R	1.70 ± 0.10**	12.6 ± 0.7**	80.1 ± 4.6**	193 ± 4.3
	S	0.45 ± 0.05	3.5 ± 0.2	288.6 ± 21.0	184 ± 7.2
3'-NO ₂	R	2.9 ± 0.3*	22.4 ± 3.3**	49.0 ± 6.6*	195 ± 15
	S	5.2 ± 0.8	47.9 ± 8.6	26.2 ± 6.5	167 ± 19
4'-CN	R	7.8 ± 0.76**	39.2 ± 3.2**	25.9 ± 2.2**	295 ± 54*
	S	0.97 ± 0.17	7.1 ± 1.5	156 ± 37	197 ± 8
4'-Cl	R	1.96 ± 0.14**	11.9 ± 1.4	88.0 ± 9.3	239 ± 11*
	S	1.22 ± 0.07	10.4 ± 1.4	103 ± 14	176 ± 38*
3'-Cl	R	2.47 ± 0.07*	5.3 ± 0.1**	190 ± 3.7**	669 ± 13**
	S	7.8 ± 1.4	25.4 ± 4.2	41.3 ± 6.2	436 ± 7
2'-Cl	R	28.3 ± 5.9**	455 ± 10**	2.4 ± 0.4**	89 ± 1.4*
	S	3.5 ± 1.0	42.5 ± 12.2	27.1 ± 0.3	116 ± 6.1
4'-Br	R	2.58 ± 0.17**	30.5 ± 4.4*	34.4 ± 5.7**	124 ± 12.2*
	S	1.30 ± 0.14	14.4 ± 1.4	70.7 ± 7.5	128 ± 1.7
3'-Br	R	1.66 ± 0.13**	7.5 ± 0.3**	132.5 ± 5.5**	214 ± 14
	S	8.16 ± 0.35	45.2 ± 1.8	22.2 ± 0.9	259 ± 16
4'-CH ₃	R	1.43 ± 0.13	9.1 ± 0.6*	111.0 ± 6.6*	226 ± 0.8**
	S	1.62 ± 0.12	15.4 ± 1.1	65.7 ± 4.4	152 ± 8.8
3'-CH ₃	R	1.56 ± 0.14**	8.8 ± 0.6**	114 ± 8.0**	252 ± 10.2*
	S	7.3 ± 2.7	57.8 ± 16	21.6 ± 5.8	177 ± 6
2'-CH ₃	R	11.0 ± 1.5**	112.2 ± 16**	9.0 ± 1.2**	140 ± 1*
	S	3.4 ± 0.12	25.6 ± 4.9	40.6 ± 7.8	194 ± 30
4'-OCH ₃	R	1.51 ± 0.2	14.3 ± 1.5	71.3 ± 6.8	149 ± 5*
	S	1.64 ± 0.2	18.8 ± 1.8	54.3 ± 5.6	121 ± 4
3'-OCH ₃	R	2.9 ± 0.2**	16.4 ± 1.8*	62.5 ± 6.4*	258 ± 25*
	S	6.0 ± 0.14	46.4 ± 5.0	22.0 ± 2.3	188 ± 16
2'-OCH ₃	R	16.0 ± 0.6**	177 ± 13**	5.7 ± 0.4**	130 ± 4.7**
	S	2.5 ± 0.3	16.3 ± 1.4	62.2 ± 5.4	215 ± 7

Data are the mean ± SEM of 3-4 rats. * and **significant difference between enantiomers P<0.05 and P<0.01 respectively.

2.4 RESULTS:

The warfarin analogues investigated, are listed in table 2.1 together with some physicochemical parameters. The pKa values of the compounds did not differ greatly, ranging between 4.8 and 5.1, indicating no influence of the substituents on the acidity of the 4-hydroxy group of the coumarin nucleus. For warfarin and 4'-nitrowarfarin (acenocoumarol), our data are in agreement with those of Van der Giesen (1982).

The partition coefficient varied with the lipophilicity of the substituent. Except for 2'-methoxywarfarin, the correlation of $\log(P_1/P_H)$ with Hansch's Π_X parameter was good indicating that the substituents did not alter the molecular conformation in such a manner that deviations of the additivity of the fragmental lipophilicity occur (Hansch and Leo, 1979).

The pharmacokinetic constants of the warfarin analogues are presented in table 2.2 and for illustration, some examples of the impact of substituents on the plasma elimination are shown in figure 2.2. Table 2.3 presents the plasma protein binding of the compounds as well as the pharmacokinetic constants of the unbound fraction, i.e. the clearance (intrinsic clearance) and the volume of distribution. Intrinsic clearances of the enantiomers of the phenyl ring substituted warfarin analogues are higher than the intrinsic clearances of the enantiomers of warfarin itself. E.g. the intrinsic clearances of S-4'-nitro- and S-4'-bromowarfarin are more than 30 times higher than the intrinsic clearance of S-warfarin; for the R-enantiomers, substitution of the 3'-hydrogen by a bromo group enhances the intrinsic clearance about 33 fold. In addition, phenyl ring substitution has drastic effects on the enantioselectivity of the intrinsic clearances. The intrinsic clearance of R-warfarin is 1.4 times higher than that of S-warfarin. 3'-Substituted warfarins show a similar pattern of enantioselectivity with R/S ratios ranging from 1.3 (3'-nitrowarfarin) to 2.9 (3'-bromowarfarin). In contrast, for the 2'-methoxy- and 2'-chloro- analogues the intrinsic clearances of the S-enantiomers are 3.2 and 1.8 times higher than those of the R-enantiomers. For 2'-methylwarfarin, there is no difference between the enantiomers. Also in the case of 4'-nitro-, 4'-cyano, 4'-chloro- and 4'-bromowarfarin, the intrinsic clearances of the S-enantiomers are 1.7 to 9 times than the intrinsic clearances of the R-enantiomers. For 4'-methyl- and 4'-methoxywarfarin, no difference between the enantiomers is observed.

Phenyl ring substitution also has clear effects on the stereochemistry of the plasma protein binding (table 2.3) of the warfarin analogues. The plasma protein binding of warfarin, the 3'-, and the 4'-substituted warfarins is 1.1 (4'-bromowarfarin) to 2.4 (3'-chlorowarfarin) times stronger for the S-enantiomers and this stereoselectivity appears to be somewhat higher for the 3'- than for the 4'- substituted analogues. 2'-Substitution however, inverses this stereoselectivity: the R-enantiomers are bound 3 (2'-methoxywarfarin) to 6 (2'-chlorowarfarin) times stronger than the S-enantiomers.

Considering plasma protein binding, there is a significant correlation between $\log P$ and $\log(\text{bound/free})$; R: $\log B/F = 0.377 \log P + 1.626$, $r=0.711$; $p<0.01$ ($n=12$) S: $\log B/F = 0.380 \log P + 1.752$, $r=0.797$; $p<0.01$ ($n=12$). N.B.: the 2'-substituted derivatives have not been included in the correlations because their mechanisms of interaction with plasma proteins may be different, i.e. inversion of binding stereoselectivity (table 2.3).

TABLE 2.3: Plasma protein binding^a and pharmacokinetic constants^b of the unbound fraction of substituted warfarins in the rat.

Analogue		fu (%)	Cl/fu (l/h.kg)	Varea/fu (l/kg)
H	R	2.26 ± 0.06**	1.4 ± 0.1*	9.5 ± 1.7
	S	1.12 ± 0.03	1.0 ± 0.1	12 ± 0.3
4'-NO ₂	R	1.22 ± 0.06**	6.6 ± 0.5**	16 ± 1.6
	S	0.94 ± 0.04	31 ± 2.6	20 ± 1.2
3'-NO ₂	R	0.64 ± 0.01**	7.6 ± 1.0	30 ± 2.4
	S	0.44 ± 0.02	6.0 ± 1.5	38 ± 4.7
4'-CN	R	1.16 ± 0.05**	2.2 ± 0.2**	25 ± 4.8
	S	0.83 ± 0.04	19 ± 2.7	24 ± 1.5
4'-Cl	R	0.77 ± 0.06**	11 ± 1.5*	31 ± 2.8
	S	0.55 ± 0.04	19 ± 2.9	32 ± 7.3
3'-Cl	R	0.72 ± 0.07**	26 ± 2.6**	93 ± 9.2**
	S	0.30 ± 0.03	14 ± 2.6	145 ± 15
2'-Cl	R	0.14 ± 0.02**	1.7 ± 0.4**	62 ± 8.9**
	S	0.91 ± 0.12	3.0 ± 0.1	13 ± 0.3
4'-Br	R	0.24 ± 0.02	14 ± 2.6**	51 ± 6.6
	S	0.22 ± 0.01	32 ± 3.7	59 ± 2.8
3'-Br	R	0.29 ± 0.01**	46 ± 2.5**	108 ± 8.0**
	S	0.14 ± 0.01	16 ± 1.3	181 ± 17
4'-CH ₃	R	0.57 ± 0.01**	20 ± 0.9	40 ± 1.7
	S	0.40 ± 0.01	17 ± 1.2	38 ± 2.4
3'-CH ₃	R	0.64 ± 0.10**	18 ± 3.1**	39 ± 6.4*
	S	0.32 ± 0.02	6.6 ± 1.8	56 ± 4.0
2'-CH ₃	R	0.29 ± 0.03**	3.1 ± 0.5	39 ± 4.0**
	S	1.14 ± 0.02	3.6 ± 0.7	17 ± 2.6
4'-OCH ₃	R	0.79 ± 0.04**	9.0 ± 1.0	19 ± 1.2
	S	0.60 ± 0.04	9.1 ± 0.1	20 ± 1.5
3'-OCH ₃	R	0.87 ± 0.06**	7.2 ± 0.1**	30 ± 3.5
	S	0.66 ± 0.05	3.3 ± 0.4	28 ± 3.3
2'-OCH ₃	R	0.20 ± 0.02**	2.9 ± 0.3**	65 ± 6.9**
	S	0.69 ± 0.03	9.0 ± 0.9	31 ± 1.7

^aThe unbound fractions fu are the mean ± S.E.M. of 4 estimates in pooled rat plasma. ^bUnbound hepatic clearance and unbound apparent volume of distribution are calculated from the data of table 2.2. ** significant difference between enantiomers P<0.01 * significant difference between enantiomers P<0.05.

Figure 2.3: The response of the plasma prothrombin complex activity (PCA) in the experiments described in figure 2.2. A: warfarin (closed circles); 4'-nitrowarfarin (open circles); B: 2'-chlorowarfarin (open triangles); 3'-chlorowarfarin (closed triangles) 4' chlorowarfarin (open squares)

All compounds affected the blood clotting activity (trombotest value). Figure 2.3 shows the effect of responses of the compounds of which the kinetics are given in figure 2.2. Compared to racemic 4'-nitrowarfarin, the effect of racemic warfarin is more intense and longer lasting, consistent with a longer residence time of the latter. The same can be argued for racemic 3'-chlorowarfarin in comparison to the other chloro-substituted analogues (figure 2.3B). Remarkably, 2'-chlorowarfarin, notwithstanding the long half-life of the R-enantiomer, showed only weak anticlotting activity. This suggests the intrinsic activity of R-2'-chlorowarfarin to be weak. Also, the other 2'-substituted warfarins showed weak activity. As the change in prothrombin complex activity (PCA) following the administration of the racemic compounds is a function of the individual enantiomeric pharmacokinetics and pharmacodynamics, a comparative quantitative evaluation of the effects of the racemates is not possible from our experiments.

2.5 DISCUSSION:

This study, presenting a series of warfarin analogues, was performed to establish the influence of substituents on pharmacokinetics, prompted by the observation that the introduction of a 4'-nitro group (acenocoumarol) greatly enhances the (metabolic) clearance of both the enantiomers, enhances the enantioselectivity of the elimination in man, and reverses the enantioselectivity of elimination in the rat (Godbillon et al, 1981; Thijssen et al, 1985, 1986). The data for R/S-warfarin and R/S-4'-nitrowarfarin are comparable to those obtained in single enantiomer studies, indicating the absence of mutual interactions. This has also been observed in man (Banfield et al, 1983; Toon et al, 1986; Gill et al, 1988).

As the warfarin analogues have in common a relatively high lipophilicity (table 2.1) and a high plasma protein binding (table 2.3), it is reasonable to assume their elimination, like warfarin and 4'-nitrowarfarin, to be solely by hepatic metabolism. With this assumption the warfarin analogues tested can be classified as low clearance drugs, i.e. their plasma clearance is much less than the liver blood flow.

Therefore, their plasma pharmacokinetics are determined by plasma protein binding and intrinsic hepatic clearance (Wingard et al, 1978; Roland and Tozer, 1980). This study shows both parameters to be determining factors for the stereoselective pharmacokinetics of the compounds. The differences between the enantiomers in protein binding appear consistent with the differences observed for Varea, e.g. for all the racemates it holds that the enantiomer having the highest protein binding shows the lowest Varea and the highest peak concentration. However, there was no significant correlation between the protein binding and Varea; $r=0.166$ and -0.32 ($p<0.05$) for the R- and S-enantiomers, respectively.

In comparison with warfarin, the introduction of substituents greatly enhanced the intrinsic clearance, and in most cases also enhanced the stereoselectivity of the intrinsic clearance. A general stereochemical pattern in intrinsic clearance is evident; $S>R$ for the 4'-substituted and 2'-substituted derivatives, and $R>S$ for the 3'-substituted. The largest differences between the enantiomers are observed for 4'-cyano, 4'-nitrowarfarin and 3'-bromowarfarin. (table 2.3). Warfarin and 4'-nitrowarfarin have been shown to be eliminated by biotransformation, which mainly involves hydroxylation of the coumarin structure (Fasco et al, 1976; Pohl et al, 1976; Dieterle et al, 1977; Toon et al, 1986; Thijssen and Baars, 1987). With the exception of the methoxy and probably the methyl derivatives (see later), this will probably also be true for the warfarin analogues we studied. If this is true this would imply that phenyl ring substitution has clear effects on the rate and stereoselectivity of hydroxylation on the coumarin site of these compounds. In addition, the diversity of P450 isozymes expressing a broad spectrum of binding and reaction selectivity (Testa, 1988) may play a role in the differences observed between the analogues. To test these hypotheses the *in vitro* metabolism of warfarin and 4'-nitrowarfarin (acenocoumarol), has been studied in greater detail as will be discussed in the next chapters. The absence of stereoselectivity in the intrinsic clearance of 4'-methoxy and 4'-methylwarfarin is probably due to the fact that their main routes of metabolism, i.e. O-demethylation for 4'-methoxy-warfarin and methyl hydroxylation for 4'-methyl are not stereoselective (own observations). For some compounds, the intrinsic clearance greatly exceeded the liver plasma flow (about $2 \text{ l h}^{-1} \cdot \text{kg}^{-1}$; Daemen et al, 1989) of the rat. The strong protein binding, however, makes them low clearance drugs.

Remarkably, depending upon the position of the substituent inversions in the enantioselectivity of the plasma protein binding are observed. Warfarin and the 4'- and 3'-substituted analogues showed greater plasma protein binding for the S-enantiomer, but the opposite was true for the 2'-substituted analogues. Furthermore, the ratio in the enantioselective binding was high for the 2'-substituted warfarins. Recent studies, using stop-flow techniques, have presented evidence for a three-step process in the warfarin-albumin interaction, a rapid association followed by a rate-limiting translocation of the drug to a binding site inside a U-shaped cleft where the final binding complex is formed (Bos et al, 1989). The restricted rotation of the ortho-substituted phenyl ring would favor the positioning

of the R-configuration in that cleft: The stronger binding of the S-enantiomers of warfarin and 4'-nitrowarfarin to rat plasma protein has been shown with ratios for unbound fraction of about 1.6 (Yacobi and Levy, 1977) and 1.1 (Thijssen et al, 1985). In human plasma S-warfarin binding is stronger (Yacobi and Levy, 1977). For 4'-nitrowarfarin, human plasma protein binding has been reported to possess the opposite enantioselectivity i.e. the binding of the R-enantiomer is stronger than that of the S-enantiomer (Gill et al, 1988). This has also been reported for immobilized human plasma albumin: the greater binding was observed for S-warfarin and for R-acenocoumarol (Fitos et al, 1989). Binding to α_1 -acid glycoprotein has been reported for some drugs to have opposite stereoselectivity compared to albumin (Walle et al, 1983; Fitos et al, 1989). However, as the 4-hydroxycoumarins are weak acids, the contribution of α_1 -acid glycoprotein, which binds mainly cationic drugs, will be of minor importance (Urien et al, 1986).

The Varea of the unbound fraction for some compounds suggests stereoselective tissue binding. However, as the distribution volumes of the analogues are small 10-30% of the body mass (Table 2.2), and mainly occupy the albumin space, the stimulated unbound distribution volume is merely the result of the mathematical operation (Rowland and Tozer, 1980). The 3'-chloro derivative might be an exception; Varea values observed of 669 and 436 ml kg⁻¹ for the R- and S-enantiomers, respectively. We have no explanation for these relatively high distribution volumes.

In summary, the study affirms stereoselectivity in plasma protein binding and in intrinsic hepatic clearance to be determining factors in the enantioselective pharmacokinetics of low clearance drugs. For the warfarin analogues, the stereoselectivity in both processes was up to a factor 3-4 for protein binding and 8-9 for the intrinsic metabolic clearance. Small structural changes, i.e. the position of the substituent, may invert the stereoselectivity. The latter observations show that care should be taken in predicting the enantiomer specific pharmacokinetics of a racemate from data of closely related racemates.

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CHAPTER 3:

COMPARISON OF THE RAT LIVER MICROSOMAL METABOLISM OF THE ENANTIOMERS OF WARFARIN AND 4'-NITROWARFARIN (ACENOCOUMAROL).

3.1 ABSTRACT:

Rat liver microsomal metabolism of the enantiomers of warfarin and acenocoumarol has been studied. The enantiomers of both compounds are hydroxylated mainly at the 6- and 7-positions. Acenocoumarol enantiomers are much better substrates for cytochromes P450 than the corresponding warfarin enantiomers; K_m values for the 6- and 7-hydroxylations are 2 to 19 times lower for R- and S-acenocoumarol than for warfarin.

Formation of the 6-, 7-, and 8- hydroxy metabolites of warfarin is stereoselective for the R-enantiomer (the R/S ratio of the total intrinsic clearance is about 3). 4'-Hydroxylation was not stereoselective. In contrast, formation of acenocoumarol metabolites is stereoselective for the S-enantiomer (the S/R ratio of the total intrinsic clearance is about 3).

The P450 inducers phenobarbitone and methylcholanthrene and the inhibitor cimetidine show different effects on the *in vitro* metabolism of the enantiomers of both compounds. Antibodies raised to P450 2C6 and 2C11, as well as antibodies raised to the P450 3A subfamily inhibit warfarin and acenocoumarol metabolism not only to different extents, but stereo- and regiochemical differences of the antibody inhibitable P450's are also observed. P450's of the 2C subfamily in microsomes from phenobarbitone treated rats primarily catalyse the 7-hydroxylation of R-warfarin but show no or very little 6- and 8-hydroxylase activity. P450 2C isozymes catalyse the 6-, 7- and 8-hydroxylation of acenocoumarol non stereoselectively or with a slight preference for the S-enantiomer.

From these data it is concluded that the kinetic differences between warfarin and acenocoumarol enantiomers can partly be explained by the diversity of isozymes involved in the metabolism of both compounds. Substitution at the phenyl ring of warfarin has major effects on the regio- and stereoselectivity of P450 isozymes and on the spectrum of isozymes that contribute to the metabolism of the 4-hydroxycoumarins.

Adapted from: Xenobiotica 21: 295-307, 1991, J.J.R. Hermans, and H.H.W. Thijssen.

3.2 INTRODUCTION:

In the use of a racemic drug knowledge of the metabolism and disposition of both enantiomers is of great importance (Williams and Lee, 1985; Eichelbaum, 1988; Testa, 1988). Enantiomers may not only follow different metabolic routes but may also interact differently when administered together with other drugs (Lewis et al, 1974; Williams and Lee, 1985 ; Eichelbaum, 1988; Testa, 1988).

The oral anticoagulants warfarin and acenocoumarol (4'-nitrowarfarin) are administered as racemates. Despite their strong structural relationship, acenocoumarol and warfarin display sharp differences in their pharmacokinetics i.e. acenocoumarol enantiomers in both

Figure 3.1: The structure of warfarin, acenocoumarol, and warfarin metabolites.

R=H: warfarin; R=NO₂: acenocoumarol. The arrows indicate sites of hydroxylation of warfarin by cytochrome P450. The dehydro metabolite possesses a double bond between C-9 and C-10.

man and rat are eliminated faster than warfarin enantiomers (Yacobi and Levy, 1974; Thijssen et al, 1985; Kelly and O'Malley, 1979; Thijssen et al, 1986; chapter 2). Furthermore, differences in the stereochemical course of warfarin and acenocoumarol clearance are observed. In the rat, R-warfarin is eliminated faster than S-warfarin (Yacobi and Levy, 1974; chapter 2), whereas for acenocoumarol the opposite is true: S-acenocoumarol is eliminated faster than the R-enantiomer (Thijssen et al, 1985; chapter 2).

These differences cannot be attributed simply to differences in the plasma protein binding of warfarin and acenocoumarol enantiomers since the unbound fraction of the R-enantiomers of both warfarin and acenocoumarol is greater than the unbound fraction of the S-enantiomers (Yacobi and Levy, 1977; Thijssen et al, 1985, also see chapter 2).

For warfarin, multiple metabolic routes have been described in both rats and man (figure 3.1): hydroxylation at the coumarin moiety (6-, 7-, and 8-hydroxylation), the phenyl moiety (4'-hydroxylation), aliphatic hydroxylation (9- (benzyl), 10-hydroxylation, formation of dehydro-warfarin) and ketone reduction. Several of these routes show stereoselectivity and have been observed *in vivo* and *in vitro* (Trager et al, 1970; Fasco et al, 1976; Pohl et al, 1976; Powell et al, 1977; Rettie et al, 1989).

In the rat, the main metabolite of R-warfarin is the 7-hydroxy metabolite (7-hydroxywarfarin) whereas 7- and 4'-hydroxylation are the main metabolic routes for S-warfarin (Pohl et al, 1976). In rat liver microsomes R-warfarin is predominantly hydroxylated to 6-hydroxywarfarin and 7-hydroxywarfarin whereas S-warfarin is hydroxylated to 4'-, 6- and 7-hydroxywarfarin (Fasco et al, 1976; Pohl et al, 1977).

COMPARISON OF THE RAT LIVER MICROSOMAL METABOLISM OF THE ENANTIOMERS OF WARFARIN AND 4-NITROWARFARIN

In the rat, acenocoumarol enantiomers are hydroxylated at the 6- and 7- positions (Thijssen and Baars, 1987). To our knowledge, the *in vitro* metabolism of the enantiomers of acenocoumarol has not been described before.

In chapter 5 stereochemical aspects of the *in vitro* ketone reduction of warfarin, acenocoumarol and 4'-chlorowarfarin in several animal species, will be described and it will be shown that this metabolic route cannot explain the observed pharmacokinetic differences between warfarin and acenocoumarol enantiomers in rats. In this study the liver microsomal monooxygenase-mediated metabolism of warfarin and acenocoumarol has been compared in an attempt to elucidate the mechanism of the observed pharmacokinetic differences between warfarin and acenocoumarol enantiomers. In addition, the effect of enzyme induction by phenobarbitone and methylcholanthrene and the effect of the P450 inhibitor cimetidine (Rendic et al, 1983) on the metabolism of the 4-hydroxycoumarins has been investigated to determine whether the isozyme spectrum involved in the metabolism of both compounds is the same. Finally, the inhibiting effect of two monoclonal antibodies raised to cytochromes P450 2C6 and 2C11 and the P450 3A subfamily has been looked upon, since the isozymes to which these antibodies are raised are known to have warfarin hydroxylase activity (Guengerich et al, 1982).

3.3 MATERIALS AND METHODS:

All chemicals were of analytical grade and were obtained from Merck (Darmstadt, West Germany) unless stated otherwise.

Preparation of the R- and S-enantiomers of warfarin and acenocoumarol:

The respective enantiomers were isolated by the method of West et al (1961). Racemic warfarin was obtained from Sigma Chemicals (St. Louis, USA), acenocoumarol as well as 6- and 7-hydroxyacenocoumarol were a kind gift of Ciba-Geigy (Basel, Switzerland). The purity of the individual enantiomers was determined by the method of Banfield and Rowland (1984) and proved to be higher than 99% and 97 % for the S- and R-enantiomers, respectively.

Preparation of microsomal fractions:

Male Wistar rats (300 gr), acclimated to 12 h light-dark cycle and having free access to standard lab food (SMR A 1210, Woerden, the Netherlands) and tap water, were killed by exsanguination under ether anaesthesia. Livers were perfused *in situ* with ice-cold saline and subsequently removed.

To obtain microsomal fractions, 4 livers were pooled and homogenized in 3 vol. ice-cold Tris-KCl-sucrose buffer, pH=7.4 (0.02 M Tris-HCl, 0.15 M KCl and 0.25 M sucrose), using a Potter homogenizer at 1500 rpm. The homogenate was centrifuged at 10,000 g for 30 min, the pellet was discarded and the supernatant was centrifuged at 110,000 g for 50 min.

The 110,000 g pellet was washed twice by resuspension in the original volume of Tris/KCl buffer and centrifugation at 110,000 g for 50 min. The final microsomal pellet was resuspended in Tris-KCl-NaCl buffer (0.02 M, 0.15 M and 1.0 M, respectively) to contain about 20 mg protein per ml homogenate.

In the enzyme induction experiment, rats (n=4 in each group) were given phenobarbitone (PB; 0.1% w/v in drinking water for 6 days) or methylcholanthrene (MC; 25 mg/kg i.p. for 3 days). Control rats did not receive any treatment. The effect of the enzyme inducers was validated by measuring the cytochrome P450 content, 7-ethoxycoumarin deethylase activity and aniline p-hydroxylase activity (Rutten et al, 1987; Macleod et al, 1973; Greenlee and Poland, 1978). Protein content was determined by the method of Lowry (Lowry et al, 1951).

The microsomes, used in the enzyme kinetic experiment, were not from the same batch as control microsomes in the induction and inhibition experiment.

Assay for warfarin and acenocoumarol metabolism:

Microsomal fractions were incubated in the presence of an NADPH-generating system at 37 °C for 10 min in test tubes. The incubation mixture consisted of 2 mg microsomal protein in 0.2 mM substrate, 20.2 mM glucose 6-phosphate, 5.8 mM MgCl₂ and 2.8 μ units of glucose 6-phosphate dehydrogenase (Sigma Chemicals, St. Louis USA) and a total volume of 310 μl (Tris-KCl buffer, pH 7.4).

The enzyme kinetic parameters in control rat liver microsomes were determined at substrate concentrations ranging from 0.02 to 1.6 mM. The incubation mixtures were preincubated at 37°C for 2 min; then the reaction was started by the addition of 50 μl of a prewarmed (37°C) NADPH solution (1.6 mM in Tris-KCl). In the cimetidine inhibition experiment cimetidine was added to the incubation medium in a final concentration of 1.1 mM. Cimetidine samples were preincubated for 5 min at 37°C before the substrate and NADPH was added. Incubations of blanks, i.e. the complete reaction mixture without NADPH were run in parallel. All incubations were carried out in triplicate. The reaction was stopped after 10 min. by the addition of 0.5 ml ice-cold acetonitrile containing an internal standard (40 μg/ml). For warfarin metabolism, acenocoumarol was used as internal standard, whereas for acenocoumarol metabolism warfarin was used. The mixture was centrifuged at 2500 rpm for 5 min to precipitate the protein. The supernatant was evaporated to dryness under a stream of nitrogen at 30°C. For extraction of metabolites from the residue, 0.5 ml 0.1 M HCl solution and 4 ml of a 1:1 (v/v) mixture of methylene chloride and petroleum ether (boiling range 40-60°C) were added. This mixture was shaken for 10 min and subsequently centrifuged. The organic phase was evaporated to dryness under a stream of nitrogen at 30 °C. The residue was dissolved in 0.3 ml of a mixture of acetonitrile and water 1:1 v/v ; 10-20 μl was analyzed by HPLC.

The HPLC system consisted of Chromspher C18 200x3 mm (Chrompack, The Netherlands) as stationary phase and a mixture of 0,1% (aq.) acetic acid and acetonitrile (7:3 v/v)

Figure 3.2: Chromatograms of extracts of incubations of S-acenocoumarol and R-warfarin with PB-treated rat liver microsomes.

Racemic acenocoumarol was used as the internal standard for the incubation of R-warfarin. Racemic warfarin was used as the internal standard for the incubation of S-acenocoumarol.

Extractions were performed with petroleum ether and methylene chloride.

Abbreviations: 4'OH, 6 OH, 7 OH, 8 OH: 4'-, 6-, 7-, and 8-hydroxylated metabolites respectively (figure 1). benz: benzyl (9)-hydroxymetabolite. W: warfarin, AC: acenocoumarol.

brought to pH 4.76 (ammonia) as mobile phase. The u.v. absorption at 303 nm was monitored on a Kratos spectroflow 783 and areas were calculated on a Hitachi D-2000 integrator. Diode array detector analysis was carried out on a Applied Biosystems 1000S detector.

Immunoinhibition of warfarin and acenocoumarol metabolism:

Mouse monoclonal antibody raised against cytochromes P450 2C6 and 2C11 was a kind gift of Dr. Ina Goldfarb and Prof. Harry V. Gelboin from the Laboratory of Molecular Carcinogenesis, National Cancer Institute, Bethesda, Maryland, USA. Rabbit serum monoclonal antibody against the cytochrome P450 3A subfamily was a kind gift of Dr. Pierre G. Kremers (Laboratoire de Chimie Medicale, Université de Liege, Belgium). 1 mg of microsomal protein (about 50 μ l) was mixed with 500 μ g of antibody (20 μ l) and was allowed to stand for 30 minutes at room temperature, then NADPH regenerating system (15 μ l) was added and samples were preincubated for 5 minutes at 37 °C before the reaction was started by addition of substrate (15 μ l: final concentration 200 μ M). Then, the same procedure as for normal incubations (as described above) was followed. The amount of antibody was about three times the amount needed for maximal inhibition of microsomal metabolism by the isozymes against which the antibodies were raised (own observations; Waxman et al, 1987); cross reactivity with various other isolated P450s was not apparent (Waxman et al, 1987; Kremers 1990: personal communication).

Statistical analysis of the data:

Vmax and Km values were determined by fitting the Michaelis-Menten equation to the data using the GPAD (GraphPAD Software, San Diego, USA) software package.

The effect of enzyme inducers and of the antibodies on warfarin and acenocoumarol metabolism was analysed for significance by the use of one way analysis of variance, followed by a LSD test (least significant difference) (Sachs, 1984).

Differences in the inhibiting effect of cimetidine on warfarin and acenocoumarol metabolism between the microsomal fractions were tested for significance using a Kruskal and Wallis test (Sachs, 1984), followed by a Nemenyi test (Sachs, 1984).

Data were analysed for normality by calculating the quotient of the range and standard deviation of the data.

Figure 3.3: Dependency of the capacity factor of the metabolites of warfarin and acenocoumarol on the pH of the eluent.

Incubations with S-acenocoumarol or R-warfarin as substrate were carried out as described. The capacity factor k' is defined as $(R_{t-m} - R_{t-i})/R_{t-i}$ in which R_{t-m} and R_{t-i} represent the retention times of the metabolite- and solvent peaks respectively.

Capacity factors were determined using the HPLC conditions as described, except that the column was held at a constant temperature of 30 °C and that the mobile phase consisted of a 9/3 v/v mixture of 0.25 % v/v aqueous acetic acid and acetonitrile. The pH was adjusted by the addition of ammonia.

TABLE 3.1:U.v. spectral data of warfarin, acenocoumarol and metabolites.

Warfarin	213 (MX,100), 245 (MN,25), 278 (MX,50), 289 (MN,40), 302 (MX,49)
Acenocoumarol	213 (MX,100), 245 (MN,38), 285 (MX,77), 292 (MN,75), 300 (MX,76)
6-Hydroxywarfarin	200 (MX,100), 245 (MN,26), 278 (MX,35), 291 (MN,33), 302 (MX,34)
6-Hydroxyacenocoumarol	201 (MX,100), 245 (MN,21), 285 (broad MX,43)
7-Hydroxywarfarin	202 (MX,100), 248 (MN,16), 281 (MX,42), 287 (MN,40), 305 (MX,49)
7-Hydroxyacenocoumarol	201 (MX,100), 250 (MN,21), 284 (MX,40), 287 (MN,39), 307 (MX,53)
8-Hydroxywarfarin	202 (MX,100), 244 (MN,13), 267 (S,32), 278 (MX,39), 290 (MN,33), 301 , (MX,36)
m6	202 (MX,100), 241 (MN,11), 272 (S,33), 287 (MX,46), 292 (MN,45), 296 (MX,46)
10-Hydroxywarfarin	205 (MX,100), 221 (BP,36), 253 (MN,13), 294 (MX,35), 301 (S,32)
m3	204 (MX,100), 212 (BP,42), 242 (MN,6), 284 (MX,57), 296 (S,54)
9-hydroxywarfarin	202 (MX,100), 247 (MN,18), 279 (MX,33), 295 (MN,29), 302 (MX,30)
m5	205 (MX,100), 244 (MN,6), 279 (MX,49), 295 (MN,38), 300, (MX,39)

Incubations of warfarin and acenocoumarol with rat liver microsomes were carried out as described in the Materials and Methods section. Extracts of these incubations were subjected to diode array detector (Applied Biosystems 1000S) analysis, using the chromatographic conditions described in the Materials and Methods section. Wavelengths are given in nm.

MX: maximum; MN: minimum; S: shoulder; BP: bending point. Relative absorptions are given in parenthesis.

3.4 RESULTS:

Figure 3.2 shows HPLC chromatograms of incubation mixtures of PB-induced rat liver microsomes with R-warfarin and S-acenocoumarol as substrates. For warfarin, 5 metabolites have been found (in order of retention times): 4'- ($R_t = 4.7$ min), 6- ($R_t = 5.8$ min), 9- ($R_t = 6.9$ min), 8- ($R_t = 7.4$ min) and 7-hydroxywarfarin ($R_t = 10.1$ min) (figures 3.1 and 3.2). At high substrate concentrations the formation of RR/SS alcohols, dehydro-warfarin and 10-hydroxywarfarin ($R_t = 2.3$, 2.8, and 3.8 min, respectively) were also observed. For acenocoumarol, next to the 6- and 7-hydroxylated metabolites ($R_t = 7.5$ and 10.4 min respectively), 5 additional metabolites have been found, annotated as m1, m2, m3, m5 and m6 ($R_t = 4.1$, 4.8, 6.7, 8.6, and 9.6 min respectively) according to their retention times on the HPLC system used (figure 3.2).

At high substrate concentrations, the formation of alcohol 2 ($R_t = 2.1$ min) is also found. Reduction of the nitro group has not been observed. The structure of m1 has been shown to be identical with dehydro-acenocoumarol by co-chromatography with the dehydro-acenocoumarol synthesised by the method of Fasco et al (1978).

For tentative identification of acenocoumarol metabolites, the u.v. spectra of warfarin and acenocoumarol metabolites (using diode array detector analysis), have been recorded and the effect of change of pH of the mobile phase on their retention times is determined.

The u.v. spectral data are shown in table 3.1. The data suggest a structural relation between m6 and 8-hydroxywarfarin: the spectra show a typical shoulder at 267 (8-hydroxywarfarin) and 272 nm (m6) respectively. The u.v. spectrum of m3 shows similarities to that of 10-hydroxywarfarin: a typical bending point and a typical shoulder are observed at 212 and 296 nm (m3) and at 221 and 301 nm (10-hydroxywarfarin) respectively. The spectra of m5 and 9-hydroxywarfarin show great similarities to the spectra of their parent compounds; however, the minima at 289 (warfarin) and 292 nm (acenocoumarol) are shifted to 295 nm; furthermore, the relative absorption in this region is changed for the metabolites, resulting in a substantially higher maximum at 279 nm than at 300 nm, when compared with the parent compounds.

The effect of pH of the mobile phase on the retention times of the metabolites has been established in the pH range of 4.40 to 4.75 as shown in figure 3.3. In this pH range the retention times of the 6- and 7-hydroxylated metabolites of both warfarin and acenocoumarol show a linear decline with rising pH. In contrast, the change in retention times of 8-hydroxywarfarin and m6 show an inverse sigmoid pattern (bending points at a pH of 4.57 and 4.68 respectively). The retention times of 9-hydroxywarfarin, m3 and m5 do not change drastically in this pH range.

Based on the u.v. spectral data and the chromatographic properties of the metabolites, m3, m5, and m6 are tentatively identified as 10-hydroxy-, 9-hydroxy-, and 8-hydroxyacenocoumarol, respectively. Additional support is given by the fact that incubation of rat liver microsomes with warfarin and acenocoumarol, in the presence of tert-butylperoxide resulted in the formation of two products for each substrate: in the case of warfarin, the dehydro- and

Figure 3.4: Substrate dependency of the rates of formation of the 6- and 7-hydroxylated metabolites of warfarin and acenocoumarol enantiomers.

The Michaelis-Menten curve was fitted to the data by non-linear regression. Data are expressed as the mean $\pm$ SD of three incubations. Pooled liver microsomes of 4 untreated rats were used as enzyme source.

the 9-hydroxymetabolite were formed (Fasco et al, 1978), whereas for acenocoumarol m1 (dehydroacenocoumarol) and m5 (9-hydroxyacenocoumarol) were formed.

The formation of warfarin and acenocoumarol metabolites is linear with time for at least 30 min. Metabolite formation rates are linear with protein content in the range of 0.8 to 12 mg of protein per ml of incubation mixture.

The formation rates of the metabolites of both warfarin and acenocoumarol as a function of substrate concentrations have been established. With the exception of the 9-hydroxy metabolites of both substrates, the formation of all metabolites follows Michaelis-Menten kinetics. V vs. $[S]$ plots for the 6- and 7-hydroxylations are given in figure 3.4. The formation of the 8-hydroxylated metabolite of R-acenocoumarol shows biphasic kinetics (figure 3.5), indicating two distinct enzyme systems. The enzyme kinetic parameters are summarized in table 3.2. The data show acenocoumarol to be a better substrate for the rat liver mixed function oxygenases than warfarin since the apparent K_m values of acenocoumarol enantiomers are

TABLE 3.2: Enzyme kinetic parameters of rat liver microsomal warfarin and acenocoumarol metabolite formation.

Metabolite	R-Warfarin			S-Warfarin		
	Vmax	Km	Cl _i	Vmax	Km	Cl _i
4'-Hydroxy	56.3	134	403	46.8	120	384
6-Hydroxy	360	123	2927	155	171	906
8-Hydroxy	30.5	33.8	900	12.1	74.4	163
7-Hydroxy	350	125	2800	57.0	85.6	666
total			7030			2119

Metabolite	R-Acenocoumarol			S-Acenocoumarol		
	Vmax	Km	Cl _i	Vmax	Km	Cl _i
10-Hydroxy	nd	nd	nd	112	142	788
6-Hydroxy	220	57.1	3853	237	50.4	4710
8-Hydroxy	12.1	7.9	1532	27.4	9.8	2796
	15.5	120	129 *			
7-Hydroxy	52.2	10.1	5168	96.5	4.5	21444
total			10682			29738

Enzyme kinetic parameters were determined by non-linear regression. Vmax is the maximal reaction rate (pmol/mg protein x min). Apparent Km values are given in μ M, the intrinsic clearances (Cl_i) in nl/mg protein x min . The totals are the sum of intrinsic clearances. nd = not detected (detectable at the highest substrate concentrations only)

* The formation of the 8-hydroxy metabolite of R-acenocoumarol was biphasic and was analyzed using an Eady-Hofstee plot (figure 3.5) and linear regression analysis.

lower than those of the corresponding warfarin enantiomers. The Km values for the major routes of metabolism, viz., 6- and 7-hydroxylation, are 2 to 19 times lower for acenocoumarol than for warfarin. The Vmax values of the microsomal hydroxylation reactions are 1.5- to 2-fold higher for S-acenocoumarol than for S-warfarin. The opposite is true for the R-enantiomers.

Figure 3.5: Eady-Hofstee plot of the rate of formation of the 8-hydroxylated metabolite of the R-enantiomer of acenocoumarol.

Data are expressed as the mean of 3 incubations ; error bars indicate SD values.
In the experiment pooled liver microsomes of 4 control rats were used.

The total (*in vitro*) intrinsic clearance of R-acenocoumarol is about 1.5 times the intrinsic clearance of R-warfarin. The difference in the total clearances is far more pronounced for the S-enantiomers: the total intrinsic clearance of S-acenocoumarol is 15 times higher than the intrinsic clearance of S-warfarin.

Concerning substrate stereoselectivity, the data clearly show the hydroxylation of the coumarin site (to 6-, 7-, and 8-hydroxywarfarin) to be stereoselective for the R-enantiomer of warfarin (the intrinsic clearances being 3 to 5 times higher for the R-enantiomer). In the case of acenocoumarol, stereoselectivity for the S-enantiomer is observed (S/R ratios of the intrinsic clearances being 1.5 to 4). The phenyl hydroxylation (at the 4' position) of warfarin is not stereoselective.

To establish whether the isozyme spectrum involved in warfarin and acenocoumarol metabolism is the same, the effect of some enzyme inducers and the P450 inhibitor cimetidine has been investigated.

TABLE 3.3: The effect of treatment with phenobarbitone (PB) or methylcholanthrene (MC) on the metabolism of warfarin and acenocoumarol enantiomers by rat liver microsomal preparations.

Warfarin				
Metabolite		UT#	PB	MC
6-Hydroxy	R	95.3 ± 1.2	12.0 ± 0.9*	22.7 ± 21.1*
	S	18.9 ± 3.9	18.6 ± 0.7	7.0 ± 1.2*
7-Hydroxy	R	55.3 ± 14.7	128.2 ± 7.6*	69.8 ± 17.3
	S	40.5 ± 6.4	15.2 ± 3.2*	28.7 ± 1.7
8-Hydroxy	R	7.2 ± 0.1	7.9 ± 1.4	11.2 ± 0.1*
	S	7.9 ± 0.1	6.0 ± 1.4	nd *
9-Hydroxy	R	43.3 ± 3.2	75.5 ± 2.2*	23.4 ± 11.0
	S	11.7 ± 2.2	48.4 ± 1.1*	9.0 ± 2.7

Acenocoumarol				
Metabolite		UT#	PB	MC
6-Hydroxy	R	135.8 ± 5.4	375.4 ± 11.9*	185.1 ± 17.6*
	S	117.6 ± 3.9	263.8 ± 27.8*	168.4 ± 4.7*
7-Hydroxy	R	39.4 ± 1.8	80.6 ± 1.1*	51.5 ± 2.6*
	S	114.2 ± 7.2	147.8 ± 12.9	127.8 ± 13.2
8-Hydroxy	R	6.4 ± 5.4	39.4 ± 2.2*	nd
	S	13.2 ± 3.1	40.3 ± 3.8*	14.7 ± 3.6
9-Hydroxy	R	8.9 ± 8.9	40.5 ± 14.3	4.3 ± 3.4
	S	14.0 ± 1.8	33.0 ± 4.6*	11.0 ± 2.2

Data are expressed as pmol of metabolite formed /min per mg of protein, and are the mean ± SD of 3 incubations (pooled liver microsomes of 4 rats in each group),

UT: microsomes from untreated rats.

* significantly different from untreated rat liver microsomes

P<0.001 (ANOVA, LSD).

nd = not detectable.

The effect of the enzyme inducers phenobarbitone (PB) and methylcholanthrene (MC) on the *in vitro* warfarin and acenocoumarol metabolism is shown in table 3.3. PB-treated rat liver microsomes show significantly higher rates of formation ($P<0.001$) of 9-hydroxywarfarin for both enantiomers. The 6-hydroxylation of R-warfarin is lowered by PB treatment ($P<0.001$), whereas the 6-hydroxylation of S-warfarin is not affected by PB treatment. A clear difference between enantiomers in the response to PB treatment is seen in the formation of 7-hydroxywarfarin, which is significantly enhanced for the R-enantiomer but depressed for the S-enantiomer ($P<0.001$).

For both acenocoumarol enantiomers, PB-treated rat liver microsomes show significantly ($P<0.001$) higher rates of formation of 6-hydroxy and 8-hydroxyacenocoumarol, whereas 7-hydroxylation is enhanced for R-acenocoumarol only. The opposite is true for the 9-hydroxylation, which is induced by PB for S-acenocoumarol only. In general, PB treatment has a greater enhancing effect on the rates of formation of metabolites of acenocoumarol than of warfarin.

Microsomes from MC-treated rats generally show lower rates of formation of warfarin metabolites than control rat liver microsomes, which is significant for the 6-hydroxylation of R- and S-warfarin and the 8-hydroxylation of the S-enantiomer. In contrast, the 8-hydroxylation of R-warfarin is significantly induced ($P<0.001$) by MC pretreatment.

Unlike warfarin metabolism, the rates of formation of acenocoumarol metabolites are not depressed by treatment with MC but are enhanced or not affected. The 6-hydroxylation of R- and S-acenocoumarol, as well as the 7-hydroxylation of R-acenocoumarol is significantly enhanced by MC ($P<0.001$).

Table 3.4 summarizes the effect of cimetidine on microsomal warfarin and acenocoumarol metabolism for the various microsomal preparations. The data are expressed as % inhibition. As shown, the effect of cimetidine is dependent on the substrate and on the microsomal source used, e.g. the metabolism of S-warfarin is inhibited to a greater extent than the metabolism of R-warfarin. This effect is most pronounced for 6- and 7-hydroxywarfarin. The formation of the 6-hydroxymetabolite of S-warfarin is inhibited to a lesser extent in MC microsomes than in control microsomes ($P<0.01$).

The inhibition of acenocoumarol metabolism by cimetidine does not show a general stereoselective pattern. Like warfarin, the extent of inhibition of acenocoumarol metabolism by cimetidine is dependent on the microsomal source used. The formation of 7- and 8-hydroxyacenocoumarol for both enantiomers is inhibited to a significantly lesser extent in PB-treated than in control microsomes. In contrast, inhibition of the formation of the 6-hydroxymetabolite of R-acenocoumarol in PB-treated microsomes is significantly greater ($P<0.01$).

In order to obtain more detailed information on the nature and selectivity of the isozymes being involved in warfarin and acenocoumarol metabolism, the effect of two monoclonal

TABLE 3.4: Inhibition of rat liver microsomal metabolism of warfarin and acenocoumarol enantiomers by cimetidine.

Warfarin				
Metabolite		UT	PB	MC #
6-Hydroxy	R	57 ± 11	45 ± 13	62 ± 13
	S	92 ± 19	74 ± 7	4 ± 3 *
7-Hydroxy	R	45 ± 13	65 ± 12	23 ± 12
	S	100	95 ± 8	100
8-Hydroxy	R	100	100	100
	S	100	100	nd
9-Hydroxy	R	54 ± 19	33 ± 11	63 ± 13
	S	92 ± 22	26 ± 16	100

Acenocoumarol				
Metabolite		UT	PB	MC#
6-Hydroxy	R	79 ± 6	95 ± 3 *	79 ± 8
	S	82 ± 10	78 ± 8	86 ± 9
7-Hydroxy	R	87 ± 10	55 ± 9 *	67 ± 22
	S	93 ± 10	38 ± 10 *	85 ± 18
8-Hydroxy	R	100	57 ± 9 *	nd
	S	100	33 ± 13 *	100
9-Hydroxy	R	58 ± 11	12 ± 8	5 ± 3 *
	S	86 ± 7	74 ± 5	100 *

Incubation mixtures were preincubated with cimetidine (final concentration 1.1 mM) at 37 °C for 5 min before the substrate (final concentration 0.2 mM) was added. The data are expressed as the percentage inhibition (mean of 3 incubations) ± SD (pooled microsomes n=4 in each group).

UT= untreated, PB= phenobarbitone, MC= methylcholanthrene.

* significantly different from untreated rat liver microsomes P<0.01 (Kruskall and Wallis, Nemenyi test).

nd = not detectable without inhibitor.

TABLE 3.5: The effect of the monoclonal antibody against P450 2C6/2C11 on rat liver microsomal warfarin and acenocoumarol metabolism.

Substrate	R-WARFARIN			S-WARFARIN		
metabolite	UT	PB	MC	UT	PB	MC
4'-hydroxy	18 ± 4	25 ± 4*	15 ± 2*	7 ± 2	14 ± 4	26 ± 4*
6-hydroxy	-3 ± 4	-11 ± 3	-5 ± 4	-5 ± 3	4 ± 7	5 ± 4
7-hydroxy	22 ± 5*	44 ± 4*	-1 ± 5	26 ± 1*	45 ± 6*	10 ± 11
8-hydroxy	-70 ± 10*	-61 ± 8*	-56 ± 12*	-21 ± 2*	-19 ± 3*	nd
9-hydroxy	22 ± 4*	44 ± 3*	-2 ± 7	12 ± 5	6 ± 8	-5 ± 9
Substrate	R-ACENOCOUMAROL			S-ACENOCOUMAROL		
metabolite	UT	PB	MC	UT	PB	MC
6-hydroxy	-3 ± 8	14 ± 1*	4 ± 7	19 ± 1*	17 ± 1*	28 ± 9*
7-hydroxy	16 ± 1*	30 ± 1*	24 ± 2*	18 ± 1*	30 ± 2*	30 ± 6*
8-hydroxy	46 ± 12*	31 ± 1*	nd	27 ± 9*	32 ± 5*	13 ± 4
9-hydroxy	0 ± 9	4 ± 1	2 ± 3	-4 ± 9	11 ± 5	1 ± 5

Data are expressed as mean % inhibition ± SD. Positive values imply an inhibition, negative values imply an enhancement. UT, PB, MC, microsomes from untreated, phenobarbitone treated and methylcholanthrene treated rats (n=4 per group) respectively.

* Significant inhibition, $P < 0.025$, ANOVA, LSD

antibodies raised against P450 isozymes, has been investigated. Antibody 2C was raised against P450 2C6/C11, whereas antibody 3A was raised against the P450 3A subfamily. These P450's are known to have warfarin hydroxylase activity (Guengerich et al, 1982). Table 3.5 shows the effect of monoclonal antibody 2C on warfarin and acenocoumarol metabolism in various rat liver microsomal fractions. Antibody 2C inhibits the 7-hydroxylation of R- and S-warfarin in microsomes from untreated or PB treated rats ($P < 0.025$) but not in microsomes from MC treated rats. The extent of inhibition does not appear to be different for R- and S-warfarin but is higher in PB microsomes than in control microsomes. The 9-hydroxylation in control and PB microsomes appears to be stereoselectively inhibited for R- ($P < 0.025$) but not S-warfarin. In contrast, the 8-hydroxylation of warfarin is enhanced about 1.6 fold for the R- and 1.2 fold for the S-enantiomer ($P < 0.025$ in both cases), in microsomes where this metabolite was found under normal (without antibody) conditions. This may be due to a decrease of competition of the 8-hydroxylase with P450 2C6 and 2C11 towards the P450 reductase or to a prevention of isozyme complexation (Kaminsky and Guengerich, 1985).

TABLE 3.6 The effect of the monoclonal antibody against the P450 3A subfamily on rat liver microsomal warfarin and acenocoumarol metabolism.

Substrate	R-WARFARIN			S-WARFARIN		
metabolite	UT	PB	MC	UT	PB	MC
4'-hydroxy	8 ± 2	13 ± 1*	9 ± 6	10 ± 3	3 ± 6	15 ± 3
6-hydroxy	-3 ± 5	18 ± 8	-24 ± 14	4 ± 4	42 ± 4*	-30 ± 16
7-hydroxy	-12 ± 9	-10 ± 7	-19 ± 9	9 ± 6	30 ± 6*	-13 ± 7
8-hydroxy	5 ± 5	2 ± 1	64 ± 20*	12 ± 1*	-1 ± 3	nd
9-hydroxy	-8 ± 5	-9 ± 9	2 ± 2	1 ± 4	-10 ± 4	-13 ± 10
Substrate	R-ACENOCOUMAROL			S-ACENOCOUMAROL		
metabolite	UT	PB	MC	UT	PB	MC
6-hydroxy	5 ± 8	23 ± 2*	26 ± 6*	1 ± 5	-6 ± 1	-6 ± 1
7-hydroxy	2 ± 1	-3 ± 1	5 ± 1	19 ± 5*	-7 ± 1	19 ± 1*
8-hydroxy	-9 ± 5	0 ± 1	nd	4 ± 2	3 ± 4	-9 ± 4
9-hydroxy	35 ± 1*	30 ± 2*	14 ± 10	-7 ± 9	19 ± 4*	16 ± 5

Data are expressed as mean % inhibition ± SD. Positive values imply an inhibition, negative values imply an enhancement. UT, PB, MC, microsomes from untreated, phenobarbitone treated and methylcholanthrene treated rats (n=4 per group) respectively.

* Significant inhibition, P<0.025, ANOVA, LSD

In the case of acenocoumarol, the 7-hydroxylation is significantly (P<0.025) inhibited by antibody 2C in all microsomes in a non-stereoselective manner. In contrast, the 6-hydroxylation of S-acenocoumarol is inhibited in all microsomes, whereas the 6-hydroxylation of the R-enantiomer is inhibited in PB microsomes only. The 8-hydroxylation of R- and S-acenocoumarol is inhibited in microsomes from untreated and PB treated rats (P<0.025).

The effect of antibody 3A is shown in table 3.6. Antibody 3A inhibits the 8-hydroxylation of R-warfarin in MC microsomes and of S-warfarin in control microsomes significantly. In addition the 6- and 7-hydroxylation of S-warfarin in PB treated microsomes is inhibited by this antibody (P<0.025).

In the case of R-acenocoumarol, the 6-hydroxylation in PB and MC microsomes and the 9-hydroxylation in control and PB microsomes is inhibited by antibody 3A (P<0.025). The 7-hydroxylation of S-acenocoumarol is inhibited by antibody 3A in MC microsomes only, whereas the 9-hydroxylation of S-acenocoumarol is inhibited in PB microsomes only (P<0.025).

Figure 3.6: Estimated formation rates of warfarin and acenocoumarol metabolites by cytochromes P450 2C6 and 2C11 in microsomes from untreated, phenobarbitone treated and methylcholanthrene treated rats.

Data are expressed as the mean of three incubations; error bars indicate SD values. 4'-OH, 6-OH, 7-OH, 8-OH, 9-OH indicate metabolites hydroxylated at the 4'-, 6-, 7-, 8-, and 9-positions.

Tables 3.5 and 3.6 give a good indication of the relative contribution of the P450's that are inhibitable by antibodies 2C and 3A to warfarin and acenocoumarol metabolism, but provide no information on the stereo- and regioselectivity and inducibility of these isozymes. Therefore, the absolute decrease of metabolite formation rates by antibody 2C and 3A, which represents the hydroxylase activity of P450 isozymes that are inhibited by the antibodies is given in figures 3.6 and 3.7.

Figure 3.7: Estimated formation rates of warfarin and acenocoumarol metabolites by cytochromes P450 from the 3A subfamily in microsomes from untreated, phenobarbitone treated and methylcholanthrene treated rats.

Data are expressed as the mean of three incubations; error bars indicate SD values. 4'-OH, 6-OH, 7-OH, 8-OH, 9-OH indicate metabolites hydroxylated at the 4', 6-, 7-, 8-, and 9-positions.

Figure 3.6 clearly shows that there are stereo- and regioselective differences in the metabolism of warfarin and acenocoumarol by P450's that are inhibited by antibody 2C. P450 2C6 is reported to be phenobarbitone inducible and to have warfarin 7-hydroxylase activity (Guengerich et al, 1982), whereas P450 2C11, a (rat) male specific, constitutive

testosterone 16 α -hydroxylase (Nebert and Gonzalez, 1987) primarily shows warfarin 4'-hydroxylase activity (Guengerich et al, 1982). Consistent with these findings we found that P450's that are inhibited by antibody 2C are able to hydroxylate warfarin at the 4' position and the 7-position. In addition the latter reaction is inducible by phenobarbitone, whereas the 4'-hydroxylation is not induced. Also, as would be expected, none of the hydroxylation reactions of warfarin and acenocoumarol is induced by MC. Next to warfarin 4'- and 7-hydroxylation, the members of the rat P450 2C subfamily show some warfarin 9-hydroxylase activity.

In the case of acenocoumarol however, rat P450 2C isozymes are also involved in the 6- and 8-hydroxylation. The 6-hydroxylation is carried out stereoselectively for the S-enantiomer in all microsomes but is significantly induced by phenobarbitone only in the case of the R-enantiomer. The 8-hydroxylation does not appear to be stereoselective for P450 2C isozymes and is induced by PB. In contrast to warfarin, the 7-hydroxylation of acenocoumarol by antibody 2C inhibitable isozymes, which is induced by PB, does not show a clear stereoselectivity.

Figure 3.7 shows the regio- and stereoselectivity of cytochrome P450 isozymes that are inhibited by the monoclonal antibody raised against the P450 3A subfamily. The rat (liver) cytochrome P450 3A subfamily consists of at least two isozymes, 3A1 and 3A2, that are inducible by pregnenolone 16 α -carbonitrile and to a lesser extent by PB (Nebert and Gonzalez, 1987, Nebert et al, 1991). P450 3A1 is shown to have 4'-hydroxylase activity (Guengerich et al 1982). Information on the catalytic activity of P450 3A2 towards warfarin enantiomers is not known. However, human P450 isozymes from the 3A subfamily have been shown to have 10- and 4'-hydroxylase activity along with some S-warfarin 7- and 6-hydroxylase activity (Rettie et al, 1992). Since antibody 3A was raised against P450's from the 3A subfamily, P450 3A1 as well as P450 3A2 will be inhibited by this antibody. Figure 3.7 shows that P450's of the 3A subfamily appear to be involved in the 4'-hydroxylation of warfarin in all microsomes; this reaction does not appear to be induced. P450 3A isozymes carry out the 6-hydroxylation of both warfarin enantiomers in PB microsomes. In contrast, P450 isozymes that are inhibited by antibody 3A conduct the 7-hydroxylation of S-warfarin but not R-warfarin; this reaction is induced by PB. In addition P450 3A isozymes possess some warfarin 8-hydroxylase activity.

In sharp contrast to warfarin, P450's 3A catalyse the 6-hydroxylation of only the R-enantiomer of acenocoumarol in PB and MC microsomes. In addition, these P450 isozymes conduct the 7-hydroxylation of acenocoumarol in a stereoselective manner for the S-enantiomer in control and in MC microsomes but, contrary to warfarin, not in PB microsomes. Finally, some 9-hydroxylase activity is observed.

3.5 DISCUSSION:

The aim of this study was to elucidate the mechanism behind the remarkable differences between the clearances of R- and S-warfarin and R- and S-acenocoumarol. Enzyme kinetics of the various metabolic routes rather than rates of formation were studied because the established parameter $V_{\max}/K_m$ is more appropriate for comparison with *in vivo* clearance data (Rane et al., 1977). This approach was recently also applied by Rettie et al (1989) to compare the *in vitro* and *in vivo* metabolism of warfarin in man.

The study shows the *in vitro* metabolism of acenocoumarol to involve the same metabolic reactions as warfarin, except for the 4'-hydroxylation, i.e. hydroxylation occurs at the coumarin moiety at the 6-, 7-, and 8-positions and at C9 (benzyl-hydroxy) and C10 (figure 3.1). However, the enantiomers of acenocoumarol appear to be better substrates for these reactions; the apparent K_m values are 2 (6-hydroxylation of R-acenocoumarol) to 19 (7-hydroxylation of the S-enantiomer) times lower (table 3.2).

The lower K_m value clearly is the main factor determining the higher intrinsic clearance of the metabolism of the enantiomers of acenocoumarol vs warfarin, which is in accordance with the *in vivo* observations (see chapter 2). Also in agreement with the *in vivo* observations is the fact that the *in vitro* clearance of warfarin is stereoselective for the R-enantiomer, whereas the *in vitro* clearance of acenocoumarol shows stereoselectivity for the S-enantiomer.

In several studies it has been shown that the hydroxylations of warfarin enantiomers can be catalysed by various cytochrome P450 isozymes (Guengerich et al, 1982 ; Kaminsky et al, 1984 ; Kaminsky, 1989). Also for the 4-hydroxycoumarin, phenprocoumon, the involvement of various P450 isozymes has been suggested (Wheeler et al, 1981). Likewise this will probably also hold for the enantiomers of acenocoumarol. The question then arises whether the set of P450 isozymes involved in the hydroxylations of R- and S-warfarin will catalyse the hydroxylations of R- and S-acenocoumarol to the same extent. The inhibition study with the P450 inhibitor (Rendic et al, 1983; Hoensch et al, 1985; Miners et al, 1988) cimetidine shows that the 7-hydroxylation in control microsomes is almost completely inhibited by cimetidine for R-acenocoumarol, but only partly for R-warfarin, despite the higher K_m value of the latter. This may indicate that different P450 isozymes are involved in the metabolism of warfarin and acenocoumarol. The induction and inhibition study also points in that direction. For instance, the 6-hydroxylation of R-warfarin is lowered by PB and MC, whereas the 6-hydroxylation of R-acenocoumarol is enhanced by PB and MC. Also, the 6-hydroxylation of R-acenocoumarol is inhibited by cimetidine to a greater extent in PB-treated microsomes than in control microsomes; this is not the case for R-warfarin. For the other metabolic routes, differences between warfarin and acenocoumarol in response to PB and MC treatment, and to inhibition (of induced microsomes vs. control microsomes) by cimetidine, can also be observed (tables 3.2 and 3.3). Taken together, these data strongly indicate that some of the differences in the metabolism of warfarin and acenocoumarol can be attributed to the diversity of enzymes involved in the major metabolic pathways of these substances; i.e. the cytochrome P450 isozymes involved in the (coumarin) hydroxylations of R- and S-warfarin

may not catalyse the same reactions for R- and S-acenocoumarol and *vice versa*, or to only minor extents.

Additional evidence for this hypothesis is provided by the immuno-inhibition experiments. P450's of the 2C and 3A subfamily are clearly involved in the metabolism of both 4-hydroxycoumarins but show a different stereo- and regioselectivity in the coumarin hydroxylation of warfarin and that of acenocoumarol (figures 3.6 and 3.7). E.g. P450 2C isozymes are able to hydroxylate acenocoumarol but not warfarin at the 6-position. On the other hand the contribution (table 3.5) of P450 2C isozymes to the 7-hydroxylation of warfarin in PB and control microsomes appears to be greater than that of acenocoumarol, whereas the opposite is true in MC microsomes. The P450 3A subfamily conducts the 6-hydroxylation of warfarin in a non-stereoselective manner but shows high stereoselectivity for the R-enantiomer of acenocoumarol; S-acenocoumarol does not appear to be hydroxylated at the 6-position by these isozymes. The relative contribution of P450 3A isozymes in the 6-hydroxylation of S-warfarin in PB microsomes is about twice as high as in the 6-hydroxylation of the R-enantiomers of warfarin and acenocoumarol (table 3.6).

These examples demonstrate that 1) cytochrome P450 isozymes are involved in the metabolism of the enantiomers of warfarin and acenocoumarol to different extents 2) even if the same (sets of) isozymes are looked upon, both substrates are hydroxylated with different stereo- and regioselectivity. This indicates that the phenyl ring of warfarin is highly involved in the interaction with the apoprotein and that the substituents additionally determine selectivity towards cytochrome P450 isozymes.

The 4-hydroxycoumarin phenprocoumon, like acenocoumarol also shows stereoselective (*in vitro*) metabolism for the S-enantiomer (Wheeler et al, 1981). Heimark and Trager (1985), in explaining this discrepancy with warfarin, suggested that the R-enantiomer of warfarin has the possibility to mimic the S-enantiomer of phenprocoumon by hemiketal formation (Valente and Trager, 1978). However, this may not be the mechanism since acenocoumarol displays the same stereoselectivity as phenprocoumon, despite its ability to form similar hemiketals as warfarin. In our opinion, the discrepancy between phenprocoumon and warfarin can be explained better by the diversity of cytochrome P450 isozymes involved in the metabolism of these compounds.

In conclusion, the study gives additional support for the viewpoint that various cytochrome P450 enzymes are involved in the regio- and stereoselectivity of the hydroxylation of R- and S-warfarin. Furthermore, the study indicates that similar hydroxylations of acenocoumarol may be catalysed by enzymes different from those transforming warfarin. This observation complicates studies aimed at the investigation of structure-biotransformation relationships.

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CHAPTER 4:

COMPARATIVE BIOTRANSFORMATION OF WARFARIN ANALOGUES IN HUMAN LIVER MICROSOMES: 4'-SUBSTITUTION MODIFIES CYTOCHROME P450 ISOZYME SELECTIVITY.

4.1 ABSTRACT:

Human liver microsomal metabolism of warfarin and acenocoumarol (4'-nitrowarfarin) has been studied. The effect of cimetidine, propafenone, and sulfaphenazole on their metabolism has been investigated in microsomes from 5 subjects.

For S-warfarin, formation of the 7-hydroxy metabolite is the main metabolic route; 6-hydroxylation and SS-alcohol formation are relatively minor routes. 50% of the metabolic clearance of R-warfarin is due to 6-hydroxylation, the other 50% mainly to 4'- and 7-hydroxylation. The enantiomers of acenocoumarol are hydroxylated at the 7- and 6- position.

Acenocoumarol is a better substrate for human liver cytochromes P450 than warfarin itself. This is due to differences in K_m values that are 4.5 to 23 times lower for R-acenocoumarol and 5 to 28 times lower for S-acenocoumarol. Human liver microsomal warfarin and acenocoumarol metabolism is stereoselective for the S-enantiomer: the S/R ratio of the intrinsic clearances is about 3 for warfarin and about 30 for acenocoumarol.

The 6- and 7-hydroxylation rates of the R-enantiomer correlate significantly with those of the S-enantiomer for acenocoumarol but not for warfarin. The 6- and 7-hydroxylation of S-acenocoumarol do not correlate with the 6- and 7-hydroxylation of S-warfarin. The same is true for the 6-hydroxylation of the R-enantiomers, but not for the 7-hydroxylation of the R-enantiomers, which correlates significantly.

Cimetidine partly inhibits the 6- and 7-hydroxylation of only the R-enantiomers of warfarin and acenocoumarol up to 40%. Propafenone inhibits the 6- and 7-hydroxylation of acenocoumarol non-stereoselectively up to 40%. In contrast, only the 7-hydroxylation of R-warfarin is inhibited by propafenone to about 40%. Sulfaphenazole stereoselectively inhibits the 7-hydroxylation of S-warfarin almost completely and in some microsomal samples also inhibits the 6-hydroxylation of S-warfarin to about 50%. In contrast, sulfaphenazole inhibits the 7-hydroxylation of both acenocoumarol enantiomers, and the 6-hydroxylation of the S-enantiomer up to about 75%. In all these cases, sulfaphenazole has similar K_i values. This may indicate the involvement of P450 isozymes of the 2C subfamily in the 6- and 7-hydroxylation of S-warfarin and S-acenocoumarol as well as in the 7-hydroxylation of R-acenocoumarol. However, the lack of a correlation between warfarin and acenocoumarol hydroxylation as well as the differential effect of the inhibitors tested, indicates that the set of P450 isozymes metabolizing warfarin is not identical to that metabolizing acenocoumarol.

The results suggest that the phenyl group of warfarin is involved in the interaction with the P450 binding sites and that ring substitution additionally discriminates for particular isozymes of this class of enzymes.

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4.2 INTRODUCTION:

The oral anticoagulants warfarin and acenocoumarol (the 4'-nitro analogue of warfarin) are administered as racemates. Although structurally related, warfarin and acenocoumarol display great differences in their pharmacokinetics. In man, the enantiomers of acenocoumarol are eliminated much faster than the enantiomers of warfarin. In addition, the ratio of elimination of the S- over the R-enantiomer of acenocoumarol is much more pronounced (the S/R ratio is about 10 *versus* 1.5 for warfarin) (Thijssen et al 1986; Kelly and O' Malley, 1979). E.g. elimination half lives are about 35-58 and 24-33 hours for S- and R-warfarin (Kelly and O'Malley, 1979), whereas elimination half lives of S- and R-acenocoumarol are 0.5-1 and 8-10 hours (Thijssen et al, 1986). Since both 4-hydroxycoumarins are eliminated by biotransformation, the pharmacokinetic differences may be explained by disparity in the metabolism of these compounds. The enantiomers of warfarin have been shown to follow different metabolic routes in man (Lewis et al, 1974; Banfield et al, 1983; Toon et al, 1987). The S-enantiomer is mainly hydroxylated to form 7-hydroxywarfarin, whereas R-warfarin is mainly reduced to its RS alcohol and hydroxylated at the 6-position. The enantiomers of acenocoumarol are hydroxylated at the 7- and, to a lesser extent, the 6-position to form 7- or 6-hydroxyacenocoumarol respectively (Thijssen et al, 1986).

In previous studies from this laboratory, it was shown that substitution at the phenyl ring of warfarin has a great impact on the pharmacokinetics and the stereoselectivity thereof in the rat (Thijssen et al, 1985; chapter 2). As shown in chapter 2, substitution of the 4'-hydrogen did not only enhance the intrinsic clearance but also led to a marked inversion of the stereoselectivity for the S-enantiomers. In contrast, 3'-substituted warfarins were cleared stereoselectively for the R-enantiomers. In chapter 3, using rat liver microsomes, it was found that warfarin and acenocoumarol follow similar metabolic routes but with K_m values that are 2 to 20 times lower for acenocoumarol. Furthermore, based on the differential effects of enzyme inducers, the P450 inhibitor cimetidine, and P450 antibodies, it was stated that the kinetic differences between warfarin and acenocoumarol can be partly attributed to the involvement of different sets of P450 isozymes in the metabolism of both 4-hydroxycoumarins. To study whether this is also true in man, the human liver microsomal metabolism of warfarin has been compared with that of its 4'-nitro analogue (acenocoumarol). Furthermore, the effect on the human liver microsomal metabolism of warfarin and acenocoumarol of three drugs (cimetidine, propafenone, and sulfaphenazole) has been described. The aspecific P450 inhibitor cimetidine was chosen since it is known to decrease the clearance of the R-enantiomer of warfarin in man (Choonara et al, 1986). Sulfaphenazole, because this compound is reported to be a potent inhibitor of human liver cytochrome P450 2C9 mediated S-warfarin 7-hydroxylation (Rettie et al, 1991). Propafenone was chosen because, although this compound is shown to decrease the clearance of warfarin in man (Kates et al, 1987), information on stereochemical aspects is still lacking.

The results show that, as in rat, 4'-substituted warfarins in humans are metabolized by different, but probably overlapping sets of liver P450 isozymes.

4.3 MATERIALS AND METHODS:

Racemic warfarin and racemic propafenone (as the HCl salt) were obtained from Sigma Chemicals (St. Louis, USA). Acenocoumarol as well as 6- and 7-hydroxyacenocoumarol were a kind gift of Ciba-Geigy (Basel, Switzerland). Cimetidine was from Smith, Kline and French Laboratories (Welwyn Garden City, UK). Sulfaphenazole was kindly provided by Dr. Vree (Dept. Clin. Pharmacy, Univ. Hospital, Nijmegen, the Netherlands). All other chemicals were of analytical grade and were obtained from Merck (Darmstadt, West Germany) unless stated otherwise.

Warfarin and acenocoumarol enantiomers were prepared as described in chapter 2.

Preparation of microsomal fractions:

Human liver samples (table 4.1) were obtained from kidney donors (I,II, and V) or were obtained post mortem (III, and IV). The use of human liver for the study had the approval of the Ethics Committee of the University Hospital. Liver samples were stored at -80 °C until processing. Data on liver sample sources are shown in table 4.1. Microsomes were prepared as described in chapter 3. The final microsomal pellet was resuspended in Tris-KCl-NaCl buffer (0.02 M, 0.15 M and 1.0 M, respectively; pH=7.4) to contain about 20 mg protein per ml homogenate.

Assay for warfarin and acenocoumarol metabolism:

The assay of warfarin and acenocoumarol metabolism was conducted essentially as previously described in chapter 3. Microsomal fractions were incubated in the presence of a NADPH-generating system at 37 °C for 15-60 min in test tubes. The incubation mixture consisted of 0.5 to 1.0 mg microsomal protein, substrate, 7.4 mM glucose 6-phosphate, 2.4 mM MgCl₂ and 1.0 μ unit of glucose 6-phosphate dehydrogenase, 0.5 mM NADPH, and 2 mM NADP⁺.

Metabolite formation rates and the inhibitor effect for the various microsomal preparations were determined at substrate concentrations of 25 μ M and 100 μ M and incubation times of 30 and 60 minutes for acenocoumarol and warfarin enantiomers respectively. Inhibitor concentrations were 25 μ M (sulfaphenazole), 100 μ M (propafenone), and 1000 μ M (cimetidine). Before the reactions were started by addition of substrate, complete reaction mixtures, containing microsomes, NADPH-generating system and inhibitor (or an equal volume of buffer in the controls) were preincubated for 15 minutes at 37 °C.

The enzyme kinetic parameters of the metabolism of acenocoumarol enantiomers by liver microsomes were determined at substrate concentrations ranging from 1 to 250 μ M and an incubation time of 15 minutes. The enzyme kinetic parameters of warfarin metabolism were determined at substrate concentrations of 25 to 800 μ M (R-enantiomer) and 2.5 to 400 μ M (S-enantiomer) and an incubation time of 30 minutes. Incubation mixtures were preincubated for 15 minutes at 37 °C before starting the reaction by the addition of substrate.

Determination of K_i values and types of inhibition were tested at R/S acenocoumarol concentrations of 2.5-25 μ M, S-warfarin concentrations of 50-400 μ M, and R-warfarin

concentrations of 100-400 μM . Inhibitor concentrations ranged from 1-25 μM for sulfaphenazole, 25-250 μM for propafenone, and 50-600 μM for cimetidine.

The metabolites were analyzed by HPLC as described in chapter 3. Protein content was determined by the method of Lowry et al (1951). Cytochrome P450 content was determined by the DT-difference method as described by Rutten et al (1987). 7-Ethoxycoumarin deethylase and aniline-p-hydroxylase activity was determined as described by Greenlee and Poland (1978) and Macleod et al (1973).

Statistical analysis of the data:

Vmax and Km values were determined by fitting the Michaelis-Menten equation to the data using the GPAD (GraphPAD Software, San Diego, USA) software package. Ki values and inhibition mechanisms were determined by the use of Dixon plots and double reciprocal plots respectively. The effect of the inhibitors on the 6- and 7-hydroxylation of warfarin and acenocoumarol was tested for significance by the use of a oneway ANOVA and least significant differences (LSD) were calculated. Spearman rank correlation coefficients were tested for significance by transformation to the Students t-distribution (Sachs, 1984).

TABLE 4.1: Information on the subjects from which liver samples were obtained.

Code	Sex	Age	Death cause	Drug History
I	male	52	Traffic accident	
II	male	18	Traffic accident	
III	female	36	Subarachnoidal bleeding	
IV	female	61	Brain damage	Alcohol abuse
V	male	57	Brain tumour	Propranolol

TABLE 4.2: Cytochrome P450 content, 7-ethoxycoumarin- (7-EC-) deethylase activity, and aniline-p- (An-p-) hydroxylase activity in the human liver microsomal preparations used.

Code	[P450] (nmol/mg)	7-EC-deethylase (nmol/mg x min)	An-p-hydroxylation (nmol/mg x min)
I	0.45 $\pm$ 0.07	0.24 $\pm$ 0.01	2.1 $\pm$ 0.6
II	0.45 $\pm$ 0.15	0.14 $\pm$ 0.01	2.5 $\pm$ 0.3
III	0.74 $\pm$ 0.06	0.21 $\pm$ 0.01	1.1 $\pm$ 0.6
IV	0.40 $\pm$ 0.12	0.21 $\pm$ 0.01	1.5 $\pm$ 0.4
V	0.51 $\pm$ 0.13	0.22 $\pm$ 0.04	2.0 $\pm$ 0.8

Data are expressed as the mean $\pm$ SD of three determinations. Cytochrome P450 content is given in nmol per mg microsomal protein. Enzyme activities are expressed as nmol of product formed per minute per mg microsomal protein.

4.4 RESULTS: AND METHODS:

Standard parameters of the human liver samples are shown in table 4.2. The obtained values are in the range of data reported in other studies (Beaune et al, 1986).

In order to obtain information on the relative contribution of the metabolites formed from warfarin and acenocoumarol the enzyme kinetic parameters V_{max} , K_m , and intrinsic formation clearances (Cl_i) have been determined for sample I (table 4.3). Next to the metabolites shown in table 4.3, small amounts of the 10-hydroxy metabolites, and the RR- and RS-alcohols are found. Remarkably, in contrast to 8-hydroxyacenocoumarol, only small amounts of 8-hydroxywarfarin are found. The 9-hydroxy- and dehydro metabolites of acenocoumarol are only detected in small amounts at the highest substrate concentrations.

Table 4.3 shows that the V_{max} values of the metabolite formation rates are 1.5 (4'-hydroxywarfarin) to 24 (6-hydroxywarfarin) times higher for R-warfarin than for S-warfarin, except for the dehydro- metabolite. Apart from 4'-hydroxylation, K_m values are 1.4 (9-hydroxywarfarin) to 65 (7-hydroxywarfarin) times higher for R-warfarin.

Based on intrinsic clearances, the formation of 7-hydroxywarfarin and dehydrowarfarin is stereoselective for the S-enantiomer, since the intrinsic clearances are 14 and 10 times higher for S-warfarin. The 4'- and 6-hydroxylation of warfarin appear non-stereoselective, whereas 9-hydroxylation is stereoselective for R-warfarin (intrinsic clearance 2.2 -fold that of S-warfarin).

For acenocoumarol, V_{max} values of the 6- and 8-hydroxylation are about 4 times higher for the R-enantiomers, whereas the V_{max} value of the 7-hydroxylation is about 2.3 times higher for the S-enantiomer. The K_m values of R-acenocoumarol are 14 (8-hydroxy metabolite) to 120 (7-hydroxy metabolite) times higher than those of S-acenocoumarol.

If intrinsic clearances are considered, the formation of the acenocoumarol metabolites is stereoselective for the S-enantiomer. Intrinsic clearances are 3.5 (8-hydroxyacenocoumarol) to 36 (7-hydroxyacenocoumarol) times higher for S- than for R-acenocoumarol.

From table 4.3, it is clear that the overall microsomal metabolism of warfarin and its 4'-nitro analogue is stereoselective for the S-enantiomer. However, the S/R ratio of the total intrinsic clearance is much higher for acenocoumarol (with a S/R ratio of about 30) than for warfarin (S/R about 3.5). Furthermore, the total intrinsic clearances of the enantiomers of acenocoumarol are considerably higher than those of the corresponding warfarin enantiomers. Intrinsic clearances of R- and S-acenocoumarol are 9 and 80 times higher than those of R- and S-warfarin. The main reason for this is that the K_m values of the acenocoumarol enantiomers are about 5-28 times lower than those of the enantiomers of warfarin itself.

Next to differences in total intrinsic clearances and in stereochemistry, there are clear differences in the regioselectivity. For S-warfarin, 7-hydroxylation is by far the most important metabolic route that accounts for about 70% of intrinsic clearance, whereas 6-hydroxylation (about 10% of intrinsic clearance) and formation of the SS alcohol (about 10 % of intrinsic clearance) are relatively minor routes. In contrast, in the case of R-warfarin, formation of the 6-hydroxy metabolite accounts for 50 % of total metabolic clearance.

TABLE 4.3: Enzyme kinetics of the human liver (sample I) microsomal metabolism of the enantiomers of warfarin and acenocoumarol.

R-WARFARIN	V _{max} (pmol/mgxmin)	K _m (μM)	Cli* (nl/mgxmin)	Cli (% total)
4'-hydroxy	3.76	370	10.2	20.9
6-hydroxy	13.8	585	23.6	48.5
7-hydroxy	2.94	336	8.8	18.1
9-hydroxy	18.7	3277	5.7	11.7
dehydro-	2.4	3619	0.4	0.8
total#			48.7	100

S-WARFARIN	V _{max} (pmol/mgxmin)	K _m (μM)	Cli (nl/mgxmin)	Cli (% total)
4'-hydroxy	2.71	371	7.3	4.4
6-hydroxy	0.55	30	18.3	11.0
7-hydroxy	0.62	5.2	120	71.9
9-hydroxy	0.41	159	2.6	1.6
dehydro-	9.79	2444	4.0	2.4
SS-alcohol	5.01	340	14.7	8.8
total			167	100

R-ACENOCOUMAROL	V _{max} (pmol/mgxmin)	K _m (μM)	Cli (nl/mgxmin)	Cli (% total)
6-hydroxy	20.6	131	157	35
7-hydroxy	3.4	14.4	236	52.6
8-hydroxy	2.2	39.4	55.8	12.4
total			449	100

S-ACENOCOUMAROL	V _{max} (pmol/mgxmin)	K _m (μM)	Cli (nl/mgxmin)	Cli (% total)
6-hydroxy	4.97	1.11	4477	33.7
7-hydroxy	7.95	0.93	8548	64.3
8-hydroxy	0.56	2.83	198	1.5
SS-alcohol	27.0	394	69	0.5
total			13292	100

*Intrinsic clearance Cli= V_{max}/K_m #total= sum of Cli's

The other 50 % are accounted for by formation of the 7-, 4'-, and 9-hydroxy metabolites.

For R- and S-acenocoumarol, 7-hydroxylation accounts for 53 and 64 % of the total metabolic clearance, whereas 6-hydroxylation accounts for about 35 %.

Since the 6- and 7-hydroxylation appear to be the main metabolic routes of warfarin and acenocoumarol in human liver microsomes, the other experiments were mainly focussed on these reactions. Figures 4.1 and 4.2 show the rates of the 6- and 7-hydroxylation of both warfarin and acenocoumarol enantiomers in 5 human liver microsomal samples.

Figure 4.1: Rates of formation of the 6-hydroxy metabolites of the enantiomers of warfarin (left) and acenocoumarol in 5 human liver microsomal prepares.

Data are expressed as the mean (pmol metabolite/ mg protein $\times$ min) $\pm$ SEM of two incubations. The five sets of bars represent microsomal fraction I (left) to V (right). Open bars: R-enantiomers; cross hatched bars: S-enantiomers.

Figure 4.2: Rates of formation of the 7-hydroxy metabolites of the enantiomers of warfarin (left) and acenocoumarol in 5 human liver microsomal preparations.

Data are expressed as the mean rate (pmol metabolite/ mg protein x min) $\pm$ SEM of two incubations. The five sets bars of represent microsomal fraction I (left) to V (right). Open bars: R-enantiomers; cross hatched bars: S-enantiomers.

The formation rates of 6-hydroxywarfarin ranged 7-fold (0.5 to 3.5 pmol/ mg x min.) for R-warfarin and 11 fold for S-warfarin (0.12 to 1.3 pmol/mg protein x min.) among the microsomal preparations. The 6-hydroxylation of R-acenocoumarol ranged 10 fold (3 to 30 pmol/mg x min), whereas that of S-acenocoumarol ranged only 4-fold (3 to 12 pmol/mg x min). The 6-hydroxylation rate of both 4-hydroxycoumarins is higher for the R- than for the S-enantiomers at the substrate concentrations chosen. This is probably due to the higher V_{max} values of the R-enantiomers (table 4.3). However, during therapy, blood levels range from 1 to 10 μ M for warfarin (Holford, 1986) and from 60 to 600 nM for acenocoumarol (Thijssen et al, 1988). The concentration of the unbound drugs will be even lower. Therefore, intrinsic clearances rather than V_{max} values will give an indication of the metabolic profile *in vivo*, since in this parameter, differences in K_m values are accounted for.

The 7-hydroxylation of all substrates appears to be less variable among the microsomes than the 6-hydroxylation. Formation rates of the 7-hydroxy metabolite range 3 to 4 fold. 7-Hydroxylation rates are generally lower for the S-enantiomer of warfarin (0.5 to 1.5 pmol/ mg x min) than for the R-enantiomer (0.7 to 2.8 pmol/mg x min.). The opposite is true for acenocoumarol (7-hydroxylation rates vary from 1.1 to 4.5 pmol/mg x min. for R-acenocoumarol versus 4.2 to 13.8 pmol/mg x min. for the S-enantiomer).

TABLE 4.4: Correlation matrix of the 6- and 7- hydroxy metabolite formation rates of R- and S-warfarin and -acenocoumarol in human liver microsomes from 5 subjects.

	R6OHW	R7OHW	S6OHW	S7OHW	R6OHAC	R7OHAC	S6OHAC	S7OHAC
R6OHW	--	--	--	--	--	--	--	--
R7OHW	0.05	--	--	--	--	--	--	--
S6OHW	0.16	-0.37	--	--	--	--	--	--
S7OHW	0.08	0.31	-0.37	--	--	--	--	--
R6OHAC	0.64	0.65	-0.24	0.03	--	--	--	--
R7OHAC	0.45	0.90*	0.12	0.41	0.92*	--	--	--
S6OHAC	0.59	0.71	0.20	0.06	0.99*	0.89*	--	--
S7OHAC	0.54	0.75	0.30	0.01	0.99*	0.90*	0.99*	--

*significant correlation $P < 0.025$, as determined by transformation to the Student's t-distribution.

TABLE 4.5:Inhibitor type and K_i values of the inhibition of the human liver (sample I) microsomal 6- and 7-hydroxylation of the enantiomers of warfarin and acenocoumarol by sulfaphenazole, cimetidine, and propafenone.

Inhibitor		6-OH-WARFARIN		7-OH-WARFARIN	
Sulfaphenazole	R-	n.i.		n.i.	
	S-	0.6 ± 0.3	Comp. 45%	0.5 ± 0.3	Comp. 95%
Cimetidine	R-	472 ± 103	Mixed 40%	1780 ± 667	Mixed 35%
	S-	n.i.		n.i.	
Propafenone	R-	n.i.		131 ± 22	Mixed 40%
	S-	n.i.		n.i.	
Inhibitor		6-OH-ACENOCOUMAROL		7-OH-ACENOCOUMAROL	
Sulfaphenazole	R-	n.i.		1.0 ± 0.4	Comp. 70%
	S-	1.3 ± 0.3	Comp. 80%	1.2 ± 0.4	Comp. 80%
Cimetidine	R-	1938 ± 328	Mixed 35%	223 ± 25	Comp. 40%
	S-	n.i.		n.i.	
Propafenone	R-	563 ± 97	Mixed 40%	231 ± 22	Mixed 40%
	S-	670 ± 170	Mixed 30%	236 ± 79	Mixed 40%

K_i values are given as mean (in μM) $\pm$ standard deviation calculated from all intersections of the obtained lines in Dixon plots. n.i., no inhibition as determined by one-way ANOVA. Comp., indicates competitive inhibition. Mixed, indicates mixed type inhibition. Types of inhibition were determined from double reciprocal plots. Percentages indicate the maximal inhibition at the lowest substrate and highest inhibitor concentrations used.

Figure 4.3: Inhibition of the 6- (above) and 7-hydroxylation of the enantiomers of warfarin and acenocoumarol by sulfaphenazole, propafenone, and cimetidine.

Inhibitor concentrations were : sulfaphenazole : 25 μ M, propafenone: 100 μ M, and cimetidine 1000 μ M. The concentration of warfarin and acenocoumarol enantiomers were 100 and 25 μ M respectively. Data are expressed as the mean $\pm$ SE of 5 human microsomal samples. * Indicates a significant inhibition: $P < 0.05$, ANOVA, LSD.

Figure 4.4: Dixon plots of the inhibition of the human liver (sample I) microsomal 7-hydroxylation of S-acenocoumarol by sulfaphenazole (graph A) and R-acenocoumarol by cimetidine and propafenone (graphs B and C).

Table 4.4 shows a correlation matrix of the 6- and 7-hydroxylation rates of warfarin and acenocoumarol enantiomers in the human liver microsomes. If the correlations between the enantiomers are considered, there appears to be no clear relationship (table 4.4) between the 6- and 7-hydroxylation of R-warfarin with those of S-warfarin. This is in sharp contrast to acenocoumarol for which the 6- and 7-hydroxylation rates of the R-enantiomer correlate significantly with those of the S-enantiomer ($P < 0.025$). Considering correlations between warfarin and acenocoumarol, only the 7-hydroxylations of the R-enantiomers correlate significantly ($P < 0.025$). For the other reactions, there is no correlation between warfarin and acenocoumarol.

Finally, a significant correlation between the 6- and 7-hydroxylation within and between the enantiomers of acenocoumarol is observed ($P < 0.025$). In contrast, these reactions do not correlate for warfarin.

The effect of sulfaphenazole, propafenone and cimetidine on the 6- and 7-hydroxylation of warfarin and acenocoumarol enantiomers by the various microsomal preparations is shown in figure 4.3. These data are presented as the mean % inhibition $\pm$ SE. Sulfaphenazole inhibits the 6-hydroxylation of S-acenocoumarol ($P < 0.05$) about 65% but has no effect on the 6-hydroxylation of R-acenocoumarol or of R- and S-warfarin. However, in the case of the 6-hydroxylation of S-warfarin, there was considerable variation in the effect of sulfaphenazole, the inhibition ranging from 0 (sample V) to 50 % (sample II). The rate of 7-hydroxylation is decreased significantly ($P < 0.05$) by sulfaphenazole for R- and S-acenocoumarol (about 55 and 75%) and for S- (about 60%) but not R-warfarin.

Propafenone significantly ($P < 0.05$) inhibits the formation of the 6- and 7-hydroxy metabolites of R- and S-acenocoumarol about 15-20%. In the case of warfarin, only the 7-hydroxylation of the R-enantiomer is inhibited ($P < 0.05$) about 25%.

Cimetidine depresses ($P < 0.05$) the 6- and 7-hydroxylation of R-warfarin (about 25%) and R-acenocoumarol (about 15 and 20%) but not of the S-enantiomers.

In table 4.5, K_i values and types of inhibition of the three inhibitors for their effect on the 6- and 7-hydroxylation of warfarin and acenocoumarol are given for sample I. However, these data should be interpreted with care since the hydroxylation reactions are probably carried out by multiple P450 isozymes, some of which may not be influenced by the inhibitors. Therefore (although estimated from the linear parts of Dixon plots as shown in figure 4.4), K_i values may be overestimated, and quasi mixed type inhibition may actually be a combination of competitive inhibitions. In fact, all inhibitions appeared to be partial (with the exception of the inhibition of the 7-hydroxylation of S-warfarin by sulfaphenazole). For that reason, the maximal inhibition (in %) at the lowest substrate and highest inhibitor concentration is given. Sulfaphenazole appears to be a competitive inhibitor of the 7- and 6-hydroxylation of S-warfarin with similar K_i values (0.6 and 0.7 μM). The 7-hydroxylation is fully inhibited by this compound, the 6-hydroxylation to 45 %. In the case of acenocoumarol,

sulfaphenazole competitively inhibits the 7-hydroxylation of both enantiomers and the 6-hydroxylation of the S-enantiomer with approximately similar K_i values (1.0-1.3 μM) and maximal inhibitions (70-80 %).

Cimetidine appears to be a mixed type inhibitor of the 6- and 7-hydroxylation of R-warfarin but with different K_i values (472 and 1780 μM respectively). In the case of R-acenocoumarol, cimetidine is a mixed type inhibitor of the 6-hydroxylation (up to 35 %, $K_i = 1938 \mu\text{M}$) but a competitive inhibitor of the 7-hydroxylation (up to 40 %, $K_i = 223 \mu\text{M}$).

Propafenone is a mixed type inhibitor of the 7-hydroxylation of R-warfarin ($K_i = 131 \mu\text{M}$) and of R- and S-acenocoumarol (K_i 's of 231 and 236 μM) and these reactions are inhibited up to 40%. Propafenone also is a mixed type inhibitor of the 6-hydroxylation of R- and S-acenocoumarol with K_i values of 563 and 670 μM and maximal inhibitions of 40 and 30 %.

4.5 DISCUSSION:

Warfarin enantiomers are often used as a probe for cytochromes P450 (Guengerich et al, 1982; Beaune et al, 1986; Kaminsky, 1989). Various P450 isozymes, displaying different regio- and stereoselectivities, have been shown to be involved in the metabolism of warfarin (Guengerich et al, 1982; Rettie et al, 1992). The variety of P450 isozymes involved in warfarin metabolism is evident if the variable stereoselectivity of pharmacokinetic interactions of other drugs with warfarin is considered. Stereoselective impairment of the clearance of only the R-enantiomer (Choonara et al, 1986; Toon et al, 1987; Sutfin et al, 1989; Abernethy et al, 1991), or only the S-enantiomer (Lewis et al, 1974; O'Reilly, 1976; O'Reilly, 1980; O'Reilly, 1982; Banfield et al, 1983) has been described, as well as non-stereoselective interactions (O'Reilly et al, 1987). All of these interactions have been attributed to inhibition of the metabolism of warfarin.

In previous studies we found that substitution at the phenyl ring of warfarin drastically changes the pharmacokinetics and the stereoselectivity thereof in the rat (Thijssen et al, 1985; chapter 2). 4'-Substitution greatly enhances the intrinsic clearances with a general preference for the S-enantiomer. E.g. substitution of the 4' hydrogen by a nitro-, cyano-, bromo- or chloro- group enhances the intrinsic clearances of the S-enantiomers 19 to 32 fold but enhances the intrinsic clearances of the R-enantiomers only 2 to 20 fold, as compared to warfarin. As a result the R/S enantiomeric ratio of 1.5 for warfarin itself is shifted to 0.2-0.4 for the 4'-substituted analogues.

This study shows that the 4'-substituent also highly influences the metabolic clearance of warfarin derivatives in human liver microsomes. The enantiomers of acenocoumarol, the 4'-nitro analogue of warfarin, display higher intrinsic clearances than the corresponding enantiomers of warfarin itself. The main reason for this is that the K_m values are 5 to 28 times

lower for acenocoumarol than for warfarin. In addition, the stereoselectivity for the S-enantiomer is much more pronounced for acenocoumarol. Interestingly, for 4'-chlorowarfarin, we also found that the microsomal metabolic clearance is much higher (12 and 120 fold for the R- and S-enantiomers) than that of warfarin, and that the S/R ratio is 10 times higher (own observations).

The observed patterns of warfarin and acenocoumarol clearance are in good agreement with those found *in vivo*. However, the clearances of the R-enantiomers may possibly be underestimated because ketone reduction, an important metabolic route of R-warfarin, is not dealt with, since the enzymes responsible for this reaction are located in the cytosol (as will be discussed in chapters 5 and 6).

As stated above, disparity in affinities of the 4-hydroxycoumarins towards human liver monooxygenases is the main reason of the differences in the metabolic clearances between the enantiomers of warfarin and acenocoumarol. In this respect, the human system behaves similarly as the rat. Different affinities to monooxygenases may arise because of the physico-chemical properties of the substrates and because of the diversity of P450 isozymes involved in their metabolism. Figure 4.3 as well as table 4.5 show clear differences in the effect of inhibitors on the 6- and 7-hydroxylation of acenocoumarol vs warfarin enantiomers. E.g., the 7-hydroxylation of R-acenocoumarol but not of R-warfarin is inhibited by sulfaphenazole. The 7-hydroxylation of S-acenocoumarol but not S-warfarin is inhibited by propafenone. The 6-hydroxylation of R- and S-acenocoumarol but not of R- and S-warfarin is inhibited by propafenone. These differences indicate that the isozymes catalysing the hydroxylation of warfarin at the 6- and 7-position do not catalyse these reactions to the same extent for acenocoumarol, although there will be some overlap. The lack of a clear relationship between the 6-hydroxylation of the enantiomers of warfarin and the corresponding acenocoumarol enantiomers and the absence of any correlation between the 7-hydroxylation of S-warfarin and S-acenocoumarol in the various microsomal preparations (table 4.4) further supports this.

Recently, Rettie et al (1992), in testing various cDNA expressed human liver cytochromes P450 for regio- and stereoselective hydroxylation of warfarin enantiomers, found that cytochrome P450 2C9 is a 7-hydroxylase of S-warfarin. In contrast, R-warfarin, which also binds to this enzyme with a relatively high affinity, is not metabolized by this enzyme (Kunze et al, 1991; Rettie et al, 1992). This P450, which is inhibited by sulfaphenazole, was stated to be responsible for the bulk of S-warfarin clearance. The enzyme was also shown to catalyse the 6-hydroxylation of S-warfarin and in human liver microsomes a correlation between the 6- and 7-hydroxylation of S-warfarin was observed (Rettie et al, 1992). However, we found that the 6-hydroxylation of S-warfarin could be only partially inhibited by sulfaphenazole in some liver samples and we did not find a correlation between the 6- and 7-hydroxylation of S-warfarin. This apparent contradiction may be explained by the fact that at least two

enzymes catalyse this reaction (Rettie et al, 1989), so that the ratio at which these enzymes are present (which may show some geographic differences) determines the actual effect of sulfaphenazole and the (lack of) correlation. In agreement with Rettie et al (1992), we find the 7-hydroxylation of S- but not R-warfarin to be inhibited by sulfaphenazole. Interestingly, sulfaphenazole inhibits the 7-hydroxylation of both R- and S-acenocoumarol. In addition, the 6-hydroxylation of S- but not of R-acenocoumarol is inhibited by sulfaphenazole. Furthermore, we found the 8-hydroxylation of R- but not S-acenocoumarol to be inhibited ($K_i = 0.6 \mu\text{M}$; data not shown). The similar K_i values would suggest these acenocoumarol hydroxylations to be carried out by the same enzyme as the 7-hydroxylation of S-warfarin. On the other hand, the acenocoumarol hydroxylation rates do not correlate with S-warfarin 7-hydroxylation, even if the data are corrected for the non-sulfaphenazole inhibitable parts. This would suggest that the hydroxylation of acenocoumarol, which is inhibited by sulfaphenazole, cannot (completely) be ascribed to cytochrome P450 2C9. The P450 2C subfamily is very complex and its isozymes often are structurally very similar (Nebert et al, 1991; Srivastava et al, 1991). E.g. it has been shown that tolbutamide is hydroxylated by at least three structurally very similar P450 isozymes 2C8-C10 (Relling et al, 1990; Srivastava et al, 1991) and that tolbutamide hydroxylation can be completely inhibited by sulfaphenazole (Miners et al, 1988). Although P450 2C8 has not been shown to conduct hydroxylation of warfarin at the coumarin ring (Rettie et al, 1992), it may well be that this isozyme or other P450s of the 2C subfamily, have this catalytic activity towards acenocoumarol. This would imply that the phenyl ring of warfarin plays an important role in the binding and in the orientation of the coumarin towards these P450 isozymes. With this respect, it is interesting to note that antibodies raised against rat liver P450 2C isozymes showed differential effects on the stereo- and regioselectivity of warfarin and acenocoumarol hydroxylations by rat liver microsomes (chapter 3), which also indicates a different binding and orientation of the 4-hydroxycoumarins.

The formation of the main metabolite of propafenone, 5-hydroxypropafenone, has been shown to be carried out by the polymorphic cytochrome P450 2D6. This may give rise to the idea that P450 2D6 catalyses the coumarin hydroxylations of warfarin and acenocoumarol since the 6- and 7-hydroxylation of both acenocoumarol enantiomers and the 7-hydroxylation of R-warfarin are inhibited by propafenone. However, the K_m value of propafenone for the formation of the 5-hydroxy metabolite is about $2 \mu\text{M}$ (Kroemer et al, 1989), whereas the K_i values determined in this study vary from 130 to $670 \mu\text{M}$. Therefore, it is very unlikely that P450 2D6 is involved in the metabolism of warfarin or acenocoumarol.

Cimetidine, an aspecific P450 inhibitor (Knodell et al, 1991) is known to decrease the *in vivo* clearance of the R-enantiomer of warfarin (Choonara et al, 1986) and acenocoumarol (Gill et al, 1989). For warfarin, this has been attributed to inhibition of the 6-hydroxylation and to a lesser extent of the 7-hydroxylation (Niopias et al, 1991), which is in accordance with the data in table 4.5 (lower K_i value of the 6-hydroxylation). The 6-hydroxylation of R-

warfarin has been shown to be carried out by cytochrome P450 1A2 (Wang et al, 1983; Rettie et al, 1992). The K_i value we found for the inhibition of this reaction by cimetidine is in good agreement with the K_i value that this substance has for purified cytochrome P450 1A2 (Knodell et al, 1991), whereas this is not the case for the 6-hydroxylation of R-acenocoumarol, possibly indicating the involvement of a different (set of) isozyme(s). In the case of acenocoumarol, cimetidine decreases the clearance of the R-enantiomer only if a relatively high dose of this drug is applied (Thijssen et al, 1986; Gill et al, 1989). The data in table 4.3 and 4.5 offer an explanation for this phenomenon, since the K_m over K_i ratios are lower for acenocoumarol than for warfarin, which implies that at equal substrate and inhibitor concentrations the extent of inhibition will be lower for acenocoumarol than for warfarin.

This study demonstrates once again that small differences in the structure of drugs can completely change their pharmacokinetics and the stereoselectivity thereof. Since a small structural change may have implications on the relative contribution of P450 isozymes in the metabolism of 4-hydroxycoumarins, differences in interactions may occur. Furthermore, ring substituted warfarin analogues may serve as probes in exploring subtle differences between the isozymes of the P450 2C subfamily.

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CHAPTER 5:

THE *IN VITRO* KETONE REDUCTION OF WARFARIN AND ANALOGUES. SUBSTRATE STEREOSELECTIVITY, PRODUCT STEREOSELECTIVITY AND SPECIES DIFFERENCES.

5.1 ABSTRACT:

The *in vitro* metabolic ketone reduction of warfarin and its analogues acenocoumarol (4'-nitrowarfarin) and 4'-chlorowarfarin has been investigated using microsomal and cytosolic fractions of several species. Both subcellular fractions show reductase activity, which is NADPH dependent. The cytosolic fractions of most species, exhibit a clear substrate- and product stereoselectivity, i.e., the R-enantiomer is preferred as a substrate to be reduced mainly to the S-alcohol. In the rat, phenobarbitone and methylcholanthrene induce the cytosolic ketone reductase activity 5 to 11 fold and 3 to 7.5 fold respectively.

The microsomal fractions also show substrate- and product stereoselectivity. Contrary to the cytosolic fractions however, a general pattern of substrate and product stereoselectivity among species is not observed. Rat liver microsomes show practically no reductase activity. Phenobarbitone and methylcholanthrene treatment appear to have no, or very little effect on the rat liver microsomal reductase activity.

Substitution of the 4'-hydrogen of warfarin results in a change of reduction rates, and in some cases even in a change of substrate- and product stereoselectivity.

Prelog's rule for product stereoselectivity of metabolic ketone reduction, does not always agree with the data presented here.

Adapted from: Biochem. Pharmacol. 38: 3365-3370, 1989. J.J.R.Hermans, and H.H.W. Thijssen,

5.2 INTRODUCTION:

Reduction of aldehydes or ketones to their corresponding alcohols plays a role in the metabolism of both endogenous (e.g. glucuronic acid, steroids) and exogenous compounds (e.g. p-nitrobenzaldehyde, daunorubicine, metyrapone, etc.) in various species (Bachur, 1976; Felsted et al, 1977; Wermuth et al, 1977; Ahmed et al, 1979; Sawada and Hara, 1978; Sawada et al, 1980). The enzymes, responsible for this type of reaction are found in microsomal and cytosolic fractions of various tissues (Bachur, 1976; Sawada and Hara, 1978; Ahmed et al, 1979; Sawada et al, 1981; Maser, 1988; Lee et al, 1988), but mainly in the liver.

The *in vivo* ketone reduction of the acetyl side-chain of the oral anticoagulant warfarin to its alcohols in man was first described by Trager et al (1970). As warfarin possesses a stereocentre, two optically active enantiomers exist: the R(+)-enantiomer and its antipode

the S(-)-enantiomer. Therefore, from racemic warfarin, four diastereomeric alcohols (figure 5.1) have been separated, alcohol 1 being the RS- or SR-alcohols and alcohol 2 being the RR- or SS-alcohols (Chan et al, 1972).

The *in vivo* and *in vitro* metabolic reduction of warfarin in the rat and in man proved to be stereoselective for both substrate preference and product formation (Chan et al, 1972; Lewis et al, 1974; Pohl et al, 1974; Moreland and Hewick, 1975; Pohl et al, 1976). In *in vitro* systems, both species prefer the R-enantiomer as a substrate, reducing it mainly to the RS-alcohol. S-warfarin is mainly reduced to the SS-alcohol. *In vivo*, in man, ketone reduction is an important metabolic route for R-warfarin, which is reduced to the RS alcohol but only a minor route for S-warfarin, which is reduced to the SS alcohol (Lewis et al, 1974). In the rat, ketone reduction is only a minor metabolic route for both warfarin enantiomers, but contrary to what is found *in vitro*, the *in vivo* reduction is stereoselective for the S-enantiomer which is mainly reduced to the SS-alcohol (Pohl et al, 1974).

The 4'-nitro analogue of warfarin, acenocoumarol, has also been shown to be reduced to its alcohols in man (Dieterle et al 1977). However, information on the stereochemistry of its metabolic ketone reduction is absent.

Warfarin analogues display great differences in the pharmacokinetics of their enantiomers (chapter 2; Thijssen et al, 1985; Thijssen et al, 1986). Therefore, the stereochemical course of the acetylonyl side chain reduction of warfarin, acenocoumarol and 4'-chlorowarfarin has been studied, using subcellular fractions of several species. Also, the effect of enzyme induction (in rats) has been looked upon. As the diastereomeric alcohols of warfarin can be quantitated easily by HPLC (Fasco et al, 1979), the enantiomers of warfarin analogues represent interesting tools for the investigation of substrate- as well as product stereoselectivity of metabolic ketone reduction.

Figure 5.1: The structure of the warfarin analogues and the corresponding alcohols. -R= H: warfarin, -R=NO₂: acenocoumarol; -R=Cl: 4'-chlorowarfarin.

THE *IN VITRO* KETONE REDUCTION OF WARFARIN AND ANALOGUES:
SUBSTRATE STEREOSPECIFICITY, PRODUCT STEREOSPECIFICITY**5.3 MATERIALS AND METHODS:**

All chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany) unless stated otherwise.

Preparation of microsomal and cytosolic fractions:

Livers of the (female) bovine ($n=1$), pig ($n=1$), horse ($n=1$) and sheep ($n=2$) were obtained from the regional abattoir and stored on ice immediately. Rat livers ($n=4$; male Wistar rats) and rabbit ($n=2$) livers were removed directly after the animals were killed (by bleeding to death under ether anesthesia).

Human liver was obtained *via* the department of Pathology of the Academic Hospital Maastricht (The Netherlands), with the approval of the Ethics Committee, from autopsy of a 36 years old female that died of brain damage.

If more livers from one species were used, these livers were pooled for preparation of microsomal and cytosolic fractions. Livers were homogenized in 3 vol. of ice-cold Tris, 0.15 M KCl and 0.25 M sucrose, brought to pH 7.4 with 6 N HCl solution), using a potter at 1500 rpm. The homogenate was then centrifuged at 10,000 g for 30 min. The pellet was discarded and the supernatant was centrifuged at 110,000 g for 50 min. The 110,000 g supernatant was used as the cytosolic fraction in the ketone reduction assay. The 110,000 g pellet was washed twice by resuspension in the original amount of Tris/KCl buffer, then homogenized and centrifuged at 110,000 g for 50 min. The final microsomal pellet was resuspended in Tris/KCl/NaCl (0.02 M, 0.15 M and 1.0 M, respectively) to contain about 20 mg of protein per ml homogenate. Rats were induced by phenobarbitone 0.1% w/v in drinking water for 6 days; $n=4$) or by methylcholanthrene (25 mg/kg i.p. for 3 days; $n=4$). The effect of administration of enzyme inducers was tested, measuring cytochrome P450 content and 7-ethoxycoumarin deethylase activity.

The protein content of the fractions was determined by the method of Lowry et al (20) using bovine serum albumin as standard. All fractions were stored at -80°C until used in the assay.

Preparation of the R- and S-enantiomers of warfarin and its analogues:

The respective enantiomers were isolated by the method of West et al (21). Racemic warfarin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). 4'-chlorowarfarin was from Aldrich Chemicals and acenocoumarol was a gift from Ciba-Geigy (Basel, Switzerland).

Purity of the enantiomers was tested by the formation of their t-BOC-L-proline diastereomers (22) and subsequent analysis by HPLC (Lichrosorb Si 60; n-hexane/ethylacetate/methanol 75/25/0.2). The purity of the enantiomers was higher than 97%.

Assay for the ketone reduction:

Microsomal and 110,000 g fractions were incubated in 55x11/12 test tubes for 10 min at 37°C at aerobic conditions. The incubation mixture contained (in Tris/KCl, pH=7.4, final vol. 360 µl): 110 µl microsomal or supernatant fraction, 0.2 mM substrate, 1.6 mM NADPH, 20.2 mM glucose-6-phosphate (G-6-P), 5.8 mM magnesium chloride, and 2.8 M units of G-6-P dehydrogenase (Sigma).

The reaction mixture was preincubated at 37°C for 2 min; then, the substrate was added. The reaction was stopped after 10 min. by the addition of 500 µl ice-cold acetonitrile, containing an internal standard. Warfarin was used as internal standard for the assay of acenocoumarol and 4'-chlorowarfarin; acenocoumarol was used as the internal standard for the assay of warfarin. The mixture was centrifuged at 2500 rpm for 5 min to precipitate the protein. The supernatant was evaporated under a stream of nitrogen at 37°C. To the residue 0.5 ml 0.1 N HCl solution was added and the warfarin analogues and their alcohols were extracted with 4 ml of a 1/1 (v/v) mixture of petroleum ether and methylene chloride. The organic phase was evaporated to dryness at 37°C under a stream of nitrogen. The residue was taken up in 1/1 (v/v) mixture of acetonitrile and water and was analyzed by HPLC. Blank incubations were run in parallel; all incubations were carried out in triplicate.

The HPLC system consisted of Chromspher C-18 (200x3 mm, Chrompack, The Netherlands) as the stationary phase and a mixture of 0.1% acetic acid and acetonitrile (7/3 v/v brought to pH 4.76 with ammonia) as the mobile phase. UV absorption at 303 nm was measured on a Kratos spectroflow 783 and areas were calculated on Hitachi D-2000 integrator.

Identification of the alcohols:

The alcohols of the warfarin analogues were synthesized for reference purposes by reduction with sodium-borohydride in methanol (Pohl et al, 1974). The alcohols were purified by the use of a two-dimensional thin-layer (silica) chromatography system. In the first dimension, the non-reduced warfarin analogue was separated from the diastereomeric alcohols, using a mixture of acetone and dichlorethane (90/10 v/v) as the mobile phase. The diastereomers in the second dimension, using diethylether/acetic acid (99/1.5 v/v) as the mobile phase. On the thin-layer system, The RS/SR alcohols (therefore also called alcohol 1) travelled faster than the RR/SS alcohols (alcohol 2). On the reversed phase HPLC system, the opposite was true.

5.4 RESULTS:

The ketone reduction of the acetyl side-chain of the warfarin analogues by liver microsomal fractions is presented in figure 5.2. Species differences in substrate (stereo)selectivity and differences in product stereoselectivity are apparent.

Figure 5.2: Rate of formation of the alcohols in microsomal fractions. Formation rates are expressed as pmol of alcohol formed per min per mg of protein (mean $\pm$ SD).

For instance, microsomes of untreated rats show practically no ketone reductase activity although minor activity is observed for S-4'-chlorowarfarin (about 30 pmol of SS alcohol is formed per mg protein per min.). The enzyme inducers phenobarbitone (PB) and methylcholanthrene (MC) do not show a clear effect on rat liver microsomal alcohol formation rates. However, PB and MC may have some enhancing effect on the formation of the alcohols of acenocoumarol. Contrary to microsomes from untreated rats, MC microsomes do not show any S-4'-chlorowarfarin reductase activity.

Liver microsomes of the other species generally show higher reductase activities. The substrate stereoselectivity varies widely, depending on the species and on the warfarin analogue. E.g., sheep microsomes show a high selectivity for the S-enantiomer of all analogues; this enantiomer is mainly reduced to the SS-alcohol. Horse microsomes also show a preference to reduce the S-enantiomers of acenocoumarol and 4'-chlorowarfarin to the S alcohols. In contrast, for warfarin, horse liver microsomes show a low reductase activity but show a preference for the R-enantiomer. Microsomal fractions of the bovine and pig show a preference for the R-enantiomer of warfarin and its 4'-chloro- analogue which is reduced to the S alcohol. Remarkably, R-acenocoumarol is a poor substrate. Rabbit liver microsomes show the highest activity for warfarin, with a preference for the S-enantiomer to be reduced to the S-alcohol. Both alcohols are found for R-warfarin. Of the analogues, only the S-enantiomer was reduced by rabbit microsomes, resulting in the formation of the S-alcohols.

Figure 5.3: Rate of formation of the alcohols in cytosolic fractions. Formation rates are expressed as pmol alcohol formed per min per mg of protein (mean $\pm$ SD).

Human liver microsomes appeared to have a preference for the R-enantiomer of warfarin to form the S-alcohol. In contrast, in the case of acenocoumarol and 4'-chlorowarfarin, a preference for the S-enantiomer is observed. The products are the S-alcohols. The microsomal ketone reductases proved to be NADPH-dependent. No activity is observed when NADH, sodium dithionite or dithiothreitol (DTT) are used as reducing agents.

Figure 5.3 shows the reduction of the warfarin analogues in the cytosolic fractions. In contrast to the microsomal fractions, in all species but the sheep, there is a clear selectivity for the R-enantiomer, which is reduced to the S-alcohol: the ratios in conversion rates between the R- and S-enantiomers range from 2.2 (4'-chlorowarfarin in the rat) to >1000 (acenocoumarol in the rabbit). Inversion of the generally observed product stereoselectivity (formation of the S-alcohols) is seen for the bovine cytosolic fraction, which reduces R-warfarin to its R-alcohol.

Inversion of the general pattern of substrate stereoselectivity (for the R-enantiomers) is observed for the sheep cytosolic fraction which shows a preference for the reduction of S-acenocoumarol and S-4'-chlorowarfarin to be reduced to the S-alcohols. Remarkably, S-warfarin is reduced to the R-alcohol by sheep cytosol.

Ketone reduction of the R-enantiomers is higher in cytosolic fractions from PB treated (5- to 11-fold, depending on the substrate used) and MC treated rats (3- to 7.4-fold) than in cytosolic fractions from untreated rats. In contrast, PB and MC treatment does not have a clear effect on the reduction of the S-enantiomers.

In general, ketone reductase activity is higher for the warfarin analogues than for warfarin itself; in most cases, acenocoumarol is the best substrate. The cytosolic ketone reductases proved to be NADPH-dependent, although small activity (10-20% of NADPH at 1 mM) is observed with NADH as cosubstrate. Microsomal and cytosolic fractions, denatured by the addition of acetonitrile do not show any reductase activity.

5.5 DISCUSSION:

Ketone reductase and aldehyde reductase activities have been observed in various tissues. The enzymes have been reported to show a broad substrate specificity and their physiological function, among others, is thought to be the reduction of steroidal ketones (Sawada et al, 1980). In comparison to other metabolic routes of xenobiotic biotransformation, carbonyl reduction up to now has obtained less attention. However, the stereochemistry of the reaction in case we are dealing with asymmetric ketones makes the study of this system worthwhile. By studying ketone substrates possessing chirality, product stereoselectivity can be easily followed by chromatographic separation of the diastereomeric products.

Ketone reduction of the acetonide side-chain of warfarin has been described before and the substrate and product selectivity has been emphasized *in vivo* (Chan et al, 1972; Lewis et al, 1974; Pohl et al, 1976) and *in vitro* (Moreland and Hewick, 1975). The present results are in agreement with these studies showing in rat and human liver cytosolic fraction substrate preference for R-warfarin and product selectivity for the S-alcohol. With the exception of the sheep, liver cytosolic ketone reductase of the other species also show a substrate preference for the R-enantiomer leading to the formation of the S-alcohol. This may suggest a possible interspecies relationship of the reductases. The order of sensitivity is generally R-acenocoumarol > R-4'-chlorowarfarin > R-warfarin. Moreland and Hewick (1975) have reported the substrate preference for R-warfarin of the ketone reductase activity in human and rat liver and kidney cytosolic fraction to be a matter of affinity rather than V_{max} . Referring to the apparent interspecies relationship of the cytosolic activities, it is likely that this observation also holds for the acenocoumarol and 4'-chlorowarfarin substrates.

In agreement with the observation of Moreland and Hewick (1975), we found the cytosolic ketone reductase activity to be sensitive for inducing agents.

Next to the cytosolic fraction, liver microsomes also exhibit ketone reductase activity. Substrate preference and product stereoselectivity are different from that of the cytosol. Solubilization of the microsomal enzyme did not change these features (own observations), excluding the possibility that the stereoselectivity is an environmental (microsomal membranes) phenomenon. Therefore, these data indicate the microsomal and cytosolic reductases to be different enzyme systems.

In enzymatic ketone reduction, product stereoselectivity is a well-known phenomenon (Prelog, 1964; Roerig et al, 1976; Kabuto et al, 1978; Prelusky et al, 1982). In studying the reduction of ketones by bacteriological systems, Prelog (1964) formulated a rule to predict the configuration of the resulting alcohol. If a ketone is projected in plane with the large group at the left, the resulting alcohol predominantly will have the configuration with the hydroxyl group above the plane (figure 5.4). Prelusky et al (1982), by studying the *in vivo* and *in vitro* reduction of some selected arylalkyl ketones in rabbits and rats affirmed the validity of Prelog's rule. For the warfarin analogues, the rule would imply the formation of the RS- and SS-alcohols from the R- and S-enantiomers, respectively. In the cytosolic systems, this

Figure 5.4: Prelog's rule for product stereoselectivity of enzymatic ketone reduction.

generally appears to be true for the R-, but not for the S-enantiomers (figure 5.3). For the microsomal systems, the S-, but less the R-enantiomers follow the rule (figure 5.2). Taking our findings together, one might raise the question whether Prelog's rule is valid for predicting product stereoselectivity of mammalian ketone reductases.

Attaching a meaning to the differences in reductase activities between the species is difficult. For human, bovine, pig and horse, only one liver could be used. This implies that for these species the data presented here are only indicative for species differences as intra-species variation cannot be ruled out. Furthermore, Moreland and Hewick (1975) showed the recovery of R-warfarin reductase activity from rat liver to decrease with post-mortem time. Only rat and rabbit livers were obtained immediately after death of the animals and the livers were perfused *in situ* with ice-cold saline before excision. Livers from the slaughter house were processed about 1-2 hr after death. The human liver was removed 3 hr after death. However, except for the human liver, all livers were put directly on ice to minimize loss of enzymatic activity. Reduction rates in both rat and human cytosolic fractions in our study are higher than in the study presented by Moreland and Hewick (1975).

Our *in vitro* data on warfarin ketone reduction in man are in accordance with the *in vivo* observations, i.e. the formation of the RS-alcohol mainly. This appears also to be the case for acenocoumarol, and therefore, the data would suggest that this metabolic route is not responsible for the great differences in pharmacokinetics between R- and S-acenocoumarol in man. The fact that R-acenocoumarol is reduced faster by the cytosolic reductases than R-warfarin may however contribute to differences in the elimination rate of the R-enantiomers of these 4-hydroxycoumarins.

In conclusion, although it is claimed that ketone reductases have a broad substrate specificity, there is a clear substrate stereoselectivity. Also, striking substituent dependencies are observed, suggesting the involvement of the benzyl moiety of the warfarin analogues in the reaction mechanism. In the next chapter (chapter 6) experiments with partially purified rabbit liver cytosolic warfarin reductases will be described.

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CHAPTER 6:

STERESELECTIVE ACETONYL SIDE CHAIN REDUCTION OF WARFARIN AND ANALOGUES: PARTIAL CHARACTERIZATION OF TWO CYTOSOLIC CARBONYL REDUCTASES.

6.1 ABSTRACT:

The reductase activity mediating the ketone reduction of the acetonyl side chain of warfarin and analogues has been partially purified from rabbit liver cytosol. The reductase activity is resolved in two different fractions, A and B, by DEAE-Sephacel chromatography. Both fractions reduce the acetonyl group of warfarin analogues with marked substrate- (R-enantiomers) as well as product stereoselectivity (alcohols of the S-configuration). The reductases are NADPH dependent, which is absolute for fraction B. The enzyme kinetics of the R-enantiomers of warfarin and three 4'- derivatives (4'-nitro-, 4'-chloro-, and 4'-methoxywarfarin) has been investigated. In contrast to fraction B, fraction A is sensitive in its K_m for 4' substitution: the K_m values of 4'- nitro- and chloro- analogues are about 6 times lower than the K_m values of the 4'- methoxy- analogue or warfarin itself. On the other hand, the V_{max} values of fraction A are all in the range of about 1 to 2 nmol/mgxmin, whereas the V_{max} values of fraction B vary from about 1 (4'-methoxywarfarin) to 12 (the 4'-nitro-analogue). The intrinsic activities (V_{max}/K_m) of both enzymes show the same rank order: 4'-nitro > 4'-chloro > 4'-methoxy = warfarin.

Warfarin reductase activity of both enzymes is not inhibited by pyrazole, sodium-barbitone or dicoumarol but strongly inhibited by quercetin, indomethacin, furosemide, and prostaglandin E2 (PGE2). In addition, fraction A is inhibited by menadione and androsterone; fraction B is inhibited by estrone. Various compounds were tested as substrates for these enzyme fractions. Menadione, haloperidol, glyceraldehyde, and estrone are substrates for fraction A but not for B. In contrast, androsterone is a (weak) substrate for fraction B only. p-Nitrobenzaldehyde, p-nitroacetophenone, and PGE2 are reduced by both enzymes. The K_m values for the latter substrate agree well with the K_i values that PGE2 has as (competitive) inhibitor of R-4'-nitrowarfarin reduction by both enzyme fractions. The product from PGE2 reduction by both enzyme fractions is prostaglandin F2 α .

The highly stereoselective acetonyl side chain reduction of warfarin analogues by rabbit liver cytosol is carried out by at least two enzymes. Endogenous substrates for both enzymes may be prostaglandins and steroids.

Adapted from: Drug Metab. Dispos. 20: 268-274, 1992. J.J.R. Hermans and H.H.W. Thijssen,

6.2 INTRODUCTION:

In man, the oral anticoagulant warfarin is extensively metabolised by hydroxylation reactions as well as reduction of the acetyl side chain (Lewis et al, 1974). About 15-20 % of an oral dose of warfarin is recovered as the alcohols produced by side chain reduction. Also for the warfarin analogue acenocoumarol (4'-nitrowarfarin) ketone reduction occurs (Dieterle et al, 1977).

Since warfarin possesses a chiral centre and ketone reduction introduces a second, reduction of the acetyl group will result in the formation of two pairs of diastereomeric alcohols: alcohol 1, being the RS/SR alcohols, and alcohol 2, the RR/SS alcohols (Chan et al, 1972). In man, ketone reduction is more important for the R-enantiomer than for the S-enantiomer of warfarin, and the alcohols formed are mainly in the RS- or SS- configuration (Lewis et al, 1974; Moreland and Hewick, 1975), demonstrating that the enzymes involved, show substrate- and product stereoselectivity.

Carbonyl reductases are a class of enzymes which are present in a great variety of species and tissues. These enzymes are able to reduce carbonyl groups of numerous xenobiotics as well as of endogenous compounds (Ahmed et al, 1979; Felsted and Bachur, 1980; Lee et al, 1988; Inaba et al, 1989; Iwata et al, 1990).

Carbonyl reduction has been shown to be involved in various inactivation processes of toxic compounds e.g. quinones (Wermuth et al, 1986), and drugs e.g. warfarin (Lewis et al, 1974; Moreland and Hewick, 1975). Also, the alcohols formed, may be pharmacologically active, as is the case for the alcohols of adriamycin (Loveless et al, 1978), naloxone (Roerig et al, 1976), and pentoxifylline (Ward and Clissold, 1987), thereby prolongating the pharmacological effect of the mother compound. Furthermore, a compound may be activated by this metabolic pathway; this is the case for the carcinogenic compound aflatoxin B1 (Wong and Hsieh, 1978) and may probably also be the case for keto-metabolites of 3-methylcholanthrene (Myers et al, 1989).

Next to its role in the metabolism of xenobiotics, carbonyl reduction plays an important role in the synthesis, interconversion and biodegradation of steroids (Pietruszko and Chen, 1976), prostaglandins (Hara et al, 1982), bile acids (Kudo et al, 1990), vitamin A (Felsted and Bachur, 1980), glucuronic acid (Felsted and Bachur, 1980), etc.

In chapter 5 we showed the reduction of the acetyl group of warfarin and some of its 4'- derivatives to be carried out by microsomal, as well as cytosolic NADPH dependent reductases. The cytosolic reductase activity was shown to exert a strong substrate- as well as product stereoselectivity (similar to that observed *in vivo* in man) and was shown to be present in a variety of species. In order to investigate this stereoselective metabolic pathway of warfarin in more detail, the cytosolic enzymes responsible for this metabolic pathway have been partially purified and an attempt has been made for characterization. The kinetics of various 4'- substituted warfarin analogues have been determined. Rabbit liver was used as the reductase source.

6.3 MATERIALS AND METHODS:

p-Nitroacetophenone, p-nitrobenzaldehyde, and haloperidol were obtained from Janssen (Beerse, Belgium). The prostaglandins E2 and F2 α were from Upjohn (Crawly, USA). All other chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany) unless stated otherwise.

Isolation of enantiomers and identification of the alcohols:

Warfarin was obtained from Sigma Chemicals (St Louis, MO), 4'-chlorowarfarin was from Aldridge Chemicals, and 4'-nitrowarfarin was a gift from Ciba-Geigy (Basel, Switzerland). 4'-Methoxywarfarin was synthesised as described in chapter 2.

Enantiomers were isolated by the method of West et al (1961). Purity of the enantiomers was tested by HPLC as described in chapter 2 and was higher than 98.5 %. Alcohols were identified by TLC as described in chapter 5.

Purification of the reductases:

All purification steps were carried out at 0 °C. 200 grams of (male) rabbit liver, obtained from the local poultry, were homogenized in 300 ml of buffer A (10 mM sodium phosphate buffer, pH=6.2, containing 1 mM dithiothreitol (DTT) and 1 mM EDTA) using a Potter homogenizer at 1500 rpm. The homogenate was centrifuged at 10,000 x g for 30 min. Subsequently, the supernatant was centrifuged for 50 min at 110,000 x g to obtain the cytosolic fraction.

The cytosolic fraction was passed through a short (10x1.0 cm) Biorex T-70 (sodium form, 200-400 mesh, obtained from Biorad, Veenendaal, the Netherlands) column, preequilibrated with buffer A, to remove hemoglobin (to avoid this protein to decrease flow rates of the next steps by aggregation). The reductase activity was not absorbed.

The pH of the effluent was brought to 7.5 by gradual addition of 0.1 M ammonia. Ammonium sulphate was added to 40 % saturation and the solution was stirred for 30 min, during which the pH was kept constant. The mixture was centrifuged at 10,000 x g for 10 min. To the supernatant, ammonium sulphate was added to obtain a solution of 80 % saturation. This fraction was stirred, kept at a pH of 7.5, and after 30 min centrifuged at 10,000 x g for 10 min.

The 40-80 % precipitate was dissolved in a minimal volume buffer B (10 mM sodium phosphate buffer pH=7.5, containing 1 mM DTT) and subjected to gel filtration on a Sephadex G-100 (Pharmacia fine chemicals, Uppsala, Sweden) column (100x2.6 cm) equilibrated with buffer B. Elution was carried out with buffer B at a flow rate of 10 ml/h. Fractions of 5 ml were collected and tested for reductase activity and protein content.

Active fractions from the G-100 step were pooled and applied to a DEAE Sephacel (Pharmacia fine chemicals, Uppsala, Sweden) column (40x5.0 cm) equilibrated with buffer B. After application, the column was washed with 2 l of buffer B. Elution was carried out at a flow rate of 100 ml/h using a linear NaCl gradient by gradual mixing of buffer B (1 l) with a 500 mM NaCl solution in buffer B (1 l). 8 ml fractions were collected and tested for activity

and protein content. This chromatographic step revealed two distinct active fractions (denoted as fraction A and B).

These fractions were concentrated by ammonium sulphate precipitation (at 95 % saturation) as described above. The precipitates of both fractions were dissolved in a minimal volume of buffer C (10 mM sodium phosphate buffer, pH=7.2, containing 2 mM DTT) and were desalted on short (5x1.5 cm) Sephadex G-25 columns equilibrated with buffer C.

The concentrated, desalted fractions were applied to a hydroxy-apatite column (12x1.0 cm) equilibrated with buffer C. Under these circumstances the reductase activity of fraction A was recovered in the void volume of the column. The reductase activity of fraction B was eluted from the hydroxy-apatite column using a linear sodium phosphate gradient by gradual mixing of 200 ml buffer C with 200 ml of a 150 mM sodium phosphate solution, pH=7.2, containing 2 mM DTT. 5 ml fractions were collected and tested for reductase activity.

The active fractions from this final step were pooled, and diluted with phosphate buffer to yield solutions containing 1 mg protein/ml in 50 mM sodium phosphate buffer. These solutions were used as enzyme fractions A and B in the experiments described. The fractions were stored at -80 °C until use. No loss of activity, due to storage, was observed. All experiments described, were carried out using fractions A and B from one single preparation.

Assay for the alcohol formation of warfarin and analogues:

During the purification procedure, fractions were tested for warfarin reductase activity as described in chapter 5.

Enzyme kinetic studies and the inhibitor studies were carried out almost similarly but there were some minor modifications. The complete reaction mixture consisted of 20 µl of enzyme fraction, substrate, NADPH, 7.0 mM MgCl₂, 25 mM glucose-6-phosphate (G-6-P) and 3.4 µ units G-6-P-dehydrogenase (Sigma Chemicals, St Louis MO) in a total volume of 60 µl. For determination of V_{max} and K_m values of warfarin and analogues the NADPH concentration was 1.0 mM; substrate concentrations were varied from 0 to 2.5 mM. In the kinetic mechanistic study NADPH was varied from 0 to 100 µM, at R-nitrowarfarin concentrations of 0.125, 0.25, 0.50, and 1.00 mM. Substrate- and product stereoselectivity was determined at an NADPH concentration of 1.0 mM and a substrate concentration of 2.5 mM. In the inhibition experiment, the inhibitor concentration was 1.0 mM, except for quercetin (0.1 mM), R-nitrowarfarin was used as a substrate at 0.5 mM. Inhibitors were either dissolved in Tris/KCl buffer or in 1.5 % ethanol in Tris/KCl buffer. The final concentration of ethanol did not exceed 0.5%; reductase activity was not influenced by this concentration of ethanol.

The complete reaction mixture, without the enzyme, was preincubated for 2 min at 37 °C. The reaction was started by the addition of the prewarmed enzyme fractions. After 15-25 min of incubation at 37 °C, the reaction was stopped by the addition of 0.5 ml 0.1 N sodium-acetate buffer pH=4.5. 20 µl of internal standard (a 1.2 mM solution of nitrowarfarin or warfarin,

depending on the substrate used) was added and was extracted with 2 ml of a 1/1 (v/v) mixture of petroleum-ether/methylene chloride. The organic phase was evaporated to dryness under a stream of nitrogen at 37 °C. The residue was taken up in 0.30 ml of a 1/1 mixture of acetonitrile and water. 20 µl was used for assay on HPLC.

The mobile phase of the HPLC system used consisted of a 3/7 (v/v) mixture of acetonitrile and 0.1 % (v/v) acetic acid. The pH of the eluent was 4.76 (adjusted with ammonia) for the assay of warfarin and nitrowarfarin alcohols and 5.10 (adjusted with ammonia) for the assay of chlorowarfarin and methoxywarfarin alcohols. The stationary phase consisted of Chromspher C-18 (Chrompack, the Netherlands). Column dimensions were 200x3 mm for the assay of warfarin and nitrowarfarin alcohols and 100x3 mm for the assay of methoxywarfarin and chlorowarfarin alcohols. The flow rate was 1.5 ml/min. The absorbance at 303 nm was recorded.

Enzyme kinetic parameters were obtained by direct fitting the Michaelis-Menten equation to the data using the software package GPAD (GraphPAD Software, San Diego, CA).

Other assays:

Protein content was determined by the method of Lowry et al (1951). The reduction of other substrates was assayed as follows: 20 µl enzyme fraction was incubated with substrate (1 mM) and NADPH (1 mM) at 37 °C for 30 minutes. The reaction was stopped by the addition of 0.5 ml 0.1 M sodium-acetate buffer pH=4.5. These mixtures were centrifuged at 1500 rpm for 1 minute. The supernatant was used to determine the amount of oxidised NADPH spectrophotometrically. Appropriate blanks were run in parallel. This method was validated by determining the amount of alcohols of R-nitrowarfarin, p-nitrobenzaldehyde and p-nitroacetophenone on HPLC (using the same column and eluent as for R-nitrowarfarin but measuring the absorption at 280 nm) and comparing it with the amount of oxidized NADPH as described above.

6.4 RESULTS:

Purification results are given in table 6.1. Quick processing of the fractions was found to be necessary since warfarin reductase activity appeared to be inactivated rapidly (a decrease of activity of about 40 % was observed after 12 hours of storage at 0°C).

DEAE-Sepharcel chromatography revealed two distinct fractions of warfarin reductase activity (figure 6.1). Fraction A eluted from the column at a NaCl concentration of about 0.1 M; fraction B eluted at a concentration of about 0.2 M. Fraction A was not absorbed by hydroxy-apatite, whereas fraction B was absorbed and eluted at a sodium phosphate concentration of about 50 mM. At this stage, the specific activity was enlarged 92- and 240 fold for enzyme fraction A and B respectively at recoveries of 6 and 17 %. Attempts for further purification of the reductases appeared troublesome because of the rapid loss of reductase activity at this stage of purification.

TABLE 6.1: Purification results.

Fraction		Total activity (nmol/min)	Total protein (g)	Specific activity (pmol/mgxmin)	Purification (fold)	Recovery (%)
Cytosol		691	26.8	26	--	100
Biorex T-70		636	19.7	32	1.3	92
Amm.sulf prep. 75% s.		572	10.9	53	2.0	83
Sephadex G-100		531	6.7	79	3.1	77
DEAE-Sephacel	A	90	0.5	180	7.0	13
	B	155	0.2	775	30	21
Hydroxy-appatite	A	47	0.02	2350	92	6
	B	125	0.02	6250	240	17

Figure 6.1: DEAE fractionation pattern of the rabbit liver cytosolic warfarin reductases.

Chromatography was carried out as described in the materials and methods section. The fractions are annotated A and B in order of their elution from the DEAE sephacel column.

The obtained enzyme fractions prefer NADPH as a cofactor for reduction of the acetyl side chain of warfarin. NADPH is obligatory for fraction B, whereas fraction A shows 15 times lower reduction rates if NADH instead of NADPH is used. Reduction rates were linear with time and the amount of enzyme added. Even at high alcohol- (up to 5 mM) and NADP⁺ concentrations, the reversed reaction (oxidation of the alcohols to the corresponding ketones) was not observed for both fractions.

TABLE 6.2: Substrate- and product stereoselectivity of the reduction of the acetyl group of 4'-substituted warfarins by the rabbit liver cytosolic fractions.

FRACTION A	S-alcohols	R-alcohols
R-warfarin	8.60 ± 1.21	0.10 ± 0.02
R-nitrowarfarin	17.50 ± 0.52	0.34 ± 0.01
R-chlorowarfarin	9.40 ± 0.52	0.14 ± 0.03
R-methoxywarfarin	13.00 ± 0.61	0.65 ± 0.04
S-warfarin	nd	nd
S-nitrowarfarin	0.97 ± 0.08	nd
S-chlorowarfarin	0.78 ± 0.06	nd
S-methoxywarfarin	nd	nd
FRACTION B	S-alcohols	R-alcohols
R-warfarin	16.00 ± 1.77	0.10 ± 0.01
R-nitrowarfarin	102.00 ± 6.63	2.26 ± 0.17
R-chlorowarfarin	62.00 ± 2.19	6.20 ± 0.19
R-methoxywarfarin	11.25 ± 0.91	0.67 ± 0.08
S-warfarin	0.47 ± 0.05	nd
S-nitrowarfarin	5.18 ± 0.21	nd
S-chlorowarfarin	1.12 ± 0.21	0.39 ± 0.14
S-methoxywarfarin	2.62 ± 0.28	nd

Alcohol formation was assayed as described in the methods section at substrate concentrations of 2.5 mM and a NADPH concentration of 1.0 mM. Data are expressed as nmol alcohol formed per 10 min per mg protein and are the mean of 3 incubations ± standard deviations. nd: not detected

Both enzyme fractions display a high degree of substrate- and product stereoselectivity in reducing the acetyl side chain of warfarin and analogues (table 6.2). The R-enantiomers of all warfarin analogues are clearly preferred as substrate over the S-enantiomers (R/S ratios of alcohol formation rates are 15 to >250 and 5 to 35 for fraction A and B respectively). For both the R- and S-enantiomers the alcohols produced are mainly in the S-configuration. RS-alcohol formation rates were 20 to 86- (fraction A) and 10 to 160 (fraction B) times higher than RR alcohol formation rates. Formation of the SR alcohol could only be observed for enzyme fraction B, using S-4'-chlorowarfarin as substrate. Since the product of S-warfarin analogues (the SS-alcohols) is easily separable by HPLC from the main product of the R-enantiomers (the RS-alcohols) we were able to discover that the S-enantiomers of the warfarin analogues are no or only very weak inhibitors of the reduction of the R-enantiomers (less than 5 % inhibition at 15 fold excess of S-vs R-).

The kinetic parameters of the S-alcohol formation of the R-enantiomers of four warfarin analogues are shown in table 6.3. The Vmax values of enzyme fraction A range from about 1 to 2 nmol/mg protein x min . The Vmax values of enzyme fraction B are more variable for the warfarin analogues; they range from about 1.3 (4'-methoxywarfarin) to about 12 nmol/mgxmin (4'-nitrowarfarin).

TABLE 6.3: Enzyme kinetic parameters of both carbonyl reductase fractions for the formation of RS alcohols using various warfarin 4'-derivatives as a substrate

SUBSTRATE	ENZYME FRACTION A			ENZYME FRACTION B		
	Vmax	Km	Cli	Vmax	Km	Cli
R-warfarin	1.33	1.34	1.0	1.75	0.41	4.3
R-nitrowarfarin	1.92	0.22	8.7	11.6	0.35	33
R-chlorowarfarin	1.02	0.23	4.4	6.90	0.28	25
R-methoxywarfarin	2.04	1.46	1.4	1.28	0.34	3.8

Enzyme kinetic parameters were obtained by direct fitting of the Michaelis-Menten equation to the data (alcohol-formation rates were measured at 8 different substrate concentrations ranging from 0-2.5 mM; incubations were carried out in triplicate). Incubations were carried out as described in the Materials and Methods section. Vmax values are expressed as nmol RS-alcohol/mg xmin. Km values are given in mM. Cli: intrinsic clearance (Vmax/Km) in µl/mg protein x min.)

TABLE 6.2: Substrate- and product stereoselectivity of the reduction of the acetyl group of 4'-substituted warfarin by the rabbit liver cytosolic fractions.

FRACTION A

S-alcohol

R-alcohol

In contrast, the K_m values of enzyme fraction A appear to be more sensitive for 4'-substitution than the K_m values of enzyme fraction B: K_m 's of fraction A are about 6 times lower for nitrowarfarin (0.22 mM) and chlorowarfarin (0.23 mM) than for warfarin (1.34 mM) and methoxywarfarin (1.46 mM) whereas the K_m values of fraction B for the analogues are in the same range (0.3 to 0.4 mM).

Based on the intrinsic clearances (V_{max}/K_m) it is clear that the R-enantiomers of nitrowarfarin and chlorowarfarin are far better substrates for both reductases than the R-enantiomers of warfarin and methoxywarfarin.

The kinetics of the cofactor NADPH have been studied at four different substrate concentrations (figure 6.2). The four obtained lines intersect in the second and third quadrant respectively for enzyme fraction B and A respectively. This pattern is consistent with an ordered sequential or a quasi equilibrium random mechanism, both of which are not uncommon for carbonyl reducing enzymes (Felsted and Bachur, 1980). The 'real' K_m values for NADPH, calculated from (linear) replots are 4 (μ M) (fraction A) and 9 (μ M) (fraction B).

Figure 6.2: Double reciprocal plots of R-nitrowarfarin reduction by fraction A (left) and B at various NADPH and R-nitrowarfarin concentrations.

Data are the mean $\pm$ SD from 3 incubations.

A: [R-nitrowarfarin] = 125 μ M, B: [R-nitrowarfarin] = 250 μ M, C: [R-nitrowarfarin] = 500 μ M,

D: [R-nitrowarfarin] = 1000 μ M

TABLE 6.4: The effect of some selected inhibitors on rabbit liver cytosolic R-warfarin reductases.

Inhibitor	% inhibition (SD)	
	Fraction A	Fraction B
Pyrazole	14 (10)	4 (2)
Sodium-barbitone	2 (3)	0 (1)
Quercetin	88 (2)	88 (1)
Dicoumarol	4 (1)	5 (1)
DL-Glyceraldehyde	20 (3)	4 (4)
HgCl ₂	99 (1)	95 (1)
Furosemide	98 (1)	45 (5)
Indomethacin	75 (1)	87 (1)
Prostaglandin E ₂	62 (5)	78 (4)
Estrone	7 (2)	30 (4)
Androsterone	40 (6)	5 (5)
Menadione	74 (4)	3 (5)
Haloperidol	12 (5)	1 (3)
Metyrapone	4 (3)	3 (1)

Data are expressed as the mean $\pm$ SD of 3 incubations. The inhibitor concentration was 1.0 mM, except for quercetin (used at a concentration of 0.1 mM). R-nitrowarfarin was used as substrate at a concentration of 0.5 mM. The cofactor (NADPH) concentration was 1.0 mM. Incubations were carried out as described in the text.

In order to characterize both warfarin reductases, the effect of some selected inhibitors has been investigated. The results are summarized in table 6.4. Pyrazole (an alcohol dehydrogenase inhibitor), sodium barbitone (an aldehyde reductase inhibitor), and dicoumarol (a DT-diaphorase inhibitor) show no inhibiting effect on both reductases. In contrast, the flavonoid quercetine, indomethacin, furosemide and prostaglandin E₂ (PGE₂) are potent inhibitors of both reductase fractions. Menadione, androsterone and to a lesser extent DL-glyceraldehyde inhibit fraction A but not fraction B. Estrone decreases this reaction for reductase B but not for reductase A. Mercuri-chloride inhibits both enzymes completely. This may point to the presence of essential thiol groups.

TABLE 6.5: Reduction rates of both reductase fractions using various substrates.

Substrate	NADPH oxidized (nmol/mgxmin)	
	mean (SD)	
	Fraction A	Fraction B
R-Nitrowarfarin	1.5 (0.7)	8.5 (0.5)
p-Nitroacetophenone	22 (1.5)	6.1 (0.5)
p-Nitrobenzaldehyde	14 (2.1)	22.5 (4.3)
Prostaglandin E2	3.2 (0.5)	1.2 (0.1)
Menadione	33 (0.4)	nd
Metyrapone	nd	nd
Haloperidol	3.5 (0.5)	nd
Androsterone	nd	0.8 (0.1)
Estrone	0.6 (0.1)	nd
DL-Glyceraldehyde	3.0 (0.1)	nd

Data are expressed as the mean (SD) of 3 incubations.

nd: not detectable

Various compounds were tested as substrates for both reductase fractions (table 6.5). As shown, p-nitrobenzaldehyde and p-nitroacetophenone are relatively rapidly reduced by both fractions. Menadione, is rapidly reduced by reductase A, but is no substrate for reductase B. The same holds true for DL-glyceraldehyde, estrone, and haloperidol. In contrast androsterone is a (weak) substrate for reductase B.

The diagnostic drug metyrapone, which is shown to be reduced by quercitrin sensitive cytosolic reductases (next to a microsomal barbiturate sensitive reductase) (Maser and Netter, 1991), appears to be no substrate for both enzyme fractions.

PGE2 is reduced by both enzyme fractions. Since this compound also is a clear inhibitor of the reduction of R-nitrowarfarin by both reductase fractions, the kinetics of PGE2 reduction and the inhibition kinetics of R-nitrowarfarin reduction have been studied in some detail. PGE2 reduction follows Michaelis-Menten kinetics (figure 6.3). V_{max} values are 3.6 and 1.6 nmol/mgxmin. for fraction A and B respectively. K_m values are 0.14 and 0.16 mM. Extracts from 1 hour incubations of both enzyme fractions with PGE2 and NADPH were analyzed on HPLC, as described by Terragno et al (1981). Next to the substrate peak, an additional peak was detected, which co-eluted with prostaglandin F2 α (data not shown).

The inhibition of R-nitrowarfarin reduction by PGE2 appears to be of the competitive type (figure 6.4) with K_i values of 0.14 mM (fraction A) and 0.15 mM (fraction B).

6.5 DISCUSSION:

In chapter 5, we showed warfarin reductase activity to be present in liver microsomes and cytosol of various species. In contrast to the microsomal fractions, the cytosolic fractions showed a general pattern of substrate- and product stereoselectivity among species, which was not different for the warfarin analogues tested. Predominantly, the R-enantiomers of 4'-substituted warfarins were reduced to the S-alcohols. In the present study we were able to resolve the cytosolic NADPH dependent warfarin reductase activity from rabbit liver into two distinct fractions by the use of DEAE-Sepharcel chromatography (table 6.1).

Although the two reductase fractions both exhibit the same substrate- (for the R-enantiomers) as well as product stereoselectivity (S-alcohols), substitution at the 4' position of warfarin differently affects the kinetics of both fractions (table 6.3). Kinetic differences are also observed for the cofactor NADPH at various R-nitrowarfarin concentrations (figure 6.2). These observations, in addition with the observed differences in the substrate and inhibitor profile (tables 6.4 and 6.5), make it unlikely that warfarin reduction by the resolved fractions is in fact carried out by one single enzyme, being present in two electrical or oligomeric states. Both reductases are not inhibited by sodium-barbiturate but strongly inhibited by quercetin and should therefore be classified as ketone reductases rather than aldehyde reductases (Ahmed et al, 1979). However, although having been purified on their ability to reduce the ketonic group of warfarin, both enzymes are able to reduce carbonyl groups from a variety of compounds among which are the quinone menadione, DL-glyceraldehyde (fraction A only), p-nitrobenzaldehyde, and some ketones. The ability of ketone reductases to reduce carbonyl groups, other than ketonic groups is not uncommon for this class of enzymes (Feisted and Bachur, 1980). Therefore, the term ketone reductase appears to be rather inconsequent for these enzymes, as has been previously also stated by Wermuth et al (1981), and could perhaps better be replaced by the term barbiturate insensitive/flavonoid sensitive carbonyl reductase.

From the physiological compounds tested, PGE₂ and the steroids androsterone (fraction B only) and estrone (fraction A only) are reduced by the reductases. The K_m values for PGE₂ reduction (0.14 and 0.16 mM for fractions A and B respectively) are in the same order of magnitude as the K_i values (0.14 and 0.15 mM respectively) that PGE₂ exhibits as inhibitor of R-nitrowarfarin reduction. In addition, angiotensin I, which has been shown to activate PGE₂ reductases (Lee and Levine, 1975), was able to enhance the reduction of R-nitrowarfarin by enzyme fraction B slightly (1.4 fold) but significantly ($P < 0.01$, t-test), by increasing the affinity of the enzyme for NADPH (own observations). Furthermore indomethacin and furosemide, known prostaglandin reductase inhibitors (Chang and Tai, 1981; Wermuth 1981), strongly inhibit the alcohol formation of R-nitrowarfarin by both fractions (table 6.4). Therefore, prostaglandins and possibly steroids may perhaps be physiological substrates for both reductases. The K_m values we found for the reduction of PGE₂ are in the range of K_m values that were reported previously for NADPH dependent prostaglandin 9-keto-reductases

in several species (0.08 to 0.32 mM) (Lee et al 1975; Stone and Hart, 1975; Lin and Jabarak 1978; Chang and Tai, 1981; Wermuth, 1981; Hara et al, 1982; Iwata et al, 1989). Whether the interaction of therapeutic doses of warfarin or its analogues with the NADPH dependent 9-keto-reduction of prostaglandins may have physiological implications is unlikely, since the conversion of PGE₂ and the formation of prostaglandin F_{2a} can be carried out via alternative and more efficient routes (Lands, 1979).

NADPH dependent reductases, having the ability to reduce the 9-keto-group of prostaglandins were initially studied because of their prostaglandin convertase activity (Lee and Levine, 1975; Hassid and Levine, 1977; Lin and Jarabak, 1978). Later, it became clear that these enzymes may not (only) serve as prostaglandin convertases, but may (also) play an important role in the detoxification of ketones, aldehydes and quinones (Lin and Jabarak, 1978; Chang and Tai, 1981; Wermuth et al, 1986). This appears to be also true for the rabbit liver cytosolic enzyme fractions described in this chapter.

Reduction of prochiral ketones results in the introduction of a chiral centre in the secondary alcohol that is produced. Carbonyl reductases are known to exert product stereoselectivity (Roerig et al, 1976; Felsted and Bachur, 1980; Prelusky et al, 1982; Falgouty et al, 1990; de Carvalho et al, 1991). By using pure enantiomers as a substrate for ketone reducing enzymes, product stereoselectivity can be easily studied since the diastereomeric alcohols are separable by simple chromatographic procedures. This may be useful since product stereoselectivity may indirectly give an indication of the orientation of the substrate when bound to the metabolizing enzyme. In this respect, the enantiomers of warfarin (or its analogues) may be interesting substrates.

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CHAPTER 7:

GENERAL DISCUSSION

7.1 THE BASIS OF THE PHARMACOKINETIC DIFFERENCES OF THE ENANTIOMERS OF WARFARIN AND ACENOCOUMAROL:

The awareness of the fact that stereoisomers may behave differently in biological systems has been growing rapidly in the past decades. One of the reasons is that new techniques in the synthesis and isolation of pure stereoisomers as well as enantioselective analytical procedures have become available. Also, various authors pointed to the importance of analyzing differences that stereoisomers may exert in biological systems (Ariëns et al, 1983; Ariëns, 1984; Simonyi, 1984; Drayer, 1988; Evans et al, 1988; Williams, 1989). That this field is gaining more and more attention is demonstrated by the fact that in 1989 a journal (*Chirality*) has been launched which specifically deals with the subject of stereoisomerism in pharmacology and toxicology.

Warfarin is an example of a chiral drug whose enantiomers differ in their pharmacokinetics as well as in interactions with other drugs (see chapter 1). The 4'-nitro analogue of warfarin, acenocoumarol possesses completely different pharmacokinetic properties than warfarin. The enantiomers of acenocoumarol are not only eliminated faster than those of warfarin, but also marked stereochemical differences are observed as has been discussed in chapter 1. Substitution of the 4'-hydrogen of warfarin by an amino or acetamido group was also shown to have a great impact on the rate of elimination as well as on the stereoselectivity thereof (Thijssen and Baars, 1983; Thijssen et al, 1985). This may point to an important role of the phenyl ring of warfarin in determining the stereochemical course of its pharmacokinetics. Therefore, we studied the pharmacokinetics of various phenyl ring substituted warfarin analogues in the rat (chapter 2). Next to effects on (stereoselective) protein binding, phenyl ring substitution does not only enhance the intrinsic clearances but also has a clear effect on the stereoselectivity thereof. Generally, of the 2'- and 4'-substituted analogues, the intrinsic clearances of the S-enantiomers are higher than the intrinsic clearances of the R-enantiomers, whereas the opposite is observed for the 3'-substituted analogues. In an attempt to reveal the mechanism of the differences between warfarin analogues, we compared the *in vitro* biotransformation of warfarin with that of acenocoumarol. This compound was chosen since acenocoumarol, like warfarin, is widely used as oral anticoagulant, but studies on its *in vitro* biotransformation have not yet been conducted.

The experiments described in this thesis show that the stereoselectivity and the rate of the oxidative metabolism differ greatly for warfarin and acenocoumarol, despite the fact that apart from 4'-hydroxylation, warfarin and acenocoumarol are hydroxylated at the same positions. In chapter 3, it was shown that the rat liver microsomal metabolism of warfarin is stereoselective for the R-enantiomer, whereas that of acenocoumarol is stereoselective for the S-enantiomer. In contrast, in human liver microsomes (chapter 4) the metabolism of both compounds is stereoselective for the S-enantiomer, but the difference between the enan-

tiomers is far more pronounced for acenocoumarol than for warfarin. In addition, in both rat liver and human liver microsomes, the metabolic clearance of acenocoumarol (chapters 3 and 4) greatly exceeds that of warfarin, due to the lower K_m values of acenocoumarol towards monooxygenases. These differences between warfarin and acenocoumarol are qualitatively in agreement with the observed differences in the intrinsic clearances between these compounds *in vivo* (Yacobi and Levi, 1974; Kelly and O'Malley, 1979; Thijssen et al, 1985; Thijssen et al, 1986; Holford, 1986; chapter 2) and the results obtained for warfarin metabolism agree well with previous studies that were conducted on the microsomal metabolism of warfarin (Pohl et al, 1976a; Rettie et al, 1989).

Since it is known that the enantiomers of warfarin are metabolized by a variety of cytochrome P450 isozymes in man and in the rat (Fasco et al, 1976; Pohl et al, 1977, Guengerich et al, 1982, Wang et al, 1983; Kaminsky et al, 1985, Rettie et al, 1992), the question arose whether the same set of isozymes is involved in the microsomal metabolism of both 4-hydroxycoumarins. Experiments with enzyme inducers as well as with the cytochrome P450 inhibitor cimetidine showed that in the rat this is probably not the case (chapter 3) since the metabolism of warfarin and acenocoumarol is differently affected. Immuno-inhibition experiments revealed that rat liver microsomal P450 isozymes of the 2C and 3A subfamilies possess hydroxylase activity towards both 4-hydroxycoumarins. However, marked differences in the regio- and stereoselectivity of these P450 isozymes are observed for both compounds, indicating that the 4-hydroxycoumarins are differently oriented to the catalytic site of the cytochromes P450. For instance, if coumarin ring hydroxylation is considered, P450 isozymes of the 2C subfamily only catalyse the hydroxylation of warfarin at the 7-position. In the case of acenocoumarol however, these isozymes catalyse the hydroxylation at the 6-, 7-, and 8-positions. This also shows that at least a part of the coumarin hydroxylations of the enantiomers of warfarin and acenocoumarol is catalyzed by different enzymes.

Similarly, in human liver microsomes warfarin and acenocoumarol also appear to be metabolized by different sets of isozymes. Cytochrome P450 2C9 has been shown to be a S-warfarin 7-hydroxylase which accounts for the main part of the metabolic clearance of S-warfarin (Rettie et al, 1992). R-warfarin was shown to be no substrate for this cytochrome P450 isozyme. Consequently, sulfaphenazole, a specific competitive inhibitor of cytochromes P450 2C8-C10 (Guengerich, 1992), inhibits the 7-hydroxylation of S- but not of R-warfarin in human liver microsomes (chapter 4). However, in the case of acenocoumarol the 7-hydroxylation of both enantiomers and the 6-hydroxylation of S-acenocoumarol is inhibited by sulfaphenazole up to 70%. This proves that contrary to warfarin, a substantial part of the metabolic clearance of R-acenocoumarol in man is due to P450 isozymes of the 2C subfamily.

GENERAL DISCUSSION

Furthermore, we showed that the (sulfaphenazole inhibitable) acenocoumarol hydroxylations do not correlate with the 7-hydroxylation of S-warfarin. This may indicate that different members of the human liver cytochrome P450 2C subfamily are involved in the coumarin hydroxylations of warfarin and acenocoumarol. The different effect of the inhibitor propafenone on the metabolism of the 4-hydroxycoumarins also shows that at least a part of the metabolism of both compounds is conducted by different P450 isozymes.

Taken together, the experiments in chapter 3 and 4 show that the differences that warfarin and acenocoumarol show in their oxidative metabolism can (at least partly) be explained by the fact that the 4-hydroxycoumarins are metabolized by different sets of isozymes. Substitution at the phenyl ring of warfarin does not only influence the stereo- and regioselectivity of the monooxygenases but also determines which isozymes metabolize the 4-hydroxycoumarin.

Another metabolic pathway of warfarin is reduction of its acetyl side chain to its diastereomeric alcohols (Lewis et al, 1974; Banfield, 1974). The reductive route has been shown to be of only minor importance in the biotransformation of warfarin in the rat (Pohl et al, 1976b). The reason for this is that the intrinsic clearance of the formation of the alcohols is relatively low as compared to the hydroxylated metabolites of warfarin (Pohl et al, 1976a; Moreland and Hewick, 1975). In man however, formation of the diastereomeric alcohols is an important metabolic route for R- but not S-warfarin and product stereoselectivity for the formation of the S-alcohol is observed (Lewis et al, 1974; Banfield et al, 1983). In agreement with Moreland and Hewick (1975), we found that the R-warfarin reducing reductase activity is mainly located in the cytosol (chapter 5); the rat and human liver microsomal reduction of warfarin and acenocoumarol, appeared to be of only minor importance in the metabolic clearance of the 4-hydroxycoumarins. Contrary to liver microsomal fractions, a general pattern of substrate- (reduction of the R-enantiomers) and product stereoselectivity (formation of the S-alcohols) is observed in the liver cytosolic fractions of various species. This stereoselectivity is not different for the 4'-substituted warfarin analogues tested. Acenocoumarol and chlorowarfarin were shown to be reduced faster by the cytosolic fractions than warfarin itself. In chapter 6, we showed that in rabbit liver cytosol, the acetyl side chain reduction of warfarin analogues is conducted by at least two carbonyl reductases having a similar substrate (R-enantiomers) and product (S-alcohols) stereoselectivity. These enzymes appear to be related, based on their similar inhibitor pattern. For both enzymes, acenocoumarol and 4'-chlorowarfarin are better substrates than warfarin or its 4'-methoxy analogue. However, the basis of the different intrinsic activities of the enzymes towards the 4-hydroxycoumarins is different. In the case of carbonyl reductase fraction A, the 4-hydroxycoumarins possess different K_m values but similar V_{max} values. In the case of carbonyl reductase fraction B, the differences in the intrinsic activities between the substrates are a matter of their different V_{max} values.

Thus, although the cytosolic ketone reduction of warfarin and its analogues is conducted with similar stereoselectivity, differences between the 4-hydroxycoumarins exist in the rate of reduction of the R-enantiomers. This may probably contribute to the differences in the rate of elimination of the R-enantiomers of warfarin and acenocoumarol.

In conclusion, the differences that warfarin and acenocoumarol show in the rate and stereoselectivity of their intrinsic clearances, can largely be explained by the differences of the enantiomers of these 4-hydroxycoumarins in the rate of their metabolism. These metabolic differences can be ascribed to the fact that the 4-hydroxycoumarins are able to interact with a variety of metabolizing enzymes possessing different stereochemical and physico-chemical demands. The stereoselectivity of these enzymes and their contribution to the clearance of the warfarin analogue is influenced by substitution at the phenyl ring.

7.2 COMPLEXITY OF ENANTIOMERIC DIFFERENCES

The experiments described in this thesis clearly show that differences between enantiomers in biological systems are due to a combination of their different three-dimensional structures and the variety of biomolecules with which they can or cannot interact. For a chiral compound, the overall stereoselectivity of its effect will be determined by the stereochemical demands of the biomolecules with which the compound interacts, as well as the contribution of these biomolecules in the resulting effect.

Clearly, predicting the stereoselectivity of the overall effect will be more difficult if the amount of biomolecules, with which enantiomers interact is large. In addition, larger interindividual and interspecies differences can be expected. However, even the interaction of enantiomers with only one biomolecule is more complex than originally stated by Easson and Stedman (1933). Although their 3-point attachment model (also see the introduction section) proved its value in the explanation of various enantiomeric ligand-receptor interactions (Ruffolo et al, 1983), the model has some limitations as has been discussed by Testa (1990):

- 1) The model only gives a qualitative description but no quantitative information (on e.g. eudismic ratios).
- 2) Only attractive, not repulsive interactions (e.g. steric hindrance) between receptors and ligands are dealt with.
- 3) The model only deals with the binding of ligands, not with their intrinsic activities.

To obtain insight in the factors that determine the different interaction of enantiomers with biomolecules, quantitative structure-activity studies using the biomolecules in a purified homogeneous form should be performed. Nevertheless, this only provides information on the interaction of chiral compounds with one type of biomolecule. Clearly, extrapolation to more complex systems, where the enantiomers interact with a wider range of biomolecules (and

may therefore show differences in their pharmacokinetics, as well as in the effects they may exert) will be a difficult matter.

Taken together, the difficulties of predicting the effects of racemates in biological systems, validate the statement of Ariëns (1983, 1984, 1987, 1991) that racemic drugs, should in fact be considered as mixtures of two different drugs, despite the similar physico-chemical properties of the enantiomers.

7.3 ENANTIOMERS AS TOOLS IN BIOMEDICAL SCIENCE:

The fact that enantiomers interact differently with biomolecules, but have similar physico-chemical properties, makes them useful experimental tools in biomedical sciences (Williams, 1989).

Because enantiomers possess similar physico-chemical properties, but often show large differences in their interaction with receptors, the distomer of a chiral drug may serve as a tool to investigate whether a certain type of receptor is involved in certain effects of the drug. For instance, the effects of the β -adrenoceptor blocking agent propranolol that are associated with its membrane stabilizing properties do not show stereoselectivity, in contrast to its effects *via* β -adrenoceptors (Rahn, 1983; Simonyi, 1984). The absence or presence of enantiomeric differences in the effect of β -adrenoceptor blocking agents thus provides insight whether or not certain pharmacological effects are indeed mediated by β -adrenoceptors (Rahn, 1983).

Stereoselectivity in ligand-receptor interactions or in substrate-/inhibitor-enzyme interactions may give information on the orientation of the ligand, when bound to the biomolecules, and may be of help in designing more efficient ligands for a specific receptor or enzyme (Testa, 1988). For instance, the study of the regio- and stereoselective metabolism of benzo(a)pyrene, provided information of the spatial requirements of cytochrome P450 1A1 (Jerina et al, 1985).

In the case of stereospecific active or facilitated transport, the enantiomer which is not subjected to this type of transport may give information on the role of passive diffusion in the disposition of the drug. An example of this is methotrexate, of which the D-enantiomer has been suggested as a probe to estimate entero-hepatic cycling of the clinically used L-enantiomer (Hendel and Brodthagen, 1984).

Furthermore, the stereoselectivity in the metabolism of compounds may provide more sensitive tools for the investigation of genetic polymorphisms as is for example shown for mephenytoin: the urinary S/R ratio of this compound appears to be an equally accurate but less time consuming screening method for phenotyping (Guttendorf and Wedlund, 1992) than the classical approach (determination of the urinary ratio of the 4-hydroxy metabolite of S-mephenytoin *versus* unchanged S-mephenytoin).

Finally, in enzyme catalysed reactions, in which an additional chiral element is introduced in the product, enantiomers offer the advantage that the products are diastereomeric and as such more easily separable by chromatographic procedures than the single enantiomers formed from an achiral substrate (Testa, 1986). An example is provided by the metabolic reduction of the ketone group of warfarin and analogues, as described in chapters 5 and 6.

7.4 ENANTIOMERICALLY PURE *VERSUS* RACEMIC DRUGS:

Since many chiral drugs were developed in an era in which knowledge of the differences between stereoisomers was less sophisticated and in which synthesis or isolation of pure stereoisomers was technically very difficult, if not impossible, about 25% (Ariëns, 1991) of the drugs currently marketed, are mixtures of stereoisomers. However, in the last decade, it is questioned by various authors whether or not chiral drugs should be marketed as mixtures of stereoisomers or as pure stereoisomers (Ariëns et al, 1983; Ariëns, 1984; Kumkumian, 1988; Birkett, 1989; Williams, 1989; Lennard, 1991). Currently, no clear answer has been given to this question and the debate is still going on. Some considerations concerning the use of racemic or enantiomerically pure drugs will be put forward.

For chiral drugs various possibilities have to be considered (Ariëns, 1984):

- 1) The enantiomers undergo chiral inversion or racemization.
- 2) The enantiomers differ quantitatively in their (desired) pharmacological effect.
- 3) The enantiomers differ qualitatively in their pharmacological effect.

Three types of chiral inversion may occur: I) the eutomer is transformed to the distomer II) the distomer is transformed to the eutomer (e.g. NSAIDs of the arylpropionic acid type; Caldwell et al, 1988), and III) racemization.

Extensive transformation of the eutomer to the distomer does not seem desirable, especially if the eudismic ratio is high. Transformation of the distomer to the eutomer may at first sight appear to be in favor of the use of the racemate, but problems may arise since inversion may be not complete and may show large interindividual differences. Therefore, if the racemate is to be applied, therapeutic drug monitoring may be difficult, and requires thorough evaluation.

If (chemical) racemization occurs (e.g. in an acidic environment like the stomach), the application of a pure enantiomer as well as of a racemate implies that two enantiomers will enter the body. In addition, the possibility exists that the enantiomers are stable under certain conditions in parts of the body. Therefore, the fate and effects of both enantiomers should be evaluated. In general, if racemization occurs, application of a pure enantiomer will have no advantage above the racemate. This is however not always the case, as is demonstrated by arbaprostil ((15-R)-15-methyl-prostaglandin E₂), which is used for the treatment of gastric

ulcers. This stereoisomerically pure drug is an inactive prodrug. In an acidic environment it is racemized (actually epimerized) to give a mixture of the active 15-S and the inactive 15-R form. This only occurs as the pH falls below 5 (Reele, 1985), and thus the amount of active drug is controlled by the gastric pH (Cox et al, 1986; Williams 1990). In this case, racemization is used for specific drug targeting of the active enantiomer.

Quantitative differences may range from extreme i.e. the distomer has very low activity (β -adrenoceptor blocking agents; Rahn, 1983; Ruffolo, 1983) to modest i.e. the distomer is nearly as active as the eutomer (warfarin; Wingard et al, 1978; Choonara et al, 1986). If the eudismic ratio is large, the contribution of the distomer to unwanted side effects or toxicity should be evaluated. This is exemplified by β_2 -adrenoceptor sympathomimetic drugs as isoprenaline, salbutamol and terbutaline that are used as bronchodilators in asthma therapy. These compounds are currently administered as racemates. Their sympathomimetic activity resides in the R-enantiomers. However, the S-enantiomers were shown to induce increased airway responsivity to histamine and leukotriene C₄ (Chapman et al, 1992) and are believed to contribute substantially to the bronchospastic hyperreactivity to inhaled allergens that is associated with the use of β_2 -sympathomimetics. Therefore, Chapman et al (1992) proposed the use of only the eutomers (the R-enantiomers) in the treatment of asthma. Another example of a chiral drug for which the application of only one enantiomer would be beneficial because the enantiomers show quantitative differences is timolol. This β -adrenoceptor blocking agent is marketed as the (-) enantiomer and is (among others) used for the treatment of glaucoma. One of its disadvantages for this application is that when it is administered ocularly, the drug may enter the circulation and as a result may have (undesired) systemic effects. When applied as eye drops in humans, 15 times higher doses of (+)-timolol were required to elicit similar systemic effects (as assessed by inhibition of isoprenalin induced bronchodilatation) as the (-)-enantiomer (Richards and Tattersfield, 1985). However the (+)-enantiomer is as effective as the (-)-enantiomer in the treatment of glaucoma (Keates and Stone 1984). Therefore, these authors proposed the (+)-enantiomer as a therapeutic agent for glaucoma treatment since less systemic side effects than for (-)-timolol should be expected. On the other hand if systemic β -adrenoceptor blockade is wanted, the (-)-enantiomer could be used.

At a high eudismic ratio, even in the case that the distomer does not contribute to side effects, it should be considered that for racemic drugs a dose is required that is twice as high as that of the eutomer; in that case the distomer can be considered 'isomeric ballast' since it serves no therapeutic purpose (Ariëns, 1984).

If the eudismic ratio is small and the contribution of both enantiomers to side effects is similar (or even absent), there may be also good reasons to market only one enantiomer (Birkett, 1989). The enantiomers may follow different metabolic routes and/or may be metabolized by different sets of isozymes (e.g. warfarin and acenocoumarol: chapters 3 to 6).

As a result, the enantiomers may interact differently with other drugs (as has been described for warfarin: see paragraph 1.5.5 and chapter 4) and large interindividual differences in the enantiomeric plasma concentration ratios should be expected (Drayer, 1988). Clearly, therapeutic drug monitoring will be more difficult for the racemate than for one of the enantiomers (Drayer, 1988). This point of consideration is of course most important for drugs with a relatively narrow therapeutic index.

Qualitative differences between enantiomers may arise because: 1) enantiomers interact with a variety of receptors to different extents 2) enantiomers interact with the same receptors in a different manner (agonist-antagonist). In both cases, the pharmacological profile of the racemate may be very complex, especially if the enantiomers differ in their pharmacokinetics (Mehvar, 1992) and should be evaluated carefully. Qualitative differences may concern toxicity or undesired side effects of only enantiomer. An example thereof is provided by propranolol. Its β -adrenoceptor blocking potency can be mainly attributed to the S-enantiomer. However, only the R-enantiomer depresses the conversion of thyroxine to triiodothyronine (Stochitzki et al, 1992).

On the other hand, the qualitative differences in the action of enantiomers may sometimes be beneficial since the enantiomers may have complementary or synergistic effects. Indacrinone offers an example of a drug of which the different pharmacological properties of the enantiomers may be of advantage in clinical practice. Indacrinone is a potent diuretic. In man, the (-)-enantiomer of indacrinone is a more potent natriuretic agent than the (+)-enantiomer; in contrast, both enantiomers are equipotent as uricosoric agents (Tobert et al, 1981). Tobert et al (1981) tested the effects of various combinations of the enantiomers of indacrinone and found that its therapeutic effect can be improved (i.e. diuresis without accumulation of uric acid in the plasma, a side effect of many diuretics) by giving indacrinone in an enantiomeric (+)/(-) ratio of 1/4.

Another, intriguing example of a drug for which the application of mixtures of stereoisomers appears to have some advantage above the application of a pure stereoisomer is labetalol. Its RR-enantiomer (dilevalol) has been introduced as a β_2 -adrenoceptor blocking drug in 1990 in Japan and Portugal but has been withdrawn because of its hepatotoxicity (Lennard, 1991). Hepatotoxicity has also been reported for labetalol (a mixture of the RR, RS, SR, and SS diastereomers) but appears to be observed less frequent (Lennard, 1991). Nevertheless, because of the hepatotoxicity of its RR-diastereomer, one might argue that other β_2 -adrenoceptor blocking drugs should be used in stead of labetalol.

From the above, it may be clear that for a chiral drug knowledge of the fate and effect of both enantiomers is crucial. When a decision has to be made whether or not to use a chiral drug in its racemic or enantiomerically pure form, the results of this evaluation, together with

the costs of drug production, and the desirability of the drug (e.g. for the treatment of life-threatening diseases) should be taken into consideration (Birkett, 1989; Campbell, 1990).

At present, many chiral drugs are marketed as a racemate and as such have proved their value in the past (Lennard, 1992). There is however a tendency in the pharmaceutical industry to prefer enantiomerically compounds when new drugs are to be produced (Campbell 1990, Lennard, 1992). This tendency is demonstrated by the fact that in the UK in 1987 less chiral drugs were developed than in the year before and that of the chiral drugs the amount of pure enantiomers doubled (Campbell, 1990). That the issue of developing racemic or enantiomerically pure drugs has penetrated the pharmaceutical industry and is seriously dealt with may be clear from table 7.1, which is drawn from results of a questionnaire held by Cayen (1991).

Taken together, the decision whether or not to use drugs in their stereoisomerically pure form is very complex, which probably is the reason that the debate on this subject is still going on.

Indeed, enantiomers or stereoisomers should be considered different drugs but this does not necessarily imply that the use of mixtures of stereoisomers (albeit not necessarily in the ratio that is obtained from non-stereoselective synthesis) should be rejected, since in some cases the different pharmacological profile of the stereoisomers can be of advantage in therapy. In that case, mixtures of enantiomers or stereoisomers can be considered combination preparations. However, it seems reasonable to assume that the tendency to develop enantiomerically pure drugs will proceed and that the application of drugs in their racemic form (without a founded reason) will gradually disappear.

TABLE 7.1: Criteria used in the industry to market enantiomers or racemates (from Cayen, 1991):

Reasons to market enantiomers:

- 1 high eudismic ratio
- 2 the distomer is toxic
- 3 low therapeutic index
- 4 natural products are used that are already enantiomerically pure.
- 5 better physico-chemical properties of the enantiomers.
- 6 no chiral inversion (enzymatic/chemical)
- 7 ease of batch production

Reasons to market racemates/mixtures of stereoisomers:

- 1 distomer has similar pharmacological and toxicological properties
- 2 high therapeutic index
- 3 synergistic or additive pharmacological properties of the enantiomers
- 4 large scale synthesis or isolation of stereoisomers is cost prohibitive or inachievable (especially for multiple chiral centres)
- 5 life threatening indications: development of pure stereoisomers is too time consuming.

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CHAPTER 8:

8.1 SUMMARY

The fact that stereoisomers exert different effects in biology is well documented and is gaining more and more attention. Warfarin and acenocoumarol (4'-nitrowarfarin) are chiral drugs that are widely used as oral anticoagulants. In the discussion concerning chiral drugs in pharmacology, warfarin is an often cited example because of its well documented stereoselective pharmacokinetics, pharmacodynamics, and drug interactions. In this thesis, the stereochemical course of the pharmacokinetics and *in vitro* metabolism of warfarin has been compared with some of its phenyl ring substituted analogues. Special attention is given to acenocoumarol since it displays quite different pharmacokinetic properties than warfarin.

The pharmacokinetics of various warfarin analogues in rats is described in chapter 2. Phenyl ring substitution appeared to have a great impact on the pharmacokinetics and the stereochemistry thereof. The S-enantiomers of warfarin and its 4'- and 3'- substituted analogues were shown to be more tightly bound to plasma proteins than the R-enantiomers. The opposite was observed for the 2'-substituted analogues.

Phenyl ring substitution resulted in a higher intrinsic clearance than that of warfarin. Stereoselectivity of the intrinsic clearance was shown to depend on the position of the substituent: the intrinsic clearance of warfarin and its 3'- substituted analogues was higher for the R-enantiomers. In contrast, 2'- and 4'- substitution generally reversed this stereoselectivity.

In chapter 3, the rat liver microsomal metabolism of warfarin has been compared with that of acenocoumarol. The overall metabolism of warfarin was found to be stereoselective for the R-enantiomer, whereas that of acenocoumarol was shown to be stereoselective for the S-enantiomer. In addition, the total metabolic intrinsic clearances of R- and S-acenocoumarol greatly exceeded those of the enantiomers of warfarin, due to the lower K_m values of acenocoumarol towards monooxygenases. From experiments with the P450 inducers phenobarbitone and methylcholanthrene and the cytochrome P450 inhibitor cimetidine it appeared that the spectrum of isozymes involved in the metabolism of warfarin is different from that involved in the metabolism of acenocoumarol. Experiments with antibodies raised against P450 isozymes of the 2C and 3A subfamilies showed that these P450s are probably involved in the metabolism of both compounds. However, their stereo- and regioselectivity, differ greatly for warfarin and acenocoumarol.

To investigate whether similar metabolic differences between warfarin and acenocoumarol are also observed in humans, their human liver microsomal metabolism has been compared in chapter 4. The overall metabolism of both compounds was stereoselective for the S-enantiomer, but this stereoselectivity was far more pronounced for acenocoumarol. In addition, the total intrinsic clearances of the enantiomers of acenocoumarol greatly exceeded those of R- and S-warfarin. As in rats, this was mainly due to the lower K_m values of

acenocoumarol towards the metabolizing enzymes. From experiments with P450 inhibitors, and from the lack of a correlation between the formation rates of warfarin and acenocoumarol metabolites it was concluded that, as in rats, the (stereochemical) differences in the kinetics between warfarin and acenocoumarol are at least in part attributable to the fact that the metabolism of these compounds is carried out by different combinations of P450 isozymes. This suggests that the phenyl ring of warfarin is involved in the interaction with P450 binding sites and that ring substituted warfarin analogues may discriminate for particular P450 isozymes.

Another metabolic route of warfarin analogues, reduction of the acetonyl side chain to the diastereomeric alcohols is described in chapters 5 and 6. The ketone reduction of warfarin, and its 4'-nitro, and 4'-chloro analogues was investigated in microsomal and cytosolic fractions of various species (chapter 5). Ketone reduction occurred in both subcellular fractions and was shown to be NADPH dependent. In microsomes, large differences between species, as well as between the warfarin analogues were observed in alcohol formation rates and in substrate- and product stereoselectivity. No general pattern was evident. In cytosolic fractions, a general pattern of substrate- and product stereoselectivity was observed: the R-enantiomers were preferred as a substrate and the alcohols formed were mainly in the S-configuration. Contrary to the microsomal reductase activity, the cytosolic reductase activity in the rat was induced by phenobarbitone and methylcholanthrene. In cytosolic fractions, acenocoumarol was generally a better substrate than 4'-chlorowarfarin which in its turn was a better substrate than warfarin.

In chapter 6, the rabbit liver cytosolic warfarin reductase has been partially purified and characterized. DEAE-Sephacel chromatography revealed that at least two enzymes (fractions A and B) possess warfarin ketone reductase activity. Both enzymes showed similar substrate- (for the R-enantiomers) as well as product stereoselectivity (formation of the S-alcohols) but were differently influenced by 4'-substitution in their kinetics. 4'-Substitution predominantly affected the K_m values for fraction A, whereas for fraction B V_{max} values were affected. NADPH was the preferred cofactor, which was absolute for fraction B. Both fractions were able to reduce carbonyl groups from a variety of compounds, including prostaglandin E2 and steroids, but fraction A showed a broader substrate spectrum and was e.g. also able to reduce the quinone menadione. Both fractions were inhibited by the flavonoid quercetin, but not by sodium-barbitone.

Finally, in chapter 7, the results of this thesis, the value of enantiomers in biomedical science, and some considerations concerning the use of enantiomerically pure or racemic drugs will be discussed.

The experiments described in this PhD thesis show that the different effects of enantiomers in biological systems is not only due to the distinct 3-dimensional structure of the enantiomers, but also to the multiplicity of biomolecules with which they can (or cannot) interact.

8.2 SAMENVATTING

Het feit dat stereoisomeren verschillende effecten vertonen in biologische systemen kent vele voorbeelden en begint steeds meer aandacht te krijgen. Warfarine en acenocoumarol (4'-nitrowarfarine) zijn chirale verbindingen die algemeen worden toegepast als orale anticoagulantia. Ten aanzien van de rol van chirale verbindingen in de farmacologie wordt warfarine vaak genoemd als voorbeeld omdat hiervan de stereochemische aspecten in de farmacokinetiek, farmacodynamiek, alsook in interacties met andere farmaca uitgebreid zijn beschreven. In dit proefschrift wordt de stereoselectiviteit van de farmacokinetiek en van het in vitro metabolisme van warfarine en enkele phenyl ring gesubstitueerde warfarine analoga met elkaar vergeleken. Hierbij wordt extra aandacht geschonken aan acenocoumarol omdat dit geneesmiddel farmacokinetisch gezien vrij sterk van warfarine verschilt.

De farmacokinetiek van enkele warfarine analoga in de rat wordt beschreven in hoofdstuk 2. Substitutie aan de phenyl ring bleek sterke effecten te hebben op de farmacokinetiek en de stereoselectiviteit hiervan. Zo bleken b.v. de S-enantiomeren van warfarine en diens 3'- en 4'- gesubstitueerde analoga sterker aan plasma eiwitten gebonden te zijn dan de R-enantiomeren. Voor de 2'- gesubstitueerde warfarine analoga bleek echter het omgekeerde het geval te zijn.

Phenyl ring substitutie leidde in alle gevallen tot een verhoging van de intrinsieke klaring. De stereoselectiviteit van de intrinsieke klaring bleek af te hangen van de positie van de substituent. De intrinsieke klaring van warfarine en zijn 3'- gesubstitueerde analoga was hoger voor de R-enantiomeren. Daarentegen bleken 2'- en 4'- substitutie i.h.a. te leiden tot een hogere intrinsieke klaring van de S-enantiomeren.

In hoofdstuk 3 wordt het rattelever microsomaal metabolisme van warfarine vergeleken met dat van acenocoumarol. De totale metabole intrinsieke klaring van warfarine bleek hoger voor de R-enantiomeer te zijn, terwijl in het geval van acenocoumarol juist de S-enantiomeer het snelst gemetaboliseerd werd. Bovendien bleek dat de intrinsieke klaring van R- en S-acenocoumarol veel hoger was dan de intrinsieke klaring van beide warfarine enantiomeren. Dit bleek met name te wijten aan de lagere K_m waarden die acenocoumarol heeft t.o.v. het monooxygenase systeem. Experimenten met de cytochroom P450 inducers phenobarbital en methylcholanthreen en de P450 inhibitor cimetidine duiden erop dat het spectrum van isoenzymen dat betrokken is bij het metabolisme van acenocoumarol verschilt van dat van warfarine. Uit experimenten met antilichamen, opgewekt tegen P450's van de 3A en 2C subfamilies, bleek dat deze P450's waarschijnlijk betrokken zijn bij het metabolisme van beide stoffen. Echter, hun stereo- en regioselectiviteit en hun relatieve bijdrage aan de totale metabole klaring bleek te verschillen voor warfarine en acenocoumarol.

Om te onderzoeken of dergelijke metabole verschillen tussen warfarine en acenocoumarol ook in de mens waargenomen worden, werd hun metabolisme in humane lever microsomen met elkaar vergeleken. Het totale metabolisme bleek voor beide stoffen stereoselectief te zijn voor de S-enantiomeer, maar deze stereoselectiviteit was veel sterker in het geval van

acenocoumarol. Ook bleek, net als in de rat, de totale intinsieke klaring van de acenocoumarol enantiomeren veel groter te zijn dan in het geval van R- en S-warfarine. Ook nu weer was dit te wijten aan de lagere K_m waarden van acenocoumarol t.o.v. de metaboliserende enzymen. Uit experimenten met P450 inhibitoren, en uit het gebrek aan correlatie tussen de vormingssnelheden van de metabolieten van warfarine en acenocoumarol werd geconcludeerd dat de kinetische verschillen tussen de warfarine analoga in ieder geval deels zijn terug te voeren op het feit dat verschillende combinaties van isoenzymen deze stoffen metaboliseren. Hieruit blijkt dat de phenyl ring van warfarine betrokken is bij de interactie met P450 bindings-plaatsen en dat phenyl ring gesubstitueerde warfarine analoga onderscheid kunnen maken tussen bepaalde P450 isoenzymen.

Een andere metabole route van warfarine analoga is de reductie van de acetonyl zijketen waarbij diastereomere alcoholen worden gevormd. De keton reductie van warfarine, en diens 4'-nitro en 4'-chloor analoga door (lever) microsomale en cytosolaire fracties van verschillende species werd bestudeerd in hoofdstuk 5. Deze reactie werd door beide subcellulaire fracties uitgevoerd en bleek NADPH afhankelijk te zijn. In microsomen werden grote verschillen tussen de species en de warfarine analoga waargenomen wat betreft reactie snelheden alsook substraat- en product stereoselectiviteit. Een algemeen patroon werd niet waargenomen. In cytosol fracties werd er echter wel een algemeen patroon van substraat- en product stereoselectiviteit waargenomen: de R-enantiomeren werden geprefereerd als substraat en de gevormde alcoholen bleken vooral in de S-configuratie te zijn. De cytosolaire keton reductase activiteit bleek, in tegenstelling tot de microsomale keton reductase activiteit, in de rat geïnduceerd te worden door phenobarbital en methylcholanthreen. Voorts bleek dat in de cytosolaire fracties acenocoumarol een beter substraat was dan 4'-chloorwarfarine, dat op haar beurt weer een beter substraat was dan warfarine.

In hoofdstuk 6 wordt de partiële zuivering en karakterisering beschreven van lever cytosolaire enzymen uit het konijn die de keton groep van warfarine kunnen reduceren. Uit DEAE Sephacel chromatografie bleek dat deze reactie door minstens twee enzymen wordt gecatalyseerd (fractie A en B). Beide fracties vertoonden een zelfde substraat- (voor de R-enantiomeren) en product (vorming van vooral de S-alcoholen) stereoselectiviteit maar substitutie van de 4'-groep van warfarine had voor beide fracties verschillende effecten op de enzym kinetiek. In het geval van fractie A bleek 4'-substitutie met name effect te hebben op de K_m van het warfarine analogon, terwijl in het geval van fractie B met name de V_{max} beïnvloed werd. Beide fracties waren in staat carbonyl groepen van verschillende stoffen (waaronder prostaglandine E2 en steroiden) te reduceren maar de substraat specificiteit bleek het breedst voor fractie A. Deze fractie kon b.v. ook het chinon menadion reduceren. Het flavonoid quercetine was een inhibitor van beide enzym fracties, terwijl natrium barbital geen effect had.

In hoofdstuk 7 tenslotte, worden de resultaten van dit proefschrift, het nut van enantiomeren als gereedschap in de biomedische wetenschappen, en enige overwegingen ten aanzien van het gebruik van racemische of zuiver enantiomere geneesmiddelen bediscussieerd.

De experimenten die in dit proefschrift beschreven worden, laten zien dat de verschillen die waargenomen worden in de biologische effecten van enantiomeren niet alleen terug te voeren zijn op de verschillende 3-dimensionale structuur van de enantiomeren, maar ook op de veelheid van biomoleculen waarmee enantiomeren al dan niet een interactie aangaan.

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