Chirality in Clinical Pharmacology: Studies with Ketoprofen

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

by

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Contents

Abstract

- Ketoprofen is a chiral nonsteroidal anti-inflammatory drug which is marketed for clinical use as a racemic mixture. It is predominantly eliminated, in man, as acyl-glucuronides in urine.

- To examine aspects of the potential enantioselective pharmacokinetics and pharmacodynamics of this drug in humans, methods were developed for quantifying total (bound plus unbound) and unbound ketoprofen enantiomers in plasma.

- Both (R)- and (S)-ketoprofen were extensively (>99%) bound to plasma protein. In healthy young volunteers, *in vitro* studies showed there to be no difference between the unbound fractions in plasma of the ketoprofen enantiomers. Further, at clinically relevant total drug concentrations in plasma, the unbound fraction of each enantiomer was independent of concentration and was not influenced by the presence of either its optical antipode or the acyl-linked glucuronide metabolites of ketoprofen.

- *In vitro* studies were conducted with the biosynthetic (R)- and (S)-ketoprofen glucuronides to assess the hydrolysis, rearrangement and reversible protein binding of these conjugates in a variety of incubation media. It was apparent that albumin, rather than plasma esterases, catalysed (stereoselectively) the hydrolysis of the conjugates and moreover, from the nature of the observed stereoselective protein binding, it appeared that separate binding and catalytic sites were situated on the albumin molecule for these metabolites. In protein-free incubation media at physiological pH and temperature, rearrangement of the acyl-glucuronides to positonal conjugate isomers predominated quantitatively, over the deconjugation reaction for both (R)- and (S)-ketoprofen glucuronides.

- The pharmacodynamics (antiplatelet effects) of ketoprofen enantiomers were assessed *in vitro*, by monitoring the inhibition of thromboxane B_2 (TXB_2) generation during controlled blood clotting. There was a close relationship between the concentration of serum unbound (S)-ketoprofen and the degree of inhibition of cyclo-oxygenase, according to a sigmoidal concentration-effect model. (R)-Ketoprofen was devoid of such activity and did not modify the potency of its optical antipode.

- Single oral doses of racemic ketoprofen were administered to fifteen elderly patients with rheumatoid arthritis and varying degrees of renal impairment. Both the pharmacokinetics and pharmacodynamics of ketoprofen enantiomers, and the influence thereon of renal function, were assessed in these patients. Significant negative correlations were observed between the area under the plasma concentration-time profile and creatinine clearance, for both total and unbound (S)-ketoprofen. Corresponding significant relationships were also observed for total and unbound (R)-ketoprofen. *In vitro* studies conducted with pre-dose blood from these patients (all of whom were receiving concurrent medication) revealed: (*i*) a higher unbound fraction in plasma for (S)-ketoprofen compared to the corresponding value for (R)-ketoprofen, (*ii*) a concentration-dependent unbound fraction for the (S)-enantiomer of ketoprofen and (*iii*) a lack of correlation between the unbound concentration of (S)-ketoprofen in serum required to inhibit platelet TXB_2 generation by 50% (EC_{50}) and creatinine clearance. It was concluded from this clinical study, that diminished renal function was associated with an increased exposure to pharmacologically active unbound (S)-ketoprofen. This was postulated to be due to regeneration of parent aglycone arising from the hydrolysis of systemically accumulated acyl-linked conjugates. And further, it was concluded that the apparent sensitivity of platelet cyclo-oxygenase to the inhibitory effect of unbound (S)-ketoprofen was not influenced by renal function.

Declaration

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. I consent to this thesis being made available for photocopying and loan when deposited in the University Library.

Peter John Hayball

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Publications in Support of this Thesis

- Hayball, P.J., Nation, R.L., Bochner, F. and Le Leu, R.K. Enantiospecific analysis of ketoprofen in plasma by high-performance liquid chromatography. *Journal of Chromatography* **570**, 446-452, 1991.

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- Hayball, P.J., Nation, R.L., Bochner, F., Sansom, L.N., Ahern, M.J. and Smith, M.D. The influence of renal function on the enantioselective pharmacokinetics and pharmacodynamics of ketoprofen in patients with rheumatoid arthritis. *British Journal of Clinical Pharmacology*, in press, 1993.

Abbreviations and Symbols

The following abbreviations and symbols have been used in the text of this thesis. Wherever practical, the pharmacokinetic symbols comply with the guidelines suggested by Rowland and Tucker (1982). For illustrative purposes, enantioselective symbols pertaining only to the (S)-enantiomer of ketoprofen are defined below.

$Ae_{(S)}$	cumulative urinary excretion (0-24 h) of (S)-ketoprofen as combined agylcone plus acyl-glucuronide
$AUC_{(S)}$	area under the plasma concentration-time profile from zero to infinite time for total (bound plus unbound) (S)-ketoprofen
$AUCu_{(S)}$	area under the plasma concentration-time profile from zero to infinite time for unbound (S)-ketoprofen
B.P.	British Pharmacopœia
$[B]_{(S)}$	concentration of (S)-ketoprofen in blood
$[B]_{(S)}/[S]_{(S)}$	blood to serum concentration ratio of (S)-ketoprofen
°C	degrees celsius
CL_{CR}	creatinine clearance
CLu_{int}	intrinsic clearance of unbound drug
C_{max}	maximum plasma concentration
CoA	coenzyme *A*
conc	concentration
cpm	cycles (revolutions) per minute
$Cu_{(S)}$	concentration of unbound (S)-ketoprofen in serum
CV	coefficient of variation
D	dose of drug
$dpm_{(S)}$	disintegrations per minute (radioactivity) of (S)-ketoprofen
E	measured effect of a drug
E_0	basal effect in the absence of drug
EC_{50}	concentration of drug required to produce 50% of maximal effect
E_{max}	maximum effect produced by a drug
f_a	fraction of oral dose absorbed from the gastrointestinal tract
$fu_{(S)}$	fraction unbound of (S)-ketoprofen
$fu_{(S)}(\%)$	percentage unbound of (S)-ketoprofen
$fu_{(unresolved)}$	fraction unbound of unresolved ketoprofen
g	rotational centrifugal force
GC-MS	gas chromatography - mass spectroscopy
h	hours
HETE	hydroxyeicosatetraenoic acid

HPETE	hydroperoxyeicosatetraenoic acid
HPLC	high-performance liquid chromatography
HSA	human serum albumin
% Inhibition	percentage inhibition elicited by a drug
l	litre
LSD	(Fischer's) least significant difference repeated measures statistical test
M	molar strength (mole per litre)
mg	milligram
min	minutes
ml	millilitre
mm	millimetre
m/z	mass to charge ratio
µCi	microcurie
µg	microgram
µl	microlitre
µm	micrometre
µM	micromole per litre
µmol	micromole
n	slope factor of the sigmoidal E_{max} equation (Hill equation)
ND	not detected
ng	nanogram
nm	nanometre
NMR	nuclear magnetic resonance
NSAID	nonsteroidal anti-inflammatory drug
$O_2^{\cdot -}$	superoxide free radical
P	statistical probability value
PBS	phosphate buffered (0.067 *M*, pH7.4) saline
PTFE	polytetrafluoroethylene (teflon)
r	correlation coefficient from linear least-squares regression analysis
r_s	Spearman rank-order correlation coefficient
SD	standard deviation
SE	standard error
$t_{½(S)}$	apparent first-order elimination half-life of (S)-ketoprofen
$t_{½(S)\text{-gluc}}$	net hydrolysis $t_{½}$ of (S)-ketoprofen acyl-glucuronide
TMS	tetramethyl silane
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2
UDP	uridine diphospho glucuronic acid
$\%Unbound_{(S)\text{-gluc}}$	percentage unbound of (S)-ketoprofen acyl-glucuronide
UV	ultra-violet wavelength
yr	years

Chapter 1

Introduction: Enantioselective Clinical Pharmacology

1.1 Terminology

An extensive and at times somewhat confusing nomenclature has developed around the subject of stereoisomerism. Figure 1.1 provides a clarification of the various structural relationships embodied in this term.

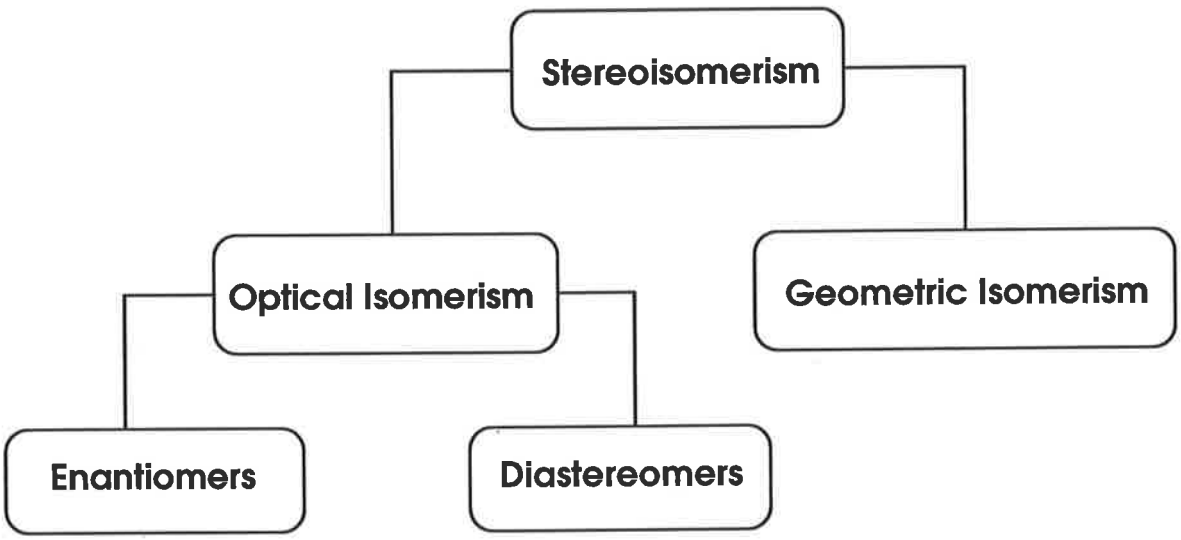


Figure 1.1 Relationships between stereoisomers and delineation of stereochemical categories.

Of the two main groups of stereoisomers the subject of this thesis focuses on optical isomers and their subclassifications. Optical isomerism arises from the property of three-dimensional objects known as chirality (from the Greek *cheir*, meaning hand). Chirality is essentially the sense of "handedness" which dissymmetric objects exhibit. Chiral objects are related as mirror images of each other and are not superimposable. Chirality on the molecular level occurs not only in molecules containing asymmetric atoms but also in molecules which possess other structural features which confer dissymmetry, for instance, in allenes or highly *ortho*-

substituted biphenyls with consequent restricted rotation about the ring bridge. In these latter cases, no single atom is chiral, but rather the overall molecule possesses a "handedness" or chirality. Such chirality due to restricted rotation about bonds is relatively uncommon amoung drug substances although the hypnotic methaqualone is an example (Figure 1.2). These types of optical isomerism also raise the sometimes confused distinction between "asymmetry" and "dissymmetry" (Lee and Williams, 1990a). Chirality is synonymous with dissymmetry and nonsuperimposability. In the case of allenes, such molecules possess an axis of symmetry and are therefore not asymmetric, however are still chiral or dissymmetric. Thus all asymmetric molecules are dissymmetric, however the converse is not always upheld.

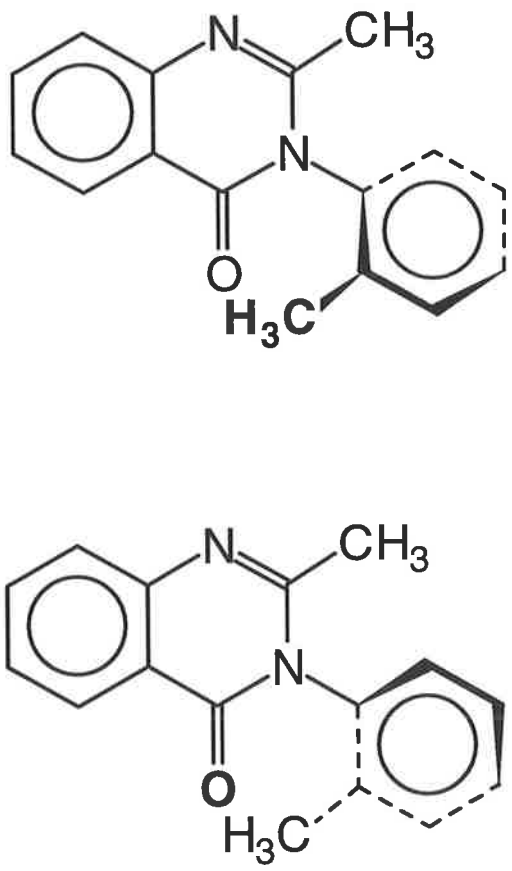


Figure 1.2 Structures of methaqualone enantiomers, optical isomerism resulting from restricted rotation around the ring bridge.

Much more common for optical isomerism in the pharmacological arena are molecules possessing a chiral centre (most commonly a saturated carbon atom) to which are covalently attached four different chemical substituents. Such molecules, which are related as mirror images (all chiral centres are inverted), are termed enantiomers or antipodes (Figure 1.3).

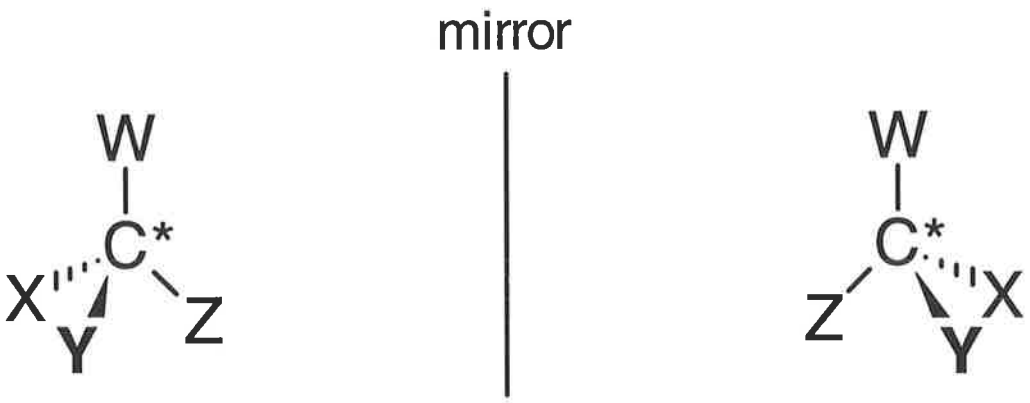


Figure 1.3 The two mirror-images (enantiomers) of a molecule possessing a single chiral centre.

Molecules containing two or more asymmetric centres which are not mirror images (since not all the chiral centres are inverted) are related as diastereomers. Diastereomers are stereoisomers that are not mirror images of each other; they have the same configuration at at least one asymmetric centre and, at the same time, different configurations at at least one asymmetric centre. Enantiomers possess identical physical and chemical properties in a symmetrical environment (for example, melting and boiling point, spectroscopic properties *etc*), in contrast to diastereomers which tend to possess different such properties. Special cases exist for molecules (such as tartaric acid) which contain two asymmetric carbons which are themselves mirror images of each other, each chiral carbon possessing the same substituents attached but in opposite configurations, thereby conferring a plane of symmetry on the molecule. Such a self-compensated molecule (a *meso*-compound) is not optically active. Tartaric acid can also exist as nonsuperimposable mirror image forms (thereby explaining

Pasteur's observations) since it has two chiral carbons giving rise to 2^n stereoisomers (*n* is the number of chiral centres), two of which are pairs of enantiomers and two are *meso* (identical) structures.

The nomenclature used for individual optical isomers was originally related to the direction of rotation of a plane of plane-polarized light induced by an enantiomer. An enantiomer rotating such light to the left (or in a counterclockwise direction) was termed *laevo*-rotary [shortened sometimes to (*l*)- and more commonly given the prefix (-)-]. A *dextro*-rotary [(*d*)-, (+)-] enantiomer rotates plane-polarized light to the right (or in a clockwise direction). This descriptor is dependent, both in magnitude and sign, on the concentration of the enantiomeric solute and the choice of solvent, respectively, through which the plane-polarized light is passed. Optical rotation gives no information about the absolute configuration of the individual enantiomers, instead being a relative term, sometimes used when the absolute configurations of the chiral centres in a molecule are not known. Optical rotation also depends on the molecular structure of the chiral molecule. For example, the enantiomeric substance ethyl-*n*-propyl-*n*-butyl-*n*-hexylmethane is optically inactive since its theoretical specific rotation (0.00001°) is far below the limits of detection by current polarimeters (Morrison and Boyd, 1983).

In order to assign absolute configuration of a chiral centre (normally determined by X-ray crystallography, nuclear magnetic resonance spectroscopy and circular dichroism experimental techniques) the Cahn-Ingold-Prelog convention or the "Sequence Rule" has been adopted and has largely superseded the (D)/(L) Fischer convention. The current convention uses (R)/(S)-prefixes to indicate the orientation of substituents around an asymmetric carbon atom. The Sequence Rule uses a sequence of priority to the four atoms or groups of atoms attached to the chiral centre, with the atom of higher atomic number having the higher priority. The chiral centre is visualized so that the group of lowest priority is directed away from the line of view and the arrangement of the remaining groups is observed. If, in proceeding from the highest priority group to the group of second priority through to the group of third priority, a clockwise direction is taken, then the configuration is specified by the prefix (R)- (from the Latin *rectus*, right) and if this direction is counterclockwise the configuration is (S)- (*sinister*,

left). In contrast, the Fischer convention relates the asymmetric centre of the molecule under consideration to the asymmetric centre of glyceraldehyde; if determined to be the same as (+)-glyceraldehyde it is given the prefix (D)-, while the (L)- prefix refers to a chiral centre related to (-)-glyceraldehyde. This dated convention has been commonly applied to descriptions of amino acid and carbohydrate stereoconfiguration; molecules which have been able to be chemically transformed back to chiral structures of known configuration. The more recent Cahn-Ingold-Prelog sequence convention has been officially adopted by *IUPAC* and will be used to identify chiral molecules, whenever possible, in this thesis. A racemic compound will be indicated by the prefix (RS)- implying an equal mixture of enantiomeric forms of the compound.

1.2 Stereoisomerism: A Historical Perspective

The part of the science of organic chemistry that deals with structure in three dimensions is termed stereochemistry. Stereoisomers differ from each other only in the form the atoms are orientated in space. Pairs of stereoisomers (specifically optical isomers) differ so little in structure (and hence properties) that all physical properties, with the exception of their ability to rotate a path of plane polarized light, are identical for each isomer. Indeed it is the ability of individual optical isomers to rotate plane polarized light in a given direction, either to the left (counterclockwise) in the case of laevorotatory (-) isomers or to the right in the case of dextrorotatory (+) isomers, that led to the discovery of enantiomerism.

The French physicist Jean-Baptiste Biot discovered the property of optical activity exhibited by certain compounds in 1815. However, it wasn't until 1848 that a fellow Frenchman Louis Pasteur made a series of observations which led to his proposals of the spatial orientation of molecules. Following on from his measurements of the optical rotation of left- and right-handed crystals of sodium ammonium tartrate he proposed that similar to the two sets of crystals, the molecules making up these crystals were mirror images of each other, thus inferring that isomers exist whose structures differ only in being mirror images of each other. This work was linked with van't Hoff's proposal of a tetrahedral carbon atom to arrive at the

concept of enantiomerism on the basis that pairs of enantiomers are the nonsuperimposable mirror-images of each other.

1.3 Optical Isomerism in Clinical Pharmacology

"*How would you like to live in Looking Glass House, Kitty? I wonder if they'd give you milk in there? Perhaps Looking Glass milk isn't good to drink...*" (quote from "Through the Looking Glass", the companion book to "Alice in Wonderland", written by Lewis Carroll in 1896).

Alice in Wonderland's speculation about Looking Glass milk has a significance greater than Lewis Carroll suspected. The milk Alice's cat so desired contains the disaccharide lactose which is composed of glucose and galactose sugar units. Dextrorotatory glucose (dextrose) is the natural sweet sugar we are accustomed to, in contrast to its mirror image form laevulose. Because the intake and processing of food involves associations between asymmetric molecules in food and asymmetric receptors or proteins in the body, there are often marked differences in the sensory perception and digestion of enantiomeric forms of chiral molecules. Indeed it is unlikely that Kitty would have found the milk particularly palatable unless, of course, Kitty was on the other side of the Looking Glass.

It wasn't until the early this century that Cushny (1908) tested the pressor effects of naturally occurring (R)-adrenaline and synthetic (RS)-adrenaline in the anaesthetized dog and deduced the discriminatory capacity of biological receptors for chiral ligands. He discovered that racemic adrenaline was half as potent as (R)-adrenaline while (S)-adrenaline was $^1/_{12}$ as active as the endogenous substrate. Chen *et al.* (1929) introduced synthetic adrenergics including ephedrine and described the pharmacology of the stereoisomers of these drugs. However, the advances in understanding the influence of optical isomerism on drug action provided by these pioneering studies of molecular geometry and adrenergic drug activity appeared to stall with the advent of major synthetic sources of pharmacological agents. Classical synthetic routes employed for the production of therapeutic agents containing chiral centres usually result in the generation of racemic product. For the preparation of enantiomerically homogeneous

molecules, synthesis of racemic material must be followed by resolution into the separate optical antipodes or alternatively, synthesis can be performed in an enantioselective (or preferably enantiospecific) fashion leading to the production of chirally-enriched products. Notably, asymmetric drug synthesis relies heavily on naturally occurring chiral amino acids, terpenes and sugars; a so-called "chiral carbon pool" (Coppola and Schuster, 1987). About 40% of synthetic drugs are chiral and of these approximately 90% are marketed as racemates (Lennard, 1991).

1.4 Definition of Enantioselectivity

Biologically active agents often show a high degree of selectivity in action related to their discriminatory capacity at molecular sites of action. This capacity requires complementarity between the bioactive agent and the molecular site of action, most commonly a protein which in turn is constructed of chiral subunits. The bioactive agent must complement its site of action both chemically and sterically. Inherent enantioselectivity in action can be accounted for on the basis of as few as three groups in the active molecule participating in a structure-specific fashion with the effector site (the so-called "three point" interaction). Thus it is hardly surprising to find enantioselectivity the rule rather than the exception in nature; since the multitude of asymmetric interaction sites present in organisms make them a veritable "chiral jungle" with which chiral drugs must come into contact. The pharmacological properties of a drug are governed by the way in which it interacts with endogenous chiral macromolecules such as proteins, polysaccharides and nucleic acids.

Differences between enantiomers in the pharmacological response to a given dose may arise from differences in the delivery of the enantiomers to the receptor sites(s) most notably, in terms of the unbound species, since it is generally accepted that only unbound drug is pharmacologically active (*i.e.*, enantioselective pharmacokinetics). Alternatively, differences may exist between enantiomers in terms of their relative complementarity and subsequent activity at the effector sites(s) (enantioselective pharmacodynamics). Both enantioselective pharmacodynamics and enantioselective pharmacokinetics are important in describing the

influence of optical isomerism on clinical pharmacology; the relative contribution of each being a complex function of many factors.

A plethora of nomenclature has grown with the increasing interest in enantioselective pharmacology. The optical isomer of a chiral drug possessing a high degree of complementarity with a given receptor type (the more active isomer at this site) is known as the eutomer with its antipode (the less active isomer) referred to as the distomer. The ratio of eutomer and distomer (of activity in the above case), the eudismic ratio, is a measure of the enantioselectivity. High activity of the eutomer implies a high degree of "goodness of fit" with the molecular site of action and hence a correspondingly poor fit for the distomer, with a large eudismic ratio resulting (Ariens, 1984). Eudismic proportion is the proportion of the concentrations of eutomer and distomer in, for example, plasma. This latter term is of particular significance in pharmacokinetics and is subject to change (from unity in the case of racemate administration) due to the often different rates of distribution, metabolism and excretion of the individual enantiomers.

1.5 Enantioselective Pharmacodynamics

In general terms, enantioselective drug action has been the subject of many review (Ariens, 1983; Ariens *et al.*, 1988; Drayer, 1986; Jamali *et al.*, 1989; Lehmann, 1983; Simonyi, 1984). Its relevance to literally hundreds of drugs places a comprehensive discussion of all chiral drugs beyond the scope of this thesis. A number of examples are cited below to provide an outline of this topic and its relevance to clinical pharmacology.

Of particular concern in this review are the commonly prescribed chiral NSAIDs, most notably, congeners of the 2-arylpropanoic acids. Extensively reviewed in the literature (Evans, 1992; Hutt and Caldwell, 1983; Williams, 1990) these particular NSAIDs are characterized by major or exclusive cyclo-oxygenase inhibition (the principal site of action of these drugs, see Section 2.2.1) elicited by the enantiomer of the (S)-configuration. Eudismic ratios ((S)/(R) relative potency) obtained with *in vitro* systems of inhibition of prostaglandin synthesis and platelet aggregation range from 16 in the case of indobufen (Cerletti *et al.*, 1990) up to 200 for

flurbiprofen (Nishizawa *et al.*, 1973). However, caution is needed in the interpretation of the actual magnitude of such ratios, since in most cases when separate enantiomers are added to the pharmacological test system, the optical purity of test stereoisomer is often not determined and assumed to be absolute. Accordingly, the biological activity recorded for the distomer (the (R)-enantiomer) may be a result of contamination of the test sample with its active antipode. The reliability of pharmacodynamic eudismic ratios for chiral NSAIDs (and other drugs) is also a function of the particular pharmacological test system and whether this system is utilized in isolation from other interdependent biological processes. When the activity of 2-arylpropanoate enantiomers is assessed *in vivo* there is often a marked disparity with data obtained by *in vitro* methods (Hutt and Caldwell, 1983). In the former case, enantiomers are subject to enantioselective dispositional processes which influence the eudismic proportion in the immediate vicinity of the effector site (see Section 1.6). Indeed members of this chiral drug class are subject to a unique metabolic transformation involving chiral inversion from the (R)-enantiomer to its pharmacologically active (S)-antipode, a process which leads to measurable pharmacological activity for the (R)-isomer arising indirectly from *in vivo* metabolism rather than as a result of a direct pharmacodynamic effect (see Sections 1.6.3 and 2.3.3).

In some cases one enantiomer of a chiral drug might be the principally active component of a racemic mixture with its optical antipode responsible for the major toxicity of the compound. Granulocytopenic reactions to racemic dopa were not seen with (L)-dopa and therefore attributed to the (D)-enantiomer (Cotzias *et al.*, 1969). For some racemic drugs enantioselectivity may be reversed for the various pharmacological properties. For instance, the (S)-enantiomer of disopyramide is the eutomer with regards to type 1 antiarrhythmic activity while (R)-disopyramide is the eutomer with respect to the drug's negative inotropic effect (Lima *et al.*, 1985). This case underscores the importance of specifically defining the pharmacological effector site at which enantioselectivity is defined or measured.

The (S)-stereoisomer of timolol is one of few β-adrenergic antagonists marketed as the pure enantiomer and is used clinically to treat glaucoma, systemic hypertension and angina pectoris. When used as an ophthalmic preparation for glaucoma, the drug is subject to a degree of systemic absorption and can lead to β-blockade in the bronchial tree with resulting

bronchoconstriction (Richards and Tattersfield, 1985). Use of such a preparation may lead to fatal bronchospasm in patients with hyper-responsive airways (Fraunfelder and Barker, 1984). In contrast, while (R)-timolol also causes a reduction in intraocular pressure (Alm *et al.*, 1990; Keates and Stone, 1984), it is markedly less potent as a β-blocker than the (S)-enantiomer; thus (R)-timolol may be a safer drug to treat glaucoma due to less risk of systemic β-adrenoceptor antagonism. The timolol example demonstrates that while one enantiomer may have a more favourable therapeutic index, the more potent enantiomer with respect to desired pharmacological effect may not necessarily be the one which is better tolerated clinically.

Far less common are examples of chiral drugs for which the enantiomers possess identical pharmacological properties in all respects (Lehmann, 1983). While the enantiomers of quinacrine and primaquine are equally active against malaria (Gause, 1945; Schmidt *et al.*, 1977) and in the case of primaquine enantiomers, equipotent as inhibitors of drug metabolism (Mihaly *et al.*, 1985), both drugs exhibit enantioselectivity in terms of their toxicity.

1.6 Enantioselective Pharmacokinetics

Interactions of chiral drugs with chiral macromolecules may occur in the absorption of the drug, its distribution, metabolism and excretion and hence each of these kinetic processes may be enantioselective (Jamali *et al.*, 1989; Lee and Williams, 1990b; Tucker and Lennard, 1990). Enantioselectivity at the dispositional level is often clouded by the complexity of the biological system. Discerning the identity of the enantioselective process in operation is not always possible due to interfering "chiral noise" (stereoselective interactions with a "silent" process; one that is not detected or measured by the investigator). Pharmacokinetic differences between enantiomers may alter pre-existing differences at the effector site, thus tempering or accentuating pharmacodynamic enantioselectivity. An exhaustive review of this topic is beyond the scope of this thesis. Accordingly, a brief discussion of possible differences between stereoisomers of a chiral drug is provided for clinically important kinetic processes.

1.6.1 Absorption

Enantiomers share identical properties in terms of aqueous and lipid solubility. For most drugs, absorption from the gastrointestinal tract is a passive process governed by achiral physicochemical properties of the compound (Rowland and Tozer, 1989). Consequently, it is to be expected that enantiomers of such drugs exhibit no differences in their relative rates of absorption. However, the oral absorption of some drugs is subject to carrier-mediated active processes implying a potential discriminatory interaction between a chiral drug enantiomer and the chiral carrier macromolecule. Such drugs tend to structurally resemble endogenous compounds which may be subject to a characteristically high degree of stereoselectivity in the absorption process. The folic acid antagonist methotrexate is subject to stereoselective absorption in favour of the (L)-enantiomer (Hendel and Brodthagen, 1984). (L)-Dopa, a metabolic precursor of dopamine used in Parkinson's disease, is absorbed much more rapidly than its optical antipode (Wade *et al.*, 1973). However, as to be expected when there is no natural restriction to passive absorption (of (D)-dopa), there is no difference between enantiomers in terms of extent of oral absorption (Lee and Williams, 1990b).

Presystemic metabolism by both the gut and liver are potentially enantioselective processes which may give rise to differences in the oral bioavailability of enantiomers of high extraction ratio drugs. Stereoselective oral bioavailability has been identified for a number of racemic drugs including verapamil (Vogelgesand *et al.*, 1984), propranolol (Von Bahr, 1982), felodipine (Eriksson *et al.*, 1991) and nitrendipine (Mast *et al.*, 1992). This metabolic process will be discussed in greater detail below (Section 1.6.3).

1.6.2 Distribution

Drug distribution involves both partitioning into tissue compartments, as well as recognition by chiral macromolecules present in both tissues and plasma. Differences between enantiomers expressed at the recognition interaction may be an important determinant of the clearance, volume of distribution and elimination half-life of the individual isomers. Enantioselective plasma protein binding, most notably for those drugs which are highly protein bound such as

the chiral NSAIDs, has been recognised as a primary determinant of the distribution profiles of individual enantiomers (Lee and Williams, 1990b). For instance, the observed preferential partitioning of (S)-ibuprofen into synovial fluid (Day *et al.*, 1988) appears to be a result of the higher free fraction in plasma recorded for the (S)-enantiomer (Evans *et al.*, 1989). Furthermore, plasma protein binding of ibuprofen enantiomers has been shown to be competitive, with the free fraction of each enantiomer increasing when in the presence of its optical antipode (Evans *et al.*, 1989). This has provided a mechanistic explanation for the observed increase in the clearance of (R)-ibuprofen when administered as a racemate compared to the clearance of this enantiomer when administered alone (Lee *et al.*, 1985). Thus, not only may intrinsic differences exist between the plasma protein binding of individual enantiomers, but enantiomer-enantiomer binding interactions may occur thereby influencing the disposition of such low intrinsic clearance drugs.

The binding of the essential amino acid (L)-tryptophan to human serum albumin occurs in a highly stereoselective fashion being *circa* two orders of magnitude more avidly bound than the unnatural (D)-enantiomer (Jahnchen and Muller, 1983; McMenamy and Oncley, 1958). In similar fashion, the binding to albumin of the hemisuccinate esters of the chiral benzodiazepine oxazepam, has been reported to occur with a high degree of enantioselectivity (Muller and Wollert, 1975).

The other major protein in human plasma to which xenobiotics, particularly basic drugs, bind to is α_1-acid glycoprotein. Enantioselective protein binding has been reported for verapamil (Eichelbaum *et al.*, 1984), methadone (Romach *et al.*, 1981) and disopyramide (Lima *et al.*, 1984; Valdivieso et al., 1988). It has been suggested that, in contrast to acidic ligands, the binding of basic drugs is relatively nonstereoselective regardless of whether to α_1-acid glycoprotein or to human plasma proteins as a whole (Drayer, 1986).

The binding of chiral drugs to extravascular protein is also a potential source of enantioselectivity. The uptake and storage of atenolol in neurosecretory cells is selective for the (S)-enantiomer (Webb *et al.*, 1988). As a result, the release of drug during sympathetic nerve stimulation is predominantly in the form of (S)-atenolol (Webb *et al.*, 1988). This

demonstrates a mechanism for pharmacodynamic differences between atenolol enantiomers arising from enantioselective drug accumulation. Enantioselective intracellular sequestration of atenolol has also been recently demonstrated for human platelets, a more accessible model of the adrenergic neurone (Walle *et al.*, 1991). Interestingly, a recent clinical study of patients receiving chronic (RS)-atenolol showed an enrichment of plasma atenolol with the pharmacologically active (S)-enantiomer after exercise, compared to the corresponding eudismic proportion in patients at rest (Stoschitzky *et al.*, 1992). The mechanism for this change remains to be elucidated.

A number of carboxylic acid xenobiotics including several 2-arylpropanoic acid NSAIDs have been shown to undergo adipose tissue incorporation in a highly stereoselective fashion (Sallustio *et al.*, 1988a; Williams *et al.*, 1986). The 2-arylpropanoates undergo metabolic activation to reactive coenzyme *A* thioesters, which in turn are presumed to be involved in the formation of hybrid triglycerides of these xenobiotics (Caldwell and Marsh, 1983). This process is specific for enantiomers of the (R)-configuration and is thought to occur via a similar mechanism to the metabolic chiral inversion process (Hutt and Caldwell, 1983, 1984; see Sections 1.6.3 and 2.3.3). Some have suggested that adipose tissue deposition of such compounds may have toxicological consequences and while this remains speculative at present (Caldwell and Marsh, 1983; Fears, 1985), it highlights the potential for drug toxicity as a result of enantioselective drug distribution.

1.6.3 Metabolism

Enantioselectivity in drug metabolism is an important determinant of possible differences in the disposition of pairs of enantiomers (Caldwell *et al.*, 1988a; Eichelbaum, 1988; Jenner and Testa, 1973; Low and Castagnoli, 1978; Testa, 1988). Such literature is replete with examples of enantioselective metabolic transformation. In general, it appears that while drug absorption and excretion do not show significant enantioselectivity (except for actively transported drugs) and plasma protein binding exhibits differences for some compounds, the largest enantiomeric differences in dispostion are derived from hepatic metabolizing activity. Examples of some of the important principles and their clinical implications are discussed below.

The stereochemical factors involved in the metabolism of racemic warfarin is classified as an example of substrate enantioselectivity (Testa, 1988). This is also an example of a low intrinsic clearance drug (Wilkinson and Shand, 1975) subject to enantioselective hepatic transformation. Warfarin is eliminated predominantly by biotransformation producing a wide variety of ring oxidation and side-chain reduction products. The pharmacologically more active (S)-warfarin is principally oxidized to form (S)-7-hydroxywarfarin and a lesser amount of (S)-6-hydroxywarfarin with a small fraction enantiospecifically reduced to (S,S)-warfarin alcohol. In contrast, (R)-warfarin is almost equally metabolized by oxidation to form predominantly (R)-6-hydroxywarfarin and some (R)-7-hyroxywarfarin, but is stereospecifically reduced to (R,S)-warfarin alcohol (Banfield *et al.*, 1983; Kaminsky *et al.*, 1984; Lewis and Trager, 1970; Lewis *et al.*, 1974). A recent study (Niopas *et al.*, 1991) examined the influence of the potent microsomal mixed-function oxidase inhibitor cimetidine on warfarin metabolism and demonstrated inhibition of the 6- and 7-hydroxylation pathways of (R)-warfarin but not of (S)-warfarin. These data demonstrate the utility of racemic drugs as stereochemical probes for *in vivo* drug metabolism studies (often performed in concert with metabolic enzyme inhibitors or inducers). The data from the above example suggest the presence of at least distinct isozymes of human hepatic cytochrome P-450 which are responsible for the 6- and 7-hydroxylation of warfarin.

The metabolism of verapamil enantiomers is an example of a high intrinsic clearance drug (Wilkinson and Shand, 1975) subject to enantioselective first-pass hepatic metabolism where the enantiomeric concentration ratio in plasma is dependent on whether the drug is given orally or intravenously (Eichelbaum *et al.*, 1984; Vogelgesang *et al.*, 1984). Accordingly, since enantioselective differences exist in the pharmacodynamics of verapamil (Echizen *et al.*, 1985a), the alteration to the eudismic proportion of drug leads to differing clinical effects as a function of route of administration (Echizen *et al.*, 1985b). The (+)/(-) ratio of plasma verapamil concentration following intravenous racemic drug administration is *circa* 2, whereas this ratio is approximately 5 after oral administration. Given that (-)-verapamil is approximately 10-fold more potent as a negative dromotropic agent than its optical antipode, the differences in plasma eudismic proportion between routes of verapamil administration

explain the observed differences in the slopes of the concentration-effect curves when the drug is given by the oral compared to the intravenous route (Echizen *et al.*, 1985a,b)

There may be genetic factors involved in enantioselective drug metabolism when a chiral drug is a substrate for a polymorphically distributed enzyme. Individuals who are deficient in a cytochrome P-450 isozyme(s) may be unable to efficiently metabolize a particular substrate which may be enantiodiscriminatory as is the case for the anticonvulsant mephenytoin. So-called "poor metabolizers" are unable to metabolize (S)-mephenytoin while "extensive metabolizers" produce a low ratio of (R)-4'-hydroxymephenytoin to (S)-4'-hydroxymephenytoin in contrast to a significantly higher such ratio in the former patient group (Yasumori *et al.*, 1990). The hydantoin class of drugs also provide an example of product (rather than substrate) enantioselectivity in drug metabolism. The aromatic hydroxylation of the achiral drug phenytoin generates a chiral metabolite (5-*para*-hydroxydiphenylhydantoin), a process which exhibits enantioselectivity favouring generation of the metabolite with the (S)-configuration (Maguire and McClanahan, 1986).

Differences in systemic exposure to potentially toxic metabolites can occur as a result of enantioselective drug transformation. The (R)-enantiomer of glutethimide is metabolized to a harmless metabolite by side-chain oxidation and is efficiently eliminated from the body; (S)-glutethimide however, is metabolized in the cyclic piperidinedione portion of the molecule yielding a toxic metabolite (Wainer *et al.*, 1987).

Metabolic chiral inversion is a highly enantioselective transformation process which has been intensively examined for the 2-arylpropanoic acid class of NSAIDs [inversion has also been described for agricultural 2-aryloxypropanoate esters (Ariens, 1988)]. The mechanism is still to be fully elucidated (Evans, 1992) but appears to involve enantiospecific generation of an (R)-acyl-thioester intermediate which may either be hydrolysed to regenerate the (R)-enantiomer or undergo epimerization to yield a thioester in which the 2-arylpropanoyl moiety has the (S)-stereoconfiguration. Subsequent hydrolysis of this sterically inverted thioester completes the inversion process. This metabolic transformation, the extent of which is dependent on both substrate and species (Hutt and Caldwell, 1983), occurs without any other

alteration to the drug molecule. Of recent interest, the location at which inversion occurs has been strongly debated, with a presystemic (gastrointestinal) site proposed on the basis of indirect evidence (Berry and Jamali, 1991; Mehvar and Jamali, 1988). In contrast, based on other studies with isolated perfused organs and tissue slices and homogenates, the liver has been suggested as the principal site of chiral inversion (Cox *et al.*, 1985; Jeffrey *et al.*, 1991; Muller *et al.*, 1990; Porubek *et al.*, 1991; Sanins *et al.*, 1990; Yamaguchi and Nakamura, 1987). While significant presystemic chiral inversion could potentially influence the systemic exposure to pharmacologically active (S)-enantiomer as a function of the gastrointestinal release characteristics of a racemic 2-arylpropanoic acid formulation, it appears that at least in a rat model, this site probably lacks sufficient metabolic capacity (Knadler and Hall, 1990). Moreover, the likelihood exists that extensive processing of organ and tissue samples may diminish the capacity of such a preparation to invert substrates (Mayer *et al.*, 1988). It needs also to be recognised that while a particular tissue might be capable of metabolic inversion, the quantitative importance of such pathways may be minimal in the presence of the high degree of protein binding that is characteristic of 2-arylpropanoates and the relatively low intrinsic capacity of this metabolic process (Hall *et al.*, 1992).

Not only are nonconjugative biotransformations of drugs amenable to substrate enantioselectivity, but so are various conjugative metabolic pathways. The stereochemical factors involved with acyl-glucuronoconjugation, a major phase II metabolic process of particular relevance to these studies, is detailed elsewhere in this thesis (Section 3.2).

1.6.4 Excretion

Both biliary and renal excretion represent final steps in drug elimination preceded by potentially enantioselective distribution and metabolism processes (and to a lesser extent enantioselective absorption steps). As a result, interfering chiral interactions at these preceding levels often makes recognition of enantioselective drug excretion difficult. The passive process involved in glomerular filtration of drugs into the renal tubular lumen suggest that renal filtration should be insensitive to the spatial orientation of chiral molecules. This process is however, dependent on the plasma protein binding of the enantiomers. Theoretically, a mechanism for

enantioselective differences in glomerular filtration of drugs, independent of binding influences, would be enantioselective effects on renal blood flow. NSAIDs are known to affect renal function by inhibiting the synthesis of vasodilating renal prostaglandins (Brater, 1988) a process which should be highly stereoselective (see Section 1.5). However, the renal elimination of unchanged drug is minor for most congeners of this pharmacological class (Upton *et al.*, 1980) which would make detection of enantioselective renal elimination difficult particularly given the coexistence (and predominance) of labile acyl-glucuronide metabolites in urine collections which themselves may be readily (and stereoselectively) hydrolyzed to the parent enantiomer of interest (see Sections 2.3.3 and 3.5).

Excretion processes such as active tubular secretion in the kidney and biliary elimination involve the interaction of drug with a chiral macromolecule, processes likely to be enantioselective. Active tubular secretion is thought to be responsible for the enantioselective renal clearances of pindolol (Hsyu and Giacomini, 1985), chloroquin (Ofori-Adjei *et al.*, 1986) and disopyramide (Lima *et al.*, 1985). The paucity of reports of enantioselective biliary excretion of drugs is possibly due to its relatively minor importance in humans as a pathway of elimination for unchanged drugs of molecular weights less than 500 (Milburn, 1970). It would appear that the majority of high molecular weight drugs, which might potentially be candidates for biliary excretion, are of semi-synthetic or natural origin and hence are used clinically as enantiomerically homogenous agents (Coppola and Schuster, 1987).

1.7 Comment

Due to the potential enantioselective nature of pharmacodynamic and pharmacokinetic properties of chiral drugs, studies of racemic compounds where drug concentrations in biological matrices must be monitored, should involve the performance of enantioselective analytical methods (see Section 4.1.1). Potentially misleading pharmacological data may be generated on racemic drugs when achiral methods of analysis are employed (Ariens, 1984; Evans *et al.*, 1988; see Section 4.1.1). Indeed the current predominance of chiral drugs marketed for clinical use as racemates highlights the therapeutic relevance of performing enantioselective pharmacology studies. Certainly, it is likely that for some drugs there will be

an improvement in the therapeutic index if the eutomer is solely marketed for clinical use rather than the racemic mixture. However, the recent enantiomer *vs* racemate debate (Ariens, 1991; Lennard, 1991), while possibly advocating the development of new drugs which are pure enantiomers or are achiral, stresses the mechanistic understanding of drug action and disposition to be gained by the enantioselective study of currently available racemic therapeutic entities.

Chapter 2

Ketoprofen: A Review of the Literature

Ketoprofen, (RS)-2-(3'-benzoylphenyl)propanoic acid, is a nonsteroidal anti-inflammatory drug (NSAID) which possesses anti-inflammatory, analgesic and antipyretic properties. In common with most congeners of the 2-arylpropanoic acid class of NSAIDs, ketoprofen is marketed for clinical use as a racemic compound. It is commonly used for the treatment of rheumatological conditions such as rheumatoid arthritis, osteoarthritis and ankylosing spondylitis and has also been used in nonrheumatological syndromes.

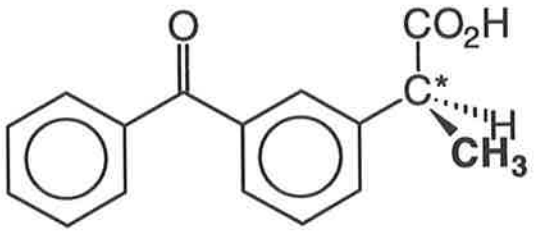
2.1 Physicochemical Properties of Ketoprofen

Ketoprofen ($C_{16}H_{14}O_3$, molecular weight: 254.3) is a white or almost white, crystalline powder which is odourless (British Pharmacopœia [B.P.], 1988, vol 1, *pp* 325-326). The pK_a of the drug is 5.9 in a 3:1 solution of methanol and water. Ketoprofen is practically insoluble in water but freely soluble in alcohols and most other organic solvents at 20°C (American Hospital Formulary Service, 1992, *pp* 1092-1097). The existence of a chiral carbon atom in the α-position to the carboxyl functional group gives rise to two possible stereoconfigurations or enantiomers of ketoprofen which are depicted in Figure 2.1.

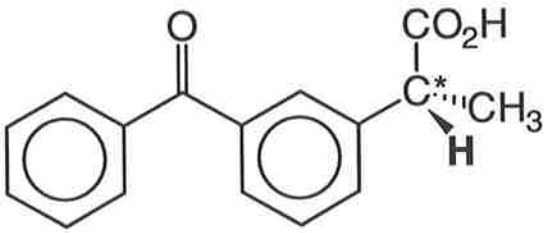
2.2 Clinical Pharmacology of Ketoprofen

Ketoprofen has pharmacological actions very similar to those of other NSAIDs or aspirin-like drugs. These drugs exhibit anti-inflammatory, analgesic and antipyretic activity. The exact mechanisms have not been clearly established, but many of the actions appear to be associated principally with the inhibition of prostaglandin synthesis. Aspirin-like drugs, such as ketoprofen, may suppress the synthesis of prostaglandins in body tissues and cells by inhibiting cyclo-oxygenase, an enzyme that catalyzes the formation of prostaglandin precursors (endoperoxides) from arachidonic acid (Vane, 1971). In addition, NSAIDs inhibit the

aggregation of platelets, again by this same mechanism (O'Brien, 1968). The markedly enantioselective nature of this inhibition of cyclo-oxygenase by congeners of the 2-arylpropanoates is discussed in detail in Section 1.5 and is assessed experimentally, for ketoprofen in Chapters 7 and 8. Alternative mechanisms of action for both the anti-inflammatory and analgesic properties of NSAIDs have been proposed and collectively, with the classical inhibition of prostanoid synthesis mechanism, are discussed below. In the first instance, these modes of action are discussed for the NSAIDs in general terms, followed by a focus on ketoprofen specifically.



(S)-ketoprofen



(R)-ketoprofen

Figure 2.1 So-called "flying-wedge" representations of (S)- and (R)-ketoprofen.

2.2.1 Mechanisms of Action of NSAIDs (General)

NSAID-mediated inhibition of the release and production of products of the arachidonic acid pathway has profound effects on a wide variety of physiopathologic processes. Overproduction of the products of this pathway, *i.e.* prostaglandins, thromboxanes and leukotrienes (collectively, referred to as "eicosanoids") occurs in a variety of pathologic states (Ferreira, 1979) accompanied by the generation and utilization of certain free oxygen radicals (Rainsford and Swann, 1984). The consequences of over-production of the eicosanoid pathways appear to depend on specific effects of: (*i*) the major products of the pathway acting on specific prostanoid receptors (including the adenylate and guanylate cyclases) and membranes and (*ii*) free radical species produced during peroxidase reactions consequent upon oxidation of arachidonate (Rainsford and Swann, 1984).

The major impetus for development of drugs to control the production of prostaglandins and thromboxanes came from the recognition in the early 1970s that certain acidic NSAIDs inhibit their production (Vane, 1971; Flower *et al.*, 1972). Vane and coworkers then embarked on an exercise to show that the inhibition of prostaglandin production was a central feature of the mode of action of anti-inflammatory/analgesic drugs. Moreover, it was recognised that inhibition of prostaglandin production could be beneficial in a wide variety of other pathophysiological conditions including prevention of thrombus formation (by inhibition of platelet aggregation), control of the menstrual cycle and correction of patent ductus arteriosus in the new-born. More recently, it has become apparent that an alternative arachidonic acid catabolic pathway generates products that profoundly affect cellular and vascular reactions in inflammation (Rainsford, 1988). This lipoxygenase pathway generates hydroperoxyeicosatetraenoic acids (HPETEs), which are reduced by peroxidase to the corresponding hydroxy-acids. One of the hydroperoxy- derivatives, 5-HPETE being a precursor of a series of biologically active compounds known as the leukotrienes. Thus a pathway diversion consequent on inhibition by NSAIDs of cyclo-oxygenase exists. Blockade of cyclo-oxygenase activity reduces production of prostaglandins and thromboxanes but consequently enhances the flow of arachidonic acid to be oxidized by the lipoxygenase pathways to possibly overproduce leukotrienes, hydroxy-fatty acids and tissue-destructive free

oxygen radical species. These eicosanoid pathways and the site of action of NSAIDs are illustrated in Figure 2.2 and may explain some of the disadvantages and potential toxic responses upon administration of congeners of this pharmacological class of agents although this is speculative at present. Moreover, theoretical advantages might be conferred upon the clinical use of agents which block both cyclo-oxygenase and lipoxygenase pathways of arachidonate catabolism (Rainsford, 1988).

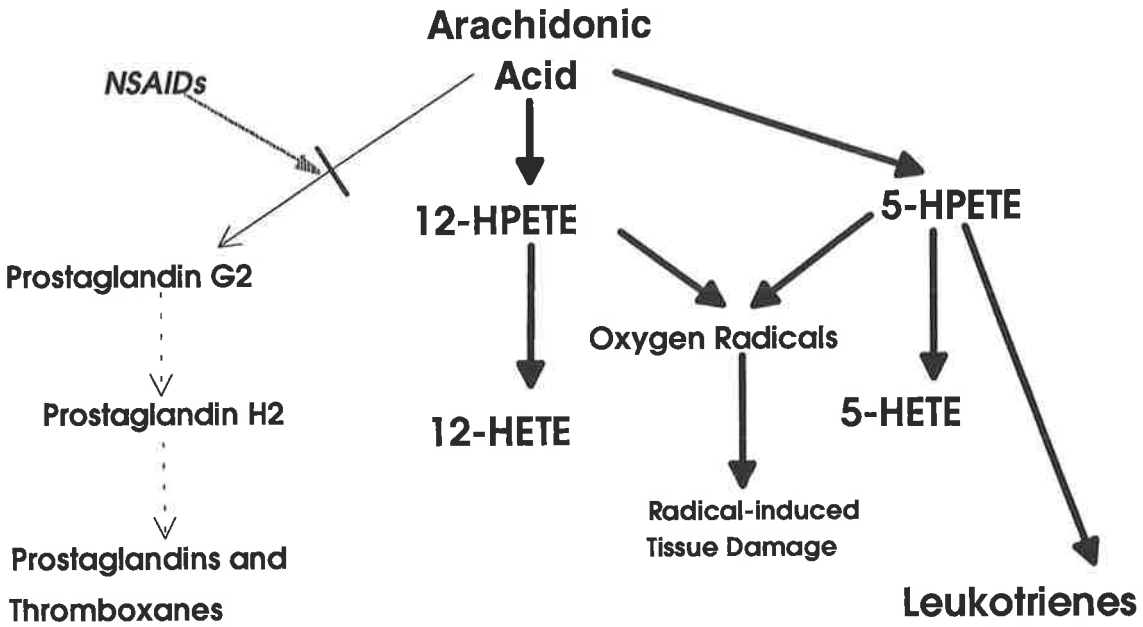


Figure 2.2 Eicosanoid pathway diversion subsequent to cyclo-oxygenase inhibition by NSAIDs (Rainsford, 1988). Blockade of this enzyme reduces prostaglandin and thromboxane production, but potentially enhances arachidonate catabolism via the lipoxygenase pathways.

The cyclo-oxygenase subunit in prostaglandin *H* synthase catalyzes the first committed step in the biosynthesis of prostaglandins (Samuelsson *et al.*, 1978). The pure synthase also has a haem-dependent peroxidase activity that is believed to initiate the cyclo-oxygenase reaction via the generation of a tyrosyl free radical (Karthein *et al.*, 1988; Kulmacz *et al.*, 1990). NSAIDs can be classified according to their effects on the cyclo-oxygenase reaction kinetics as substrate analogs that interfere with the binding of arachidonate in either: (*i*) a reversible manner (for example the arylalkanoic acids including ketoprofen) and (*ii*) a irreversible manner (*e.g.* aspirin and indomethacin) or alternatively, (*iii*) radical-quenching agents (*e.g.* paracetamol) that interfere with the initiation of the cyclo-oxygenase reaction (Lands and Hanel, 1983).

Evidence for the widely held theory of NSAID-induced cyclo-oxygenase inhibition as the means by which these drugs exert their pharmacological and toxicological actions is a reasonable rank-order correlation between inhibition of prostaglandin synthesis *in vitro* or *ex vivo* and anti-inflammatory or analgesic effects *in vivo* (Matsuda *et al.*, 1984; Ferreira and Vane, 1979; Tomlinson *et al.*, 1972). However notable exceptions to this rule have prompted speculation of NSAID pharmacological activity elicited, at least in part, by mechanisms independent of prostaglandin synthesis (Abramson and Weissmann, 1989; Day *et al.*, 1987; Evans, 1992; Goodwin 1984). Recently, Brune and coworkers (1991) established that the analgesic activity of the enantiomers of flurbiprofen were equal in an *in vivo* rat model of nociception yet (R)-flurbiprofen, in contrast to its optical antipode, had imperceptible effects on prostaglandin synthesis. These results could not be explained in terms of potential chiral inversion (see Sections 1.6.3 and 2.3.3) since this is a negligible metabolic pathway in rats for flurbiprofen and it was suggested that additional molecular mechanisms of analgesia remain to be elucidated (Brune *et al.*, 1991). Indeed the correlation between prostaglandin synthesis inhibition and analgesic effects is distorted by the nonopioid analgesics such as paracetamol and phenazone which do not inhibit prostaglandin production at analgesic concentrations (Brune *et al.*, 1981; Lanz *et al.*, 1986). Moreover, the oldest compound of this pharmacological class, salicylic acid, does not inhibit cyclo-oxygenase at analgesic concentrations (Brune *et al.*, 1981) or doses (Rosenkranz *et al.*, 1986).

NSAID postulated mechanisms of action independent of effects on prostanoid production include: (*i*) the ability of NSAIDs to lower sulphated glycosaminoglycan synthesis in articular cartilage *in vitro* (Brandt and Palmoski, 1984; McKenzie et al., 1976), [although ketoprofen has been shown not to influence proteoglycan metabolism by human cartilage (Wilbrink *et al.*, 1991)], (*ii*) their suppression of neutrophil aggregation, chemotaxis degranulation and resultant superoxide ($O_2 \cdot^-$) generation (Kaplan *et al.*, 1984; Nielsen and Webster, 1987; Shelly and Hoff, 1989; Smolen and Weissmann, 1980), (*iii*) inhibition of inflammatory oedema by an action on polymorphonuclear leukocytes (Rampart and Williams, 1986) and (*iv*) the ability of NSAIDs to inhibit mitochondrial β-oxidation of fatty acids (Freneaux *et al.*, 1990; Geneve *et al.*, 1987; Zhao *et al.*, 1992). The clinical significance of most of these alternative postulated mechanisms of action remain to be fully elucidated. However, it has been suggested (Abramson and Weissmann, 1989) that the effects of NSAIDs on stimulus-response coupling in neutrophils may explain the anti-inflammatory activity of salicylic acid (a weak inhibitor of prostanglandin synthesis) as well as the apparent pro-inflammatory actions of particular NSAIDs. Further, in a recent study (Twomey and Dale, 1992), a range of NSAIDs were examined for their effects on stimulus-induced neutrophil oxidative burst. Three distinct categories of NSAIDs were delineated according to the resultant $O_2 \cdot^-$ response. It was suggested that some NSAIDs, while providing temporary relief of arthritis symptoms, could exacerbate the underlying inflammatory condition as a result of $O_2 \cdot^-$-mediated tissue damage (Twomey and Dale, 1992).

These prostaglandin-independent effects of NSAIDs are thought to arise as a result of their ability to partition (or dissolve) into cellular phospholipid bilayers whereupon they physically disrupt intercellular signalling and subcellular protein-protein interactions (Abramson and Weissmann, 1989). This mode of action would be expected to lack the degree of structural specificity inherent in the cyclo-oxygenase mechanism of NSAID action and as suggested by Evans (1992), is likely to occur without the same degree of enantioselectivity. As detailed in Section 1.2.1 physical properties such as lipid or aqueous solubility are identical for individual enantiomers of a chiral drug and thus the process of membrane partitioning should be nonenantioselective assuming bilayer sequestered (carrier) proteins are not involved. This appears to be supported by the lack of enantioselectivity observed with the uncoupling of

oxidative phosphorylation in rat liver mitochondria and $O_2 \cdot^-$ production in macrophages induced by clindanac enantiomers (Kawai *et al.*, 1984), and a recent report of equipotent inhibition by (R)-, (S)- and (RS)-ibuprofen of human polymorphonuclear cell function *in vitro* (Villanueva *et al.*, 1993).

In summary, it is likely that inhibition of prostaglandin synthesis is the primary mechanism by which NSAIDs exert their pharmacological and toxicological actions. However, the more recent experimental endeavour in terms of prostaglandin-independent biological properties of these drugs, is beginning to contribute to a more detailed understanding of their clinical pharmacology.

2.2.2 Mechanism of Action of Ketoprofen

Specific studies of ketoprofen's mechanism of action have principally focused on its ability to interfere with arachidonic acid metabolism. Ketoprofen is a powerful inhibitor of cyclo-oxygenase being 6 to 12 times more potent than naproxen and indomethacin respectively, as judged by isolated guinea pig lung preparations perfused with arachidonic acid (Guyonnet and Julou, 1976). The same rank order of potency was established for these drugs' *in vivo* anti-inflammatory activity (carrageenin-induced inflammation) in this animal species; however, the relative potency (based on dose) of the drugs was narrower than shown for the *in vitro* studies (Guyonnet and Julou, 1976). Ketoprofen has also been demonstrated to be a potent inhibitior of prostaglandin synthesis in sheep and rat seminal vesical microsomes (Dawson *et al.*, 1982; Kubota *et al.*, 1979). Prostaglandin levels and associated paw oedema following carrageenin administration to rats were also reduced by coadministration of ketoprofen (Kubota *et al.*, 1979).

Interestingly, ketoprofen has also been reported (Kubota *et al.*, 1979) to inhibit the lipoxygenase pathway of the arachidonic acid cascade (see Figure 2.2) thereby conferring a theoretical advantage to this NSAID over cyclo-oxygenase inhibitors that facilitate increased arachidonic acid catabolism via the lipoxygenase pathway (see Section 2.2.1 and Figure 2.2). While the clinical significance of ketoprofen's inhibition of lipoxygenase is unknown, it has

been suggested that the products of this pathway (leukotrienes and hydroxy-fatty acids) facilitate tissue destruction and thus inhibition of their production should retard tissue destruction in inflamed joints (Rainsford, 1988).

In terms of prostaglandin-independent mechanisms elicited by ketoprofen, it has been shown to stabilize lysosomal membranes against osmotic damage (Migne *et al.*, 1976) and prevent the release of lysosomal enzymes that mediate inflammatory tissue degeneration (Smith, 1978).

It should be noted that with the exception of a study published in abstract form (Moreno *et al.*, 1990) none of the above studies have examined the biological activity of separate ketoprofen enantiomers but instead, have studied racemic ketoprofen as if it were a single compound. Moreno *et al.* (1990) examined inhibition of arachidonic acid-induced rabbit platelet aggregation and found preferential inhibition by the (S)-enantiomer of ketoprofen. However, this study was largely qualitative in nature having been carried out at a single enantiomer concentration with a reported 83% inhibition by (S)-ketoprofen versus 16% platelet inhibition by its optical antipode. The enantioselective pharmacodynamics of NSAIDs and other drugs are the subject of discussion elsewhere in this thesis (Section 1.5). In addition, the pharmacodynamics of ketoprofen enantiomers in man are addressed as part of experimental work carried out with *in vitro* studies in healthy subjects in the first instance (Chapter 7) and subsequently applied to patients with rheumatoid arthritis and varying degrees of renal impairment (Chapter 8).

2.2.3 Therapeutic Indications of Ketoprofen

The chief clinical application of ketoprofen has been as an anti-inflammatory agent in the treatment of musculoskeletal disorders such as the arthritides. Like all NSAIDs, ketoprofen is only likely to provide symptomatic relief from pain and inflammation associated with these conditions and will not halt the progression of pathological insult to the afflicted synovium. The clinical use of ketoprofen has been the subject of a number of reviews (Avouac and Teule, 1988; Brogden *et al.*, 1974; Kantor, 1986).

Rheumatoid arthritis

In general, when used in the symptomatic treatment of rheumatoid arthritis, ketoprofen has relieved pain and stiffness; reduced swelling, tenderness and the number of joints involved; and improved mobility and grip strength. Placebo-controlled trials have demonstrated the efficacy of ketoprofen in the treatment of rheumatoid arthritis (Brogden *et al.*, 1974; O'Brien and Grunwaldt, 1976). Double-blinded comparative studies with aspirin (Kennedy, 1976), and NSAIDs including diclofenac (Bendix *et al.*, 1983), ibuprofen (David, 1990; Mills *et al.*, 1973), indomethacin (Caldwell *et al.*, 1988b; Kirchheiner *et al.*, 1976; Viara *et al.*, 1975), naproxen (Wollheim *et al.*, 1978), piroxicam (Jalava and Junnila, 1987; Lang and Steger, 1981) and tiaprofenic acid (Valentini *et al.*, 1987) have shown ketoprofen to be at least as efficacious as the drug under comparison with most indices of clinical effectiveness. Ketoprofen has also been found to be effective in the symptomatic treatment of juvenile rheumatoid arthritis (Brewer *et al.*, 1982).

Osteoarthritis

In the symptomatic treatment of osteoarthritis, ketoprofen has relieved pain and stiffness and increased the range of motion and functional activity. In this condition, NSAIDs are principally used for their analgesic rather than anti-inflammatory properties, although inflammation may be part of the clinical presentation of osteoarthritis. Ketoprofen has been demonstrated to be more effective than placebo in controlled studies of patients with this condition of the hip and knee (Caroit *et al.*, 1976; Famaey and Colinet, 1976). It has been compared with indomethacin (Hossain, 1990), tenoxicam (Ejstrup *et al.*, 1988) and naproxen (Leopold, 1980) in double-blinded cross-over studies with few differences reported between the drugs in terms of efficacy and tolerance.

Ankylosing spondylitis

Ketoprofen has been reported as a useful drug for the symptomatic treatment of ankylosing spondylitis. Results of clinical studies suggest that the drug may be effective in relieving night pain, morning stiffness and pain at rest and in improving mobility (Kantor, 1986; Russell and Labelle, 1983). Comparative double-blinded studies with other NSAIDs in the treatment of

ankylosing spondylitis have been performed with flurbiprofen (Cherubino and Longoni, 1983) and isoxicam (Doury and Roux, 1986). In both these studies ketoprofen was found to be less effective than the comparative NSAID, although improvements from baseline to final assessment were noted after ketoprofen treatment in most clinical variables.

Anti-inflammatory ketoprofen dosage

As an anti-inflammatory agent the effective daily dose of ketoprofen has been confirmed at 200 mg/day following extensive review of the literature (Avouac and Teule, 1988) with little apparent advantage conferred by dosing more frequently than once or twice daily even though the half-life of the drug in plasma is approximately 2 h (Upton *et al.*, 1981). This may, in part, be due to the kinetics of the drug in synovial fluid (see Section 2.3.2).

Pain

Ketoprofen has been demonstrated to be effective in the symptomatic relief of mild to moderate pain, such as postoperative pain (Roaas, 1987; Turek and Baird, 1988), pain associated with oral surgery (Cooper, 1988), dysmenorrhea associated pain (Mehlisch, 1988; Montrull *et al.*, 1986) and for relief of visceral pain associated with cancer (Stambaugh and Drew, 1988; Stambaugh *et al.*, 1986). A gel-form of the drug has been successfully applied topically for the alleviation of pain associated with sporting injuries (Baixauli *et al.*, 1990; Gevi and Merlo, 1983; Noret *et al.*, 1987). The studies above have shown that ketoprofen, when used orally to relieve mild to moderate acute pain, is at least as effective as usual analgesic doses of other NSAIDs or mild opiate analgesics and more effective than placebo. It is also apparent that no additional benefit is obtained by increasing the single analgesic dose above 100 mg (Turek and Baird, 1988).

Summary

It appears that for certain rheumatological and nonrheumatological syndromes, ketoprofen is efficacious as an anti-inflammatory analgesic agent. However, it is unlikely that ketoprofen offers significant advantages over other NSAIDs. Indeed, factors such as cost and toxicity or tolerance (*vide infra*) are the more usual limiting factors in the use of such drugs. In the case of rheumatoid arthritic patients however, the variability in symptom response to individual

agents of this drug class needs to be kept in mind. Some patients will respond to one particular NSAID and not to another and moreover, the response of a given patient to a particular NSAID may vary with time.

2.2.4 Adverse Effects of Ketoprofen

The principal adverse effects of ketoprofen are those associated with the upper gastrointestinal tract. In most cases, the upper gastrointestinal effects are minor and include nausea, dyspepsia and epigastric discomfort (Kantor, 1986). Less frequent are subjective nervous system symptoms including headache, drowsiness and complaints referable to the lower gastrointestinal tract (diarrhoea, constipation and flatulence) (Kantor, 1986). In the U.S.A. trials of ketoprofen use reviewed by Kantor (1986), treatment with the drug was discontinued for adverse effects in 13% of patients. More serious gastrointestinal reactions (peptic ulceration) in patients chronically treated with ketoprofen were recorded at a rate of 0.33% compared with 1.76% in patients administered long-term aspirin (Kantor, 1986). These comparative data suggesting greater safety (in terms of gastrointestinal ulceration and bleeding) for ketoprofen compared to aspirin use are supported by additional studies (Lussier and Arsenault, 1976; Rahbek, 1976; Ranlov *et al.*, 1983).

Transient depression of renal function with associated increased serum creatinine and possible fluid retention is a characteristic adverse effect of NSAIDs (Henrich, 1983). Since prostaglandins synthesized in the kidneys are potent vasodilators (Walshe and Venuto, 1979), inhibition of their synthesis will affect renal function in certain situations. Ketoprofen has been associated with acute renal failure which was reversed upon withdrawal of the drug (Atkinson *et al.*, 1986; Sennesael *et al.*, 1986). More serious was a report of ketoprofen-induced irreversible renal failure (Pazmino and Pazmino, 1988). However, the overall incidence of ketoprofen-induced renal toxicity is very low (Kantor, 1986; Speirs, 1988).

Another relatively infrequent side effect of ketoprofen is that associated with the skin occurring at a third of the rate reported for upper gastrointestinal adverse effects (Speirs, 1988). A rash

is the principal manifestation of dermatological side effects induced by ketoprofen with less frequent pruritis, urticaria, erythaema and eczema reactions reported.

The predisposition of certain aspirin-intolerant patients to bronchoconstriction upon exposure to NSAIDs is well recognised, and for ketoprofen, the importance of this reaction (albeit rare) is highlighted by a report of life-threatening asthma, urticaria and angioedema after ketoprofen administration (Frith *et al.*, 1978).

In light of the benoxaprofen experience with fatal liver toxicity, there have been no such reports for ketoprofen following relevant literature and case-study searches (Mills and Sturrock, 1982; Widmark and Vavra, 1984).

On balance, with chronic use, ketoprofen appears to have a very low incidence of serious adverse effects on par with the better tolerated NSAIDs and notably less toxic than aspirin (Kantor, 1986).

2.3 Clinical Pharmacokinetics of Ketoprofen

Ketoprofen has been in clinical use since its development in the early 1970s in France. While it has been exclusively marketed as a racemate, most of pharmacokinetic studies on ketoprofen have been performed using nonenantioselective methods of analysis. The pharmacokinetic properties of a chiral drug determined using the results of nonenantioselective drug analysis may not reflect the true pharmacokinetics of the individual enantiomers. The limitations of such studies is addressed in Section 4.1.1. For the purpose of examining the literature in relation to ketoprofen pharmacokinetics accent will be focussed (when possible) on those studies where the disposition of individual enantiomers is described.

2.3.1 Absorption of Ketoprofen

Ketoprofen has been administered most commonly as a regular or sustained-release oral dosage form. It has also been administered by the rectal, intramuscular, topical and intravenous route.

In terms of unresolved drug, studies have shown that the rate of absorption of ketoprofen from regular-release oral formulations is rapid and almost complete (see Jamali and Brocks (1990) for a review containing these nonenantioselective studies). These authors estimated the oral bioavailability of ketoprofen to be >92% based on area under the plasma concentration-time curve (AUC) data pooled from a number of studies (Jamali and Brocks, 1990). Although an intravenous preparation is available for clinical use in some countries, no oral bioavailability investigations have been carried out in a single cohort of subjects. Not all of an orally administered dose of ketoprofen can be recovered in urine as parent drug plus metabolites (see Section 2.3.3), thereby complicating the use of urinary recovery data as an accurate index of oral absorption. The bioavailability value of >92% (Jamali and Brocks, 1990) suggests minimal metabolism of ketoprofen during first-pass through the liver and in turn, implies that the hepatic clearance of the drug is capacity limited. Concurrent ingestion of food has been reported to slow the rate of ketoprofen absorption, but not its extent (Bannwarth *et al.*, 1988).

The oral absorption of ketoprofen enantiomers appears to be very similar and suggests that as for most drugs, ketoprofen absorption is a passive process and consequently, unlikely to be influenced by drug stereochemistry (Foster *et al.*, 1988a,b). Sallustio and coworkers (1988b) examined the disposition of ketoprofen enantiomers upon chronic administration of a sustained-release dose product (Orudis® SR, Rhone-Poulenc Rorer) to elderly patients with arthritis. Some of these patients exhibited simple absorption profiles with maximum concentrations achieved at 6 h in contrast to *circa* 1 h reported for a similar patient category administered regular-release ketoprofen (Foster *et al.*, 1988a). Other patients revealed biphasic absorption profiles and some experienced maximum enantiomer concentrations at approximately 10 h however, in all cases, absorption appeared to be nonenantioselective (Sallustio *et al.*, 1988b).

The systemic absorption of a topical gel preparation of ketoprofen has been reported to be minimal with only 0.5% of the dose appearing in urine as metabolites (Gevi and Merlo, 1983). The bioavailability of intramuscular and rectal ketoprofen relative to orally administered encapsulated drug was 96% and 83%, respectively, in terms of unresolved drug (Ishizaki *et al.*, 1980).

2.3.2 Distribution Processes of Ketoprofen

Plasma protein binding

In common with its 2-arylpropanoate structural congeners, ketoprofen is classified as a restrictively cleared drug (Lin *et al.*, 1987) for which the unbound species in plasma will assume primary importance with respect to both dispositional and pharmacodynamic processes. Moreover, ketoprofen (as with most NSAIDs) is extensively bound to plasma proteins and hence the fraction unbound in plasma is sufficiently small in magnitude to make even relatively minor changes to protein binding highly relevant in terms of the potential magnitude of alterations to the fraction unbound in plasma (see Section 5.1). Using nonenantioselective methods, the degree of plasma protein binding of ketoprofen has been reported as approximately 99% (Netter *et al.*, 1987; Royer *et al.*, 1986; Williams *et al.*, 1981).

Studies of the *in vitro* binding of separate ketoprofen enantiomers to human serum albumin demonstrated a greater unbound fraction for the (S)-enantiomer (Rendic *et al.*, 1980). However, the ligand to protein concentration ratios examined in this study were orders of magnitude larger than would be expected with normal dosing in humans and hence the clinical significance of this observation is questionable (see Section 5.3). Indeed, the degree of stereoselectivity of protein binding of etodolac enantiomers has been found to be sensitive to albumin concentration with similarly designed *in vitro* studies (Muller *et al.*, 1992). Moreover, there is a lack of data pertaining to potential competition between ketoprofen enantiomers for protein binding sites which is possible given that: (*i*) the drug is administered as a racemate and (*ii*) competition between ibuprofen enantiomers (structural congeners of ketoprofen enantiomers) for protein binding sites has been observed in man (Evans *et al.*, 1989). This

latter study also demonstrated concentration-dependent protein binding of ibuprofen enantiomers and again, no such examinations have been performed for ketoprofen.

An interesting chromatographic application of the inherent chirality of human serum albumin binding of 2-arylpropanoates has been described (Noctor *et al.*, 1991). A human serum albumin-based chiral stationary phase was used for the high-performance liquid chromatographic resolution of ketoprofen enantiomers. This protein-based stationary phase (reported to retain native enantioselectivity in the binding of chiral ligands) was also applied to a study of the affinity and binding mechanism of such drugs. Ketoprofen enantiomers were resolved with this column, suggesting enantioselective protein binding (the order of elution of enantiomers was not stated). However, the physiological significance of these data is uncertain given the possible atypical binding protein conformation in the presence of a "nonphysiological" mobile phase containing 15% acetonitrile in phosphate buffer at pH6.9.

In essence, very little is known about the plasma protein binding of the individual ketoprofen enantiomers, most notably under clinically relevant conditions. This issue is addressed in subsequent experimental chapters where methodology is developed and applied to the study of the pharmacokinetics and pharmacodynamics of ketoprofen enantiomers in man.

Distribution into synovial fluid

The synovium is thought to be the site at which NSAIDs exert their chief pharmacological actions (Day *et al.*, 1988; Simkin, 1988; Wallis and Simkin, 1983). Moreover, since only unbound drug is likely to interact with biological receptors to elicit pharmacological effects, it follows that unbound drug concentrations in synovial fluid should be of importance in relation to NSAID antirheumatic properties. As a result of the ethical and technical difficulties associated with repeated sampling of this matrix, there has been a limited number of studies which describe synovial fluid concentrations of ketoprofen.

Netter and coworkers (1987) found that in terms of unresolved drug, unbound concentrations in synovial fluid approximated those in serum, however with time the ratio of synovial fluid/serum drug concentrations increased, suggesting delayed drug clearance from the former

compartment. It was apparent from studies of total (bound plus unbound) concentrations of ketoprofen enantiomers in synovial fluid that sequestration and clearance was nonenantioselective (Foster *et al.*, 1989a). Some mechanistic insights into chiral 2-arylalkanoate distribution into joints has been provided by studies with ibuprofen enantiomers where the drug appeared to diffuse into the synovial fluid in the unbound form followed by a proportion of the drug (10-20%) diffusing out of this compartment bound to albumin (Day *et al.*, 1988).

It follows that the less pronounced fluctuation in synovial fluid concentration of ketoprofen after oral dosing may explain the longer clinical dosing interval than would otherwise be predicted to be required based solely on plasma or serum drug concentrations. In addition, the profile of ketoprofen concentration in synovial fluid should not be significantly altered as a result of the drug's formulation as a sustained-release preparation (Brooks and Day, 1991).

2.3.3 Metabolism and Excretion of Ketoprofen

Upon administration to man, ketoprofen is eliminated via extensive biotransformation. Early studies of ketoprofen metabolism were performed without regard to the stereochemistry of the parent drug and its metabolites and moreover, without consideration of the chemically and biologically labile character of the principal acyl-glucuronide conjugate metabolites (see Sections 3.3 and 3.5). Accordingly, much of the early drug disposition data generated for ketoprofen need to be considered in light of these limitations.

A significant metabolic route for ketoprofen in humans is the generation of a renally eliminated acyl-glucuronide with a negligible quantity of parent drug appearing in urine as unchanged drug (Upton *et al.*, 1980). Previous reports suggested that up to 50% of the dose of ketoprofen was excreted in urine as unchanged drug (Delbarre *et al.*, 1976; Jeffries *et al.*, 1979; Lewellen and Templeton, 1976). In contrast, Upton and coworkers (1980) established that in healthy subjects 74% of the oral dose was recovered in urine as acyl-linked glucuronoconjugates and further, provided evidence that previous claims of significant renal elimination of parent drug were artefactual reflecting hydrolysis of the conjugates during urine

sample collection, storage and handling. Similar urinary recoveries of ketoprofen acyl-glucuronides have been confirmed in other studies when drug has been administered to healthy subjects as an oral formulation (Advenier *et al.*, 1983; Houghton *et al.*, 1984; Ishizaki *et al.*, 1980; Stafanger *et al.*, 1981; Upton *et al.*, 1982) and as an intramuscular and rectal product (Ishizaki *et al.*, 1980). In these studies, urinary excretion of acyl-glucuronides appeared to be complete within 24 h of dosing. Interestingly, in elderly subjects the urinary recovery of acyl-linked conjugates was significantly diminished with reported values for unresolved drug metabolites of 33% (Stafanger *et al.*, 1981) and 47% (Advenier *et al.*, 1983). This has been confirmed by more recent studies using enantioselective analytical methods (Foster *et al.*, 1988a; Sallustio *et al.*, 1988b) which in turn have shown preferential recovery of conjugates of (S)-ketoprofen compared to (R)-ketoprofen [(S):(R) ratio of *circa* 1.3:1].

The biliary or faecal elimination of ketoprofen glucuronides has been reported to be negligible (Delbarre *et al.*, 1976; Foster *et al.*, 1989b; Populaire *et al.*, 1973). Alternative metabolic transformations of ketoprofen, with the exception of chiral inversion (*vide infra*), are less well established. Early studies of the drug reported "minor" (semi-quantitative data cited only) aromatic hydroxylation of ketoprofen in humans (Populaire *et al.*, 1973).

While workers have been able to account for a substantial proportion of a given oral dose of ketoprofen (eliminated as metabolites or parent drug), there still remains to be identified a fraction of the dose which cannot be explained by incomplete drug bioavailability (Jamali and Brocks, 1990) and which becomes more substantial in elderly patients with diminished renal function. This particular issue is targeted in greater detail in one of the experimental chapters in this thesis (see Section 8.4).

Members of the 2-arylpropanoate class of NSAIDs are subject to a unique metabolic transformation involving the stereospecific inversion of the (R)-enantiomer to its corresponding optical antipode, the magnitude of which is dependent on the animal species and the particular structural congener (Caldwell *et al.*, 1988a; Hutt and Caldwell, 1983). Ketoprofen is subject to a relatively minor degree of metabolic chiral inversion with 10% of the dose of (R)-ketoprofen converted to the pharmacologically active, in terms of cyclo-oxygenase

inhibition, (S)-enantiomer (Jamali *et al.*, 1990). This process assumes greater importance for other 2-arylpropanoates in humans and has been the subject of a number of recent literature reviews (Evans *et al.*, 1992; Williams, 1990). Both the site of the inversion step (Berry and Jamali, 1991; Jeffrey *et al.*, 1991) and the mechanism of this metabolic step (Knadler and Hall, 1990; Knihinicki *et al.*, 1989, 1991; Mayer *et al.*, 1988; Porubek *et al.*, 1991; Tracy and Hall, 1992) have focussed the efforts of a number of researchers recently. Such mechanistic studies have targeted the fundamental importance of a coenzyme *A* (Co*A*) thioester metabolic intermediary although controversy as to the apparent stereospecific nature of this process still rages (see Section 1.6.3). In any case, it is unlikely that this inversion process will assume significant clinical significance with respect to the disposition of ketoprofen. Moreover, it follows that the Co*A* thioester-mediated lipid incorporation noted with extensively inverted 2-arylpropanoates (Sallustio *et al.*, 1988a; Williams *et al.*, 1986) implies that the sequestration of ketoprofen into human adipose tissue should be minimal. This assumes that the inversion process and lipid incorporation are interdependent with both relying on the generation of the Co*A* thioester of the alkanoic acid (Caldwell and Marsh, 1983; Fears, 1985; Hutt and Caldwell, 1983).

Jamali and coworkers (1990) have derived standard pharmacokinetic parameters for total (bound plus unbound) ketoprofen enantiomers upon separate oral administration of each of the stereoisomers (25 mg) and racemic ketoprofen (50 mg) to healthy young volunteers. There were no significant differences between enantiomers for either oral clearance, apparent volume of distribution or half life. Moreover, these kinetic parameters were no different whether ketoprofen enantiomers were administered alone or together as racemic drug, suggesting a lack of kinetic interaction between enantiomers. This study also confirmed the preferential urinary recovery of glucuronoconjugates of (S)-ketoprofen discovered in earlier work (Foster *et al.*, 1988a,b; Sallustio *et al.*, 1988b). The lack of enantioselectivity in the plasma concentration-time profiles of ketoprofen stereoisomers observed in each of these studies appears to be unaffected by oral dosage of racemic drug (Jamali and Brocks, 1990). Further, over a dosage range of 25 to 200 mg the AUC of each enantiomer for total (bound plus unbound) drug was proportional to oral dose, indicating that clearance (together with possibly

the unbound fraction of each enantiomer in plasma and the bioavailability of the drug) is not saturable within the normal therapeutic dose range (Jamali and Brocks, 1990).

2.3.4 Factors Which Alter the Pharmacokinetics of Ketoprofen

2.3.4.1 Disease State Influences

Arthritis

The disposition of ketoprofen appears to be independent of the rheumatic disease process, based on comparable enantioselective studies in healthy subjects (Foster *et al.*, 1988b) and patients with osteoarthritis and rheumatoid arthritis (Foster *et al.*, 1988a). It has been suggested (Jamali and Brocks, 1990) that this may be related to the fact that the patients used in Foster and coworkers' study (1988a) had chronic but "inactive" disease. Studies with ibuprofen (Aarons *et al.*, 1983) and naproxen (Van Den Ouweland *et al.*, 1987) have shown an increase in the unbound fraction of drug in plasma from arthritic patients suffering an acute flare of their inflammatory condition, presumably due to a lower albumin concentration in these patients. In contrast, Wanwimolruk *et al.* (1982) reported no difference in either ibuprofen or flurbiprofen unbound fraction in plasma from patients with rheumatoid arthritis compared to those with ostheoarthritis nor was the unbound fraction of either drug correlated with serum albumin concentration (data pooled from both patient groups). However, the inflammatory status of patients was not stated in this study (Wanwimolruk *et al.*, 1982). Clearly, the effect of chronic arthritic conditions on the pharmacokinetics of ketoprofen remains to be fully elucidated.

Hepatic disease

The effects of hepatic insufficiency on the pharmacokinetics of ketoprofen have not been expressly addressed in the literature. Advenier *et al.* (1983) reported significant increases in AUC and $t_{½}$ for total (bound plus unbound) unresolved drug in an elderly cohort of subjects (mean age of 86 years) which they attributed to diminished glucuronoconjugation ability. This postulate was questioned by Verbeeck *et al.* (1984) who suggested a renal mechanism for the observed reduction in ketoprofen clearance (Advenier *et al.*, 1983; *vide infra*). Inherent

difficulties are associated with resolving correctly the effects of diminished hepatic and renal function. The likelihood exists for elderly patients to experience a concomitant loss of both renal and hepatic function and determinations of the relative contributions in this age group of loss of the latter is made difficult in the absence of accurate hepatic function measures. It should also be noted that any examination of the influence of hepatic disease on ketoprofen disposition must involve examination of the unbound species, since protein binding changes are possible due to either decreased synthesis of albumin or accumulation of endogenous binding inhibitors (Lin *et al.*, 1987; Wilkinson, 1986).

Renal disease

Since ketoprofen is subject to negligible urinary elimination as unchanged drug (Upton *et al.*, 1980), impaired renal function would not be expected to alter renal clearance of the drug. However, ketoprofen is cleared chiefly as renally eliminated acyl-glucuronides which would be expected to accumulate in renally impaired patients or in elderly subjects in whom renal function is diminished. As detailed elsewhere in this thesis (Sections 3.3 and 3.5) acyl-glucuronides are labile compounds which can be readily hydrolysed under physiological conditions back to the parent aglycone, the so-called "futile cycle" (Meffin, 1985). Thus the elevation of parent unresolved ketoprofen concentrations in plasma observed in elderly subjects (Advenier *et al.*, 1983) and in patients with renal disease (Stafanger *et al.*, 1981) is likely to be due to reduced renal clearance of the acyl-glucuronides of ketoprofen. Additional support for this renal mechanism is afforded by the diminished clearance of ketoprofen observed in subjects co-administered probenecid (Foster *et al.*, 1989b; Upton *et al.*, 1982). According to this mechanism, probenecid is thought to compete with the glucuronides of ketoprofen for renal tubular secretion (Verbeeck, 1990; Verbeeck *et al.*, 1984). Probenecid may also competitively inhibit the glucuronosyltransferase-mediated biotransformation of ketoprofen at the enzyme level (see Section 2.3.4.2).

An enantioselective examination of ketoprofen disposition as a function of renal impairment (Sallustio *et al.*, 1988b) failed to demonstrate a relationship between the time-averaged steady-state plasma concentrations of ketoprofen enantiomers and creatinine clearance. However, unbound enantiomer concentrations of drug were not measured (Sallustio *et al.*, 1988b) and

the likelihood exists for changes to plasma protein binding (Lin *et al.*, 1987) to mask possible alterations in the AUC for unbound enantiomers. Other enantioselective ketoprofen studies have shown the presence of ketoprofen glucuronides in plasma from elderly subjects, notable by their absence in plasma from young subjects administered the drug (Foster *et al.*, 1988a,b), and diminished recovery of conjugated drug in the urine of patients with impaired renal function (Foster *et al.*, 1988a; Sallustio *et al.*, 1988b). These observations provide additional support for the postulate of renal dysfunction-induced regeneration of parent aglycone arising from the hydrolysis of systemically accumulated acyl-glucuronides and are focussed upon, experimentally, in Chapter 8.

2.3.4.2 Pharmacokinetic Drug Interactions

Pharmacokinetic drug interactions with NSAIDs have been extensively reviewed recently (Verbeeck, 1990). The alterations to the disposition of ketoprofen upon concurrent administration of probenecid involves competition between the glucuronide conjugates of both ketoprofen and probenecid for secretion by the renal tubules (Foster *et al*, 1989b; Upton *et al.*, 1982; Verbeeck *et al.*, 1984). This mechanism leads to accumulation of labile conjugates of ketoprofen in plasma with the potential for systemic deconjugation and consequent regeneration of parent compound. Probenecid may also compete with ketoprofen for glucuronidation at the enzyme level as demonstrated for naproxen conjugation (Spahn and Benet, 1987), however this remains to be unequivocally established. Interestingly, the interaction between probenecid and ketoprofen has been reported to be stereoselective with a 60% reduction in the urinary excretion of conjugated (R)-enantiomer compared to a 33% reduction in the excretion of (S)-ketoprofen conjugate (Foster *et al.*, 1989b). However, caution is needed with such an interpretation since systemic deconjugation of acyl-glucuronides can in itself be stereoselective (Iwakawa *et al.*, 1988; Knadler and Hall, 1991; Volland *et al.*, 1991) thereby confusing the possible stereoselective elimination of diastereomeric acyl-glucuronides (see Section 3.2).

Aspirin has been shown to interact with the kinetics of unresolved ketoprofen in a complex fashion (Williams *et al.*, 1981). The mechanism involved displacement of ketoprofen from

plasma protein binding sites, increased total body clearance, increased unbound clearance of ketoprofen to nonconjugated metabolite (unidentified) and decreased unbound clearance of parent drug to conjugate. These changes counter-balanced each other yielding no net alteration to unbound drug clearance in the presence of coadministered aspirin (Williams *et al.*, 1981). Given the lack of information with regards to individual enantiomers of ketoprofen, the mechanism of this interaction with aspirin remains to be elucidated.

A recent study of the effect of metoclopramide on the pharmacokinetics of regular-release ketoprofen (50 mg capsule) showed a marked reduction in AUC and C_{max} in relation to unresolved bound plus unbound drug (Etman *et al.*, 1992). The authors suggested that metoclopramide, by speeding gastric emptying, reduced the dissolution and subsequent absorption of ketoprofen from the gastrointestinal tract. Other drugs examined for their effect on (unresolved) ketoprofen kinetics for total drug include sucralfate (Caillé *et al.*, 1987), aluminium phosphate (Brazier et al., 1981) and cimetidine (Verbeeck *et al.*, 1988). With the exception of sucralfate which reduced the rate but not the extent of ketoprofen oral absorption, none of these drugs significantly altered the pharmacokinetic parameters of ketoprofen.

In terms of ketoprofen effects on the disposition of coadministered drugs there have been a number of reports of altered response to warfarin and methotrexate. Prolonged bleeding times have been noted in patients receiving both ketoprofen and warfarin (Flessner and Knight, 1988; Wilson, 1988). While the mechanism has yet to be elucidated, the possibility of a cyclo-oxygenase inhibition-mediated antiplatelet effect of ketoprofen remains. Since NSAIDs decrease the adhesiveness of platelets by inhibition of the pro-aggregatory agent thromboxane A_2 (TXA_2) production (Ferreira and Vane, 1979), conceivably the warfarin-induced prolongation of bleeding time might be enhanced by coadministration of ketoprofen. Thus the augmented hypoprothrombinaemic response to warfarin, in the presence of ketoprofen, is likely to be a pharmacodynamic rather than a pharmacokinetic interaction. The influence of ketoprofen on TXA_2 production by platelets is discussed in Section 7.1 and is addressed experimentally in Chapters 7 and 8.

Severe methotrexate toxicity has been reported in patients on high-dose methotrexate who have been administered ketoprofen (Thyss *et al.*, 1986). The mechanism for this interaction was subsequently investigated using an animal model (Perrin *et al.*, 1990). It appeared that ketoprofen significantly reduced the renal clearance of methotrexate, presumably by competition between the glucuronides of ketoprofen and methotrexate for secretion by the renal tubules. Significantly, methotrexate is sometimes used in combination with ketoprofen for the treatment of rheumatoid arthritis. However, the doses of methotrexate used result in clinically insignificant changes to plasma methotrexate concentrations due to concomitant ketoprofen administration (Ahern *et al.*, 1988).

2.3.5 Comment

Whilst racemic ketoprofen has been used clinically since the 1970s, glaringly absent from the literature are pharmacokinetic and pharmacodynamic data pertaining to the unbound enantiomeric species. Ketoprofen's classification as a restrictively cleared, low extraction drug (Lin *et al.*, 1987) implies that only the unbound species will be available for metabolic clearance or for interacting with biological receptors to elicit pharmacological effects. Ketoprofen is subject to significant metabolic transformation to acyl-glucuronide conjugates, a process which is complicated by the inherent instability of these metabolites in biological matrices. It is likely the apparent sensitivity of ketoprofen disposition to renal function is due to systemic regeneration of parent drug from accumulated conjugates (Faed, 1984). This is of particular clinical significance since anti-inflammatory drugs, such as ketoprofen, are commonly used in elderly patients in whom renal function is likely to have declined as a result of the natural ageing process (Lindeman *et al.*, 1985).

The experimental chapters of this thesis (Chapters 4-8) address the development and application of methods to describe some aspects of the enantioselective pharmacokinetics and pharmacodynamics of ketoprofen in man, most notably in terms of the unbound moieties, and the influence thereon of renal function.

Chapter 3

Acyl-Glucuronides: The Influence of Optical Isomerism

Acyl-glucuronidation plays a significant role in the elimination of many carboxylic acid xenobiotics including chiral and achiral NSAIDs such as ketoprofen and naproxen (Upton *et al.*, 1980), carprofen (Rubio *et al.*, 1980), fenoprofen (Rubin *et al.*, 1972), ximoprofen (Mayo *et al.*, 1990) and diflunisal (Tocco *et al.*, 1975). Principally, these compounds form 1-*O*-β-acyl (ester) conjugates with optically active (D)-glucuronic acid from the glucuronosyl donor, uridine diphospho glucuronic acid (UDP glucuronic acid). This transfer is catalysed by a microsomal enzyme UDP glucuronosyltransferase of which multiple forms exist (Bock *et al.*, 1983; Burchell *et al.*, 1991; Hockman and Zakim, 1983). The reaction leads to an inversion at the anomeric C-1 of the sugar moiety and the formation of the β-(D)-glucuronide. The purpose of this conjugation is to produce a strongly acidic compound which, in most cases, is more water-soluble (and hence more readily eliminated) at physiological pH than its precursor; pK_a values for most glucuronides are in the order of 3.0-3.5 (Smith and Williams, 1966). However, rather than leading to abolition of biological activity of the parent compound, it appears that this process may have pharmacological and toxicological consequences by virtue of the physiologically-labile nature of acyl-glucuronides [see Faed (1984) and Spahn-Langguth and Benet (1992) for reviews]. Moreover, since glucuronoconjugates of, for example, 2-arylpropanoic acid NSAID analogues are principally renally eliminated in man (Upton *et al.*, 1980), the disposition and biological fate of these metabolites should be sensitive to renal function or coadministered drugs such as probenecid which compete with glucuronides for elimination by the kidneys (Meffin, 1985). In the case of chiral drugs, acyl-glucuronidation generates diastereomeric conjugates which possess different physicochemical properties. Thus stereoselectivity in the biological handling and systemic reactivity of these glucuronides may result from both macromolecular (protein) interactions and/or interactions with achiral processes.

3.1 Acyl-Glucuronide Structure and Isomerism

Glucuronic acid exists in two anomeric forms, α-(D)-glucuronic and β-(D)-glucuronic acid, depending on whether the hydroxyl function at C-1 is in an axial or equatorial configuration, respectively. Accordingly, the aglycone when attached to C-1 of the pyranose ring (6-membered sugar ring), will assume either an α- or β-configuration (see Figure 3.1). For pyranose derivatives possessing neighbouring highly electronegative aglycones (*e.g.* carbonyl functions), the equatorial attachment (β-configuration of the glucuronide) is more stable and is consequently the glucuronide anomer which is biosynthesized (Pigman and Isbell, 1968). These 1-*O*-β-acyl-glucuronides may readily undergo intramolecular rearrangement by migration of the acyl group to positions C-2, -3 and -4 of the sugar moiety (Faed, 1984). Unlike the biosynthetic 1-*O*-β-acyl-glucuronide, these positonal isomers (regioisomers) are not susceptible to enzymatic (β-glucuronidase) hydrolysis (Blanckaert *et al.*, 1978). Isomerization of the sugar group is also possible yielding a 5-membered (furanose) structure in either α- or β-anomeric forms (except for the C-1 position), in addition to open chain and lactone arrangements of the moiety (Sinclair and Caldwell, 1982). Thus for chiral aglycones attached to glucuronic acid through an ester linkage, a multitude of isomers is possible for each aglycone enantiomer.

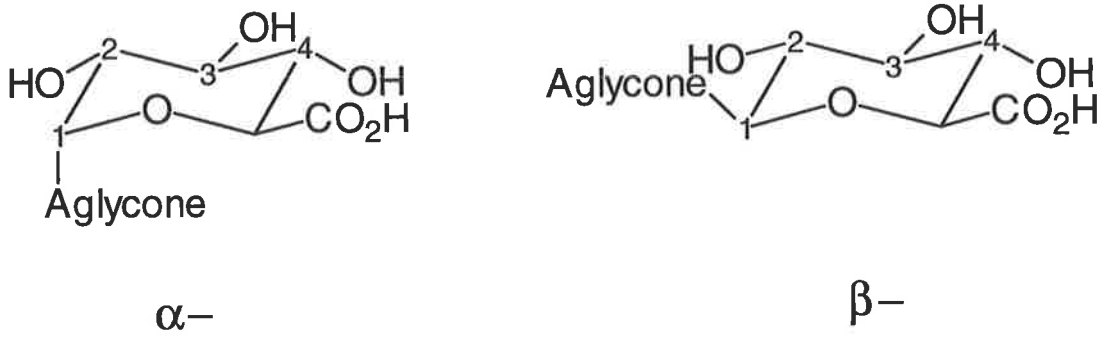


Figure 3.1 The two anomeric forms of 1-*O*-acyl-(D)-glucopyranosiduronate (-acyl-glucuronide) in the α-configuration and the β-configuration.

3.2 Enantioselective Acyl-Glucuronidation

In contrast to considerations of the glucuronoconjugation of hydroxylated chiral substrates which yield relatively stable ether glucuronides, the study of enantioselectivity in the generation of acyl-glucuronides needs to be considered in light of the intrinsic reactivity of the latter group of conjugates. Indeed the degradation (at physiological pH and temperature) of acyl-glucuronides may be stereoselective as shown for those of fenoprofen (Volland *et al.*,1991), benoxaprofen (Spahn *et al.*, 1989a), carprofen (Iwakawa *et al.*, 1988) and 2-phenylpropanoic acid (Nakamura and Yamaguchi, 1987). As highlighted by Nakamura and Yamaguchi (1987) and Spahn-Langguth and Benet (1992), significant stereoselective hydrolysis of conjugates will influence not only the total metabolite concentrations but also the (R)/(S)-acyl-glucuronide proportion in the matrix of interest; thereby, potentially leading to a misinterpretation of both the rates and (R):(S) ratios of glucuronide generation with *in vivo* and *in vitro* studies. It has been recommended that biological samples be rapidly stabilized or quenched by exposure to cold-acidic (pH3-4) conditions to prevent degradation of biosynthetic glucuronides to either hydrolyzed aglycone or to various regioisomers (Faed, 1984).

Both *in vivo* and *in vitro* studies have been used to investigate the stereochemical factors involved in the acyl-glucuronidation process. Consideration of *in vivo* studies needs to be tempered by inherent experimental complications associated with the resolution of the biotransformation process from competing "chiral noise". Interdependent dispositional processes such as distribution, competing metabolic pathways and elimination are all potentially stereoselective (see Section 1.5). For instance, caution is needed in the interpretation of urinary (R)/(S)-acyl-glucuronide recoveries as indicators of stereoselective conjugate formation (Arima, 1990; Foster *et al.*, 1988a,b; Lee *et al.*, 1985). In these studies, not only may stereoselective systemic deconjugation be occurring but renal elimination of the glucuronides may be stereoselective as shown for the conjugates of carprofen (Iwakawa *et al.*, 1989).

Investigations of substrate enantioselectivity in formation of acyl-glucuronides have been performed *in vitro* with microsomes, solubilized microsomal protein and immobilized protein

obtained from animal and human liver as sources of UDP-glucuronosyl transferase isozymes. El Mouelhi *et al.* (1987) have described species dependent enantioselective formation of conjugates of naproxen, ibuprofen and benoxaprofen. Similar results for benoxaprofen glucuronidation have been reported by Spahn *et al.* (1989a). Preferential glucuronidation of the (R)-enantiomer of both 2-phenylpropanoic acid by rat liver microsomes (Fournel-Gigleux *et al.*, 1988) and fenoprofen by a sheep liver microsome preparation (Volland and Benet, 1991) have also been noted. In each of those studies potential interference from stereoselective deconjugation was minimized by rapid sample quenching. Such was not the case in an investigation of the glucuronidation of 2-phenylpropanoic acid by rat liver slices (Nakamura and Yamaguchi, 1987) where preferential formation of the glucuronide of the (S)-enantiomer was observed. However, these workers confirmed stereoselective deconjugation of the glucuronides was occurring (hydrolysis of the (R)-acyl-glucuronide was faster in their unstabilized incubation media) and accounted for this in their interpretation of the glucuronide formation data.

Enzyme inducer studies (Fournel-Gigleux *et al.*, 1988) have shown an unchanged (R)/(S) ratio of glucuronide formation for 2-phenylpropanoic acid in rats. This suggests that both enantiomers are possibly conjugated by the same form or by very closely related forms of UDP glucuronosyltransferase. Competitive enzyme inhibition studies with probenecid have shown a similar reduction in the glucuronidation of both enantiomers of naproxen (Spahn and Benet, 1987). This has possible implications for the interpretation of the effects of probenecid on the so-called "futile cycle" (Meffin *et al.*, 1983a,b). In addition to proposed competition between glucuronides of probenecid and clofibric acid for renal elimination (Meffin *et al.*, 1983b), probenecid might also reduce aglycone clearance by direct inhibition of UDP glucuronosyltransferase. Taken together, these mechanisms describing a reduced aglycone clearance (for drugs forming acyl-glucuronides) upon coadministration of probenecid, serve to illustrate the multifactorial nature of some pharmacokinetic drug interactions. *In vitro* studies with human liver enzymes showed inhibition of (S)-naproxen glucuronidation by its optical antipode and *vice versa* (El Mouelhi *et al.*, 1987). Thus enantiomeric interference (*i.e.* inhibition of conjugation of one optical isomer by the other) could be operative in metabolism of racemic 2-arylpropanoic acids, although this is yet to be demonstrated *in vivo*.

3.3 Intramolecular Rearrangement and Hydrolysis of Acyl-Glucuronides

Acyl-glucuronides are capable of undergoing a number of structural modifications when exposed to physiological conditions. These include hydrolysis to the parent aglycone, intramolecular rearrangements (isomerization via acyl migration) and intermolecular transacylation reactions. Transacylation (or other mechanism) reactions of acyl-glucuronides in protein solutions can lead to covalent binding of the drug moiety to protein yielding a drug-protein adduct and is discussed in Section 3.4.

Studies with endogenous bilirubin-IXα glucuronides collected from bile demonstrated a time-dependent conversion of the original biosynthetic 1-*O*-β-acyl-(D)-glucopyranosiduronate (glucuronide) to 2-, 3- and 4-*O*-acyl isomers of the metabolite which, unlike the biosynthetic material, were resistant to hydrolysis by β-glucuronidase (Compernolle *et al.*, 1978). Subsequently, studies with the acyl-glucuronides of a range of chiral and achiral xenobiotics including clofibric acid (Sinclair and Caldwell, 1982), zomepirac (Hasegawa *et al.*, 1982; Smith *et al.*, 1985), valproic acid (Dickinson *et al.*, 1984), oxaprozin (Ruelius *et al.*, 1986), diflunisal (Musson *et al.*, 1985; Hansen-Moller *et al.*, 1987), isoxepac (Illing and Wilson, 1981), furosemide (Rachmel *et al.*, 1985), tolmetin (Hyneck *et al.*, 1988; Munafo *et al.*, 1990) and probenecid (Eggers and Daust, 1981), have shown acyl migration to be a general phenomenon for these metabolites with the notable exception of salicylyl-glucuronide (Bradow *et al.*, 1989). The presence of an *ortho*-phenol group in salicylyl-glucuronide has been proposed to participate in the generation of a stable cyclic lactone (see Figure 3.2) thereby preventing further acyl migration (Bradow *et al.*, 1989). Studies of the isomerization of acyl-glucuronides have shown this process to be both time- and pH-dependent with the glucuronides stabilized by lowering pH to 3 (Faed, 1984). Moreover, large variation (see Table 3.1) has been noted in the degradation (principally rearrangement *in vitro*) from highly labile glucuronides like that of tolmetin (Munafo *et al.*, 1990) to more stable species like the glucuronide of flufenamic acid (Van Breeman *et al.*, 1986); the apparent first-order degradation half-life for the latter being approximately 30-fold greater under similar incubation conditions. *In situ* mechanistic studies

with ^{1}H-NMR spectroscopy of HPLC-purified isomers have determined the order of migration to be from the biosynthetic glucuronide to the C-2 position followed by formation of C-3 and C-4 esters (via an *ortho* ester intermediate) with no reformation of the high-energy 1-*O*-acyl bond (Bradow *et al.*, 1989). All other rearrangement steps were reversible with no evidence being found for rearrangements beyond nearest-neighbour hydroxyl groups in glucuronic acid (Bradow *et al.*, 1989). Like the biosynthetic glucuronide, the migration isomers can liberate aglycone by hydrolysis at physiological pH (Watt and Dickinson, 1990).

Table 3.1 Apparent first-order degradation half-lives of representative 1-*O*-β-acyl-glucuronides *in vitro* in protein-free phosphate buffer (various molarities) at physiological pH and temperature.

Aglycone	$t_{1/2}$ (h)	*Reference*
Carprofen	(R): 1.7, (S): 2.9	Iwakawa *et al.*, 1988
Clofibric acid	7.3	Gugler *et al.*, 1979
Diflunisal	1.0	Dickinson and King, 1989
Fenoprofen	(R): 1.0, (S): 2.0	Volland *et al.*, 1991
Flufenamic acid	7.0	Van Breeman *et al.*, 1986
Ibuprofen*	3.3	Castillo and Smith, 1991
Indomethacin	1.4	Van Breeman *et al.*, 1986
Oxaprozin	1.3	Ruelius *et al.*, 1985
Tolmetin	0.26	Munafo *et al.*, 1990
5-(2-(8-phenyloctyl)phenyl)-4,6--dithianonanedioic acid	2.2	Newton *et al.*, 1992

*Degradation $t_{1/2}$ determined for unresolved acyl-glucuronides

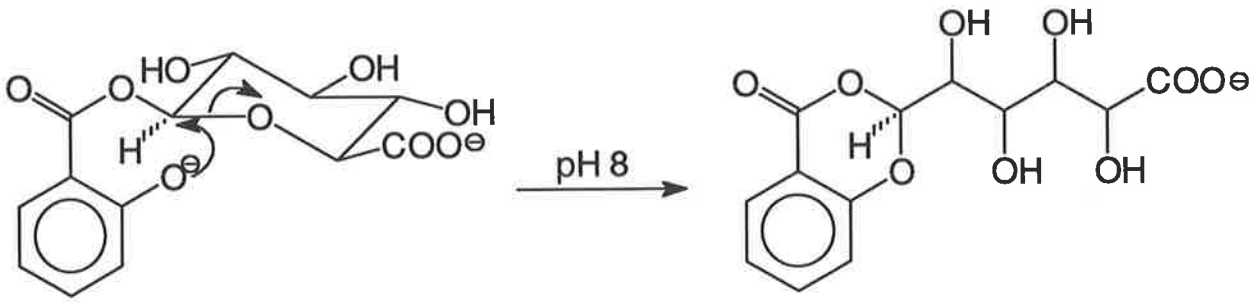


Figure 3.2 Structure of the stable isomer of salicylyl-glucuronide proposed by Bradow *et al.* (1989).

In addition to isomerization of acyl-glucuronides (the predominating reaction of these metabolites *in vitro*), *in vitro* studies with (for example) flurbiprofen (Knadler and Hall, 1991) tolmetin (Hyneck *et al.*, 1988), carprofen (Iwakawa *et al.*, 1988) and fenoprofen (Volland *et al.*, 1991) together with previously cited acyl migration studies, have shown the propensity for glucuronides and (presumptively, in some cases) their isomers to be readily hydrolysed to the parent aglycone at physiological pH and temperature. Interestingly, the presence of albumin or plasma in the incubation media can affect the rate of deconjugation of glucuronoconjugates. Studies with oxaprozin glucuronide demonstrated an esterase-like activity for human plasma and albumin with accelerated deconjugation observed in these media compared to protein-free incubations (Ruelius *et al.*, 1986). In contrast, the deconjugation of tolmetin glucuronide was slowed in the presence of human serum albumin, but catalysed by bovine serum albumin (Munafo *et al.*, 1990). While there have been relatively few *in vivo* studies which have examined rearrangement and hydrolysis of acyl-glucuronides, it appears that at least for diflunisal, animal studies have shown a predominance of hydrolysis over rearrangement (reversed for *in vitro* studies) for these metabolites (Watt *et al.*, 1991). Similar to acyl migration, the hydrolysis of glucuronides is inhibited in a cold acidic environment (Faed, 1984; Spahn-Langguth and Benet, 1992) although Dickinson *et al.* (1984) have noted acid hydrolysis of valproyl glucuronide at pH<2.

The combined processes of acyl migration and hydrolysis of glucuronides of chiral drugs have been shown to be stereoselective. Studies of the degradation of the (R)- and (S)-acyl-1-*O*-β-glucuronides of benoxaprofen (Spahn *et al.*, 1989a), carprofen (Iwakawa *et al.*, 1988), fenoprofen (Volland *et al.*, 1991) and flunoxaprofen (Spahn *et al.*, 1988) have shown the apparent first-order degradation half-life of the (S)-acyl-glucuronide to be approximately two-fold that of its corresponding (R)-acyl-glucuronide even though those incubations were carried out in media free of a chiral discriminator (such as a biological macromolecule like albumin). However, this is not surprising given the diastereoisomeric relationship between the respective glucuronides of the 2-arylpropanoic acid enantiomers. Knadler and Hall (1991) reported stereoselective hydrolysis of flurbiprofen glucuronoconjugates in a variety of incubation media at physiological pH and temperature. Notably, they found preferential hydrolysis of the conjugates of (S)-flurbiprofen in both plasma and albumin and very little conjugate hydrolysis in buffer alone. It was suggested that albumin may be a major source of hydrolytic activity in plasma (Knadler and Hall, 1991).

3.4 Generation of Covalent Drug-Protein Adducts

In addition to isomerization and hydrolysis reactions, the electrophilic character of acyl-glucuronides enables them to covalently bind to appropriate nucleophilic sites on biological macromolecules (Faed, 1984). While the irreversible binding of drugs to protein via their acyl-glucuronides is generally considered to be a quantitatively minor pathway compared to isomerization and hydrolysis, it has been proposed as a contributing mechanism for drug toxicity including hypersensitivity responses (Faed, 1984; Smith *et al.*, 1986; Spahn-Langguth and Benet, 1992).

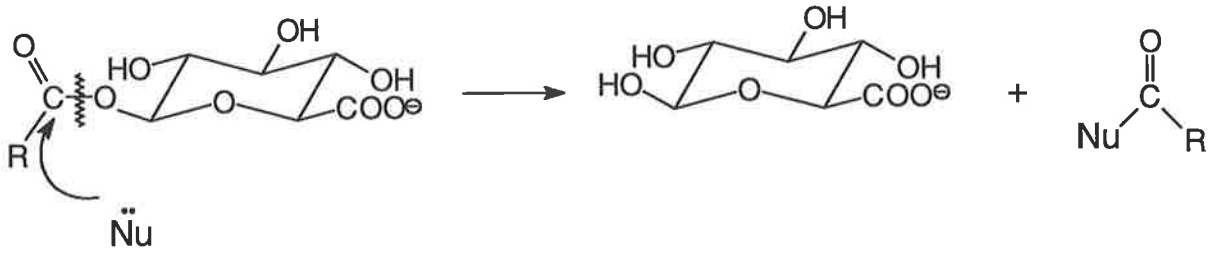
The irreversible binding of drugs to proteins via acyl-glucuronides has been reported for a range of drugs *in vitro* including clofibric acid, benoxaprofen, indomethacin and flufenamic acid (Van Breeman and Fenselau, 1985; Van Breeman *et al.*, 1986), oxaprozin (Ruelius *et al.*, 1986), tolmetin (Munafo *et al.*, (1990), zomepirac (Smith *et al.*, 1985), diflunisal (Watt and Dickinson, 1990), fenoprofen (Volland *et al.*, 1990) and etodolac (Smith *et al.*, 1992). *In vivo* studies reporting this phenomenon, have been performed with zomepirac (Smith *et al.*, 1986),

diflunisal and probenecid (McKinnon and Dickinson, 1989) and fenoprofen (Volland *et al.*, 1991).

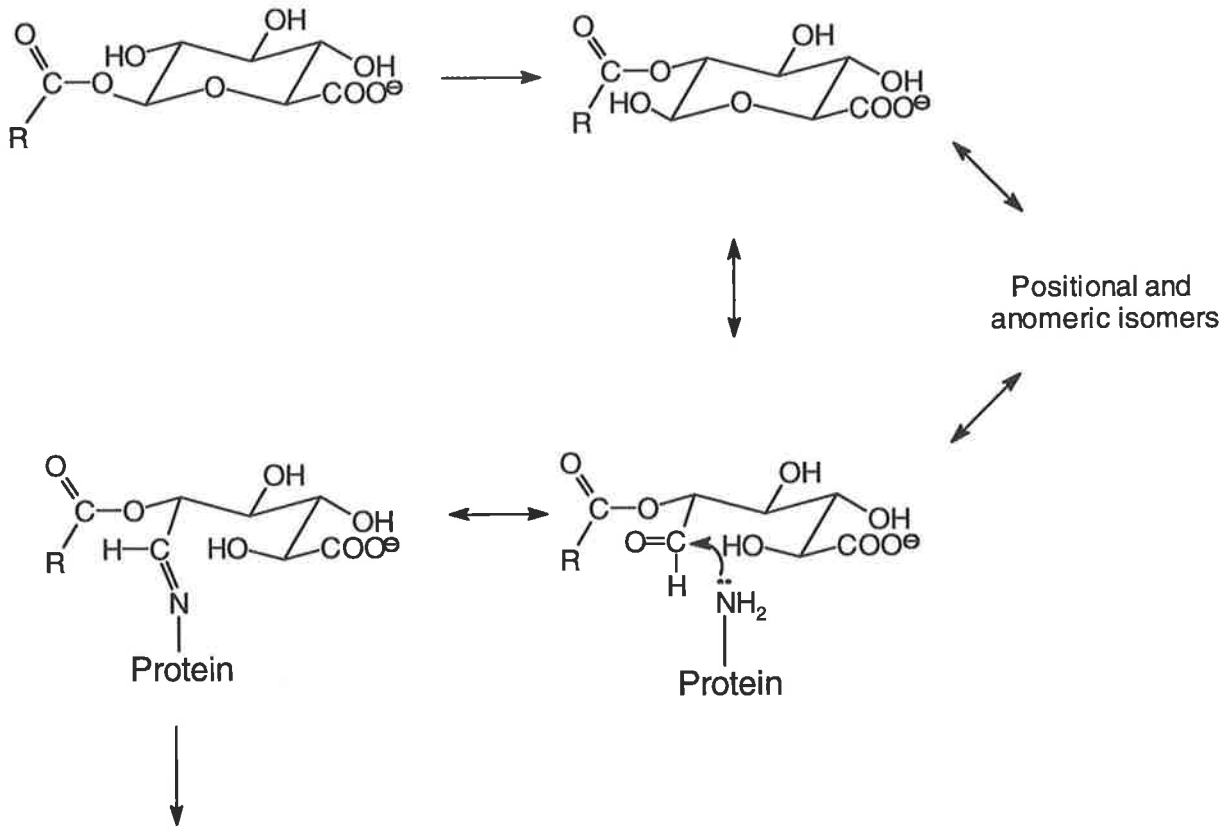
A general mechanism of formation of covalent drug-protein adducts has not been established, although two types have been proposed (see Figure 3.3). The first is a transacylation mechanism involving nucleophilic displacement of glucuronic acid from the ester group by macromolecular nucleophilic sites such as the thiol functionality of cysteine residues (Van Breeman and Fenselau, 1985), hydroxyl groups from tyrosine residues (Wells *et al.*, 1987) and primary amino groups of lysine residues (McDonagh *et al.*, 1984). The alternative mechanism is analogous to the nonenzymic glycosylation of albumin (Higgins and Bunn, 1981) and requires prior acyl migration allowing ring-opening of the sugar and subsequent generation of a stable ketoamine derivative (Smith *et al.*, 1986,1990). In the second proposed mechanism (the so-called "imine mechanism"), both the drug and glucuronic acid moieties (as the ester) bind to the macromolecule. Both mechanisms could operate together as suggested for tolmetin (Munafo *et al.*, 1990) although this issue is yet to be fully resolved.

There have been relatively few studies which have examined the potentially stereoselective nature of irreversible binding of drugs to protein via their acyl-glucuronide metabolites. Volland *et al.* (1991) described significantly more irreversible binding for the (R)-enantiomer of fenoprofen *in vitro* however, *in vivo* this stereoselectivity was reversed. This provides a clear example of competing enantioselective metabolism since fenoprofen is subject to significant chiral inversion (Hayball and Meffin, 1987; Rubin *et al.*, 1985) which will increase the exposure to (S)-fenoprofen (and subsequently its acyl-glucuronide) relative to its optical antipode *in vivo.* It has been suggested that relative irreversible binding of fenoprofen enantiomers *in vivo* is a function of the area under the respective plasma glucuronide concentration-time profile (Volland *et al.*, 1991). Investigations with the glucuronides of carprofen enantiomers *in vitro* have shown a higher extent of covalent linkage for the (S)-enantiomer after 1 hour of incubation (physiological pH and temperature) but after 24 hours the binding of the (R)-enantiomer predominated (Iwakawa *et al.*, 1988). Clearly, competing stereoselective isomerization and hydrolysis pathways for glucuronides together with possible

stereoselectivity in adduct stability necessitate caution in the mechanistic interpretation of such differences.



(**A**) Nucleophilic displacement mechanism, where Nu represents a nucleophilic group, within the macromolecule such as NH, O or S and leads to the generation of an acylated protein and liberated glucuronic acid;



(**B**) Imine mechanism, where the sugar moiety acts as a bridge between the acyl residue and the macromolecule (indicated by "Protein" in the above scheme) and becomes part of the adduct structure;

Figure 3.3 Postulated mechanisms for the irreversible binding of carboxylic acids to proteins via their acyl-glucuronides: (**A**) the nucleophilic displacement mechanism, leading to an acylated protein and liberation of (D)-glucuronic acid, and (**B**) the imine mechanism, whereby glucuronic acid acts as a bridge between the aglycone residue and the macromolecule.

3.5 Clinical Implications of Reversible Acyl-Glucuronidation

An important focus of the experimental work described in later chapters of this thesis is the physiologically labile character of acyl-glucuronides (specifically relating to those of ketoprofen enantiomers). It is evident from dispositional studies of drugs undergoing extensive acyl-glucuronidation that although renal elimination of unchanged drug may be a minor pathway, there may be reduced clearances observed for such compounds in patients with renal dysfunction and in elderly patients in whom renal function would be expected to be impaired. In addition, reduced clearances of such drugs have been demonstrated in patients coadministered probenecid. While these are unexpected observations on the grounds of minimal kidney involvement in the excretion of parent aglycone, they are consistent with the so-called "futile cycle" hypothesis (Meffin, 1985). In such a cycle, the net clearance of a drug depends on conjugation to an acyl-glucuronide and competition between renal clearance of the glucuronide and its clearance by hydrolysis back to the parent drug. Following concomitant probenecid administration or in patients with impaired renal function, the renal clearance of the glucuronide is diminished and net drug clearance falls.

Since most congeners of the chiral 2-arylpropanoic acid NSAIDs are both marketed as racemic mixtures and are extensively metabolized to acyl-glucuronides, the regeneration of parent aglycone will have unique stereochemical implications in light of the coincident metabolic chiral inversion pathway (see Sections 1.4.3 and 2.3.3). Meffin *et al.* (1986) have described such a mechanism in a renal-failure animal model. In this case, both enantiomers undergo acid-glucuronide futile cycles and the fraction of the (R)-enantiomer undergoing chiral inversion increases (compared to renally healthy controls), thereby increasing the plasma concentration of the pharmacologically active (S)-enantiomer upon racemic drug administration. Indeed, the combined effects of an increased fractional (R)- to (S)- inversion (due to the (R)-enantiomer futile cycle) and the (S)-enantiomer futile cycle in operation, may together greatly increase the exposure to total (bound plus unbound) (S)-arylpropanoate (Meffin *et al.*, 1986). Such a hypothesis is somewhat complicated however, by renal dysfunction-induced changes to enantiomer plasma protein binding (Jones *et al.*, 1986) and the possibility that the kidney may

be involved in the inversion process (Yamaguchi and Nakamura, 1987) [and hence the inversion clearance may be diminished with impaired renal function]. However, the net effect of reduced renal elimination of acyl-glucuronides should be an increased exposure to the parent aglycone (see Figure 3.4). Moreover, this phenomenon (although not specifically tested) may be accentuated during chronic drug administration (Upton *et al.*, 1984).

More recently, it appears that such a cycle of reversible acyl-glucuronidation, rather than being "futile", may lead to immunotoxicological responses via the generation of covalent drug-protein adducts (see Spahn-Langguth and Benet, 1992; for a comprehensive review of this topic). Indeed, increased exposure to acyl-glucuronides as a result of their reduced renal elimination, might possibly lead to higher systemic levels of drug-protein adducts. The reactivity of acyl-glucuronides under physiological conditions is generally accepted. However, the toxicological significance of these reactions, particularly the generation of covalent linkages between drug and macromolecule, is speculative at present. A recent study of the acyl-glucuronide metabolite of the broad spectrum investigational anti-tumour agent flavone-8-acetic acid demonstrated the propensity for the glucuronide to undergo transacylation reactions *in vitro* (Chabot and Gouyett, 1991). These workers have speculated that this novel cytotoxic drug (Corbett *et al.*, 1986) may exert at least part of its anti-tumour effect by possibly binding to nucleic acids and/or the immunomodulator nitric oxide, by virtue of the electrophilic character of the acyl-glucuronide.

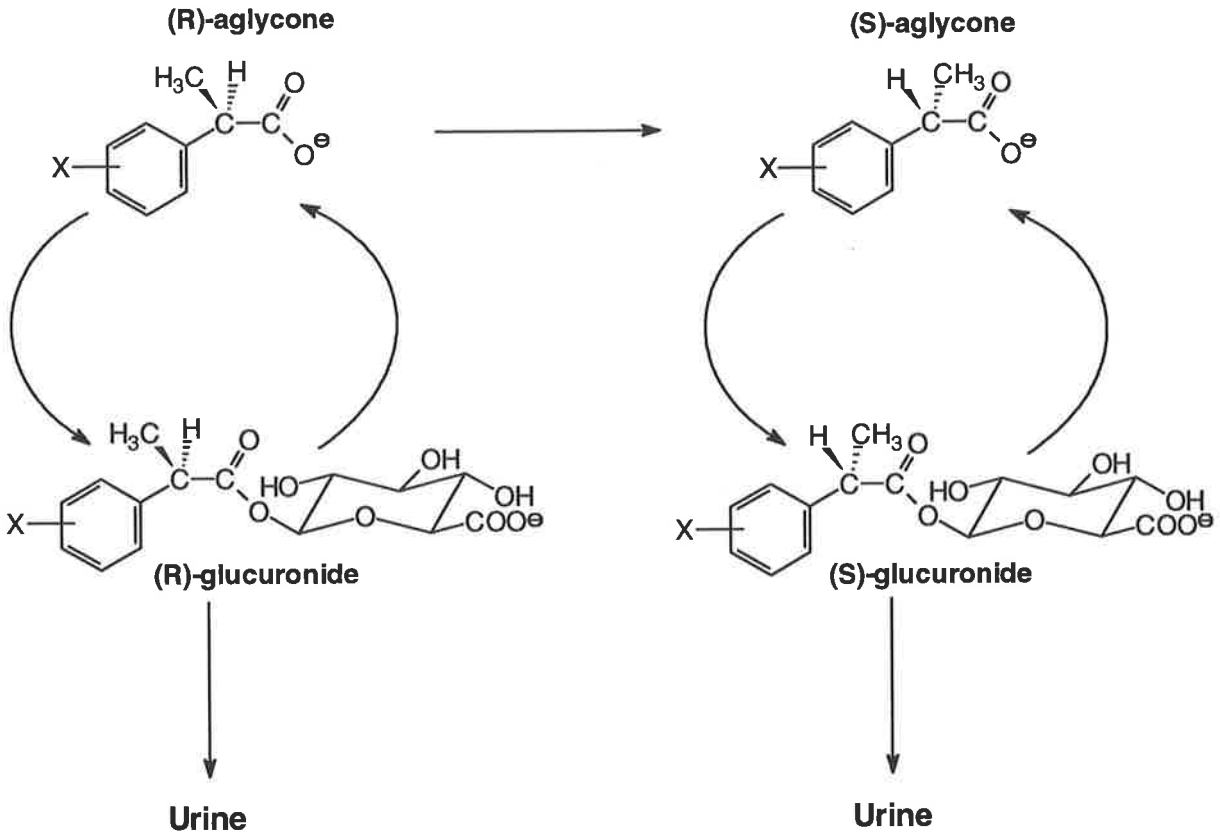


Figure 3.4 The reversible cycle proposed by Meffin *et al.* (1986) for the formation, hydrolysis and renal clearance of acyl-glucuronides of 2-arylpropanoic acid NSAIDs which undergo metabolic chiral inversion. Not shown in this simplified model are: (*i*) additional biotransformation pathways for both aglycones and glucuronides and (*ii*) regeneration of aglycone from rearranged isomers of biosynthetic glucuronides and to a lesser extent (quantitatively) from irreversibly bound drug-protein adduct.

As discussed for ketoprofen in Section 2.3.3, if biological samples containing acyl-linked conjugate metabolites are not rapidly quenched (pH and temperature adjusted), potentially misleading aglycone concentration data may be generated. The facile deconjugation of these metabolites in unstabilized plasma, urine or bile may lead to an overestimation of the concentration of nonconjugated (parent) drug. This has been highlighted by Upton *et al.* (1980) who demonstrated that the true renal elimination of unchanged ketoprofen, naproxen and probenecid was negligible and indeed even the low levels of unchanged drugs measured in urine was probably an overestimation due to possible metabolite deconjugation taking place in

the bladder. In contrast, previous studies with, for example ketoprofen (Delbarre *et al.*, 1976), had incorrectly estimated the urinary excretion of unchanged drug to be approximately 50% of the administered dose due to inadvertent metabolite deconjugation during urine sample collection, storage and analysis.

It is likely that in isolation, the intramolecular rearrangement reactions of acyl-linked glucuronides will assume little clinical significance, apart from indicating the inherent reactivity of the conjugates (Faed, 1984). Indeed, animal studies with diflunisal acyl-glucuronide have demonstrated the quantitative importance of the hydrolysis pathway over the glucuronide isomerization process *in vivo* (Watt *et al.*, 1991), the reverse order of importance observed with comparative *in vitro* studies (Watt and Dickinson, 1990). However, acyl migration has major practical implications since the glucuronide isomers (unlike the biosynthetic material) are resistant to hydrolysis by β-glucuronidase (Blanckaert *et al.*, 1978). The reliance on β-glucuronidase for the cleavage of glucuronoconjugates (for instance, in quantification of urinary acyl-linked conjugates in drug-metabolite analysis) would result in an underestimation of the true contribution of this metabolic process for those drugs whose conjugates undergo isomerization. Similarly, the use of urinary metabolite data for estimating oral bioavailability of such drugs may be in error if hydrolysis of conjugates was performed by β-glucuronidase alone. It is recommended that mild alkali be used to account for aglycone emanating from isomerization conjugates plus biosynthetic glucuronide, which together are cleaved under these conditions (Faed, 1984). In addition, when performing enantioselective studies with chiral drugs metabolized to acyl-linked glucuronides, the theoretical possibility of base-catalyzed racemization of the aglycone should be addressed.

In conclusion, the facile degradation of acyl-glucuronides under physiological conditions necessitates caution in the interpretation of rates of formation of this important biotransformation product (see Section 3.3) and of urinary/biliary metabolite recovery data for drugs subject to significant acyl-glucuronidation (see Section 2.3.3). Unless biological samples containing acyl-glucuronides are rapidly stabilized by temperature and pH control, potentially misleading dispositional data may be generated. In addition, it is likely that for chiral drugs which are predominantly metabolized to acyl-glucuronides, stereoselective reactivity of these

conjugates needs to be considered since this may contribute to the overall enantioselectivity of the pharmacokinetic profile of the parent drug. Acyl-glucuronides, rather than being necessarily elimination metabolites of no pharmacological significance, need to be considered as potential systemic sources of deconjugated parent aglycone and possibly toxic intermediaries, particularly in situations where they may accumulate systemically.

Chapter 4

Enantioselective Analysis of Ketoprofen in Plasma by High-Performance Liquid Chromatography

4.1 Introduction

4.1.1 Analysis of Enantiomers in Biological Matrices

The requirement for investigations of the stereochemical basis of drug disposition and pharmacodynamics has become self-evident (see Sections 1.5 and 1.6). Indeed for enantiomers of a chiral drug possessing enantioselective pharmacological properties which is administered as a racemate, potentially misleading drug disposition and concentration-effect data may be generated if nonenantioselective analytical methods are used (Ariens, 1984; Evans *et al.*, 1988). While achiral methods of analysis are capable of describing the behaviour of individual enantiomers when they are administered as separate enantiomers or when only one enantiomer is present in the matrix of interest, this approach has limitations. In the case of a drug marketed for clinical use as the racemate, considerable practical difficulties may arise when individual enantiomers are sought for use in human drug studies. In addition, studies involving the separate administration of pure enantiomers provide no information on the effect of one enantiomer upon the pharmacokinetic and pharmacodynamic properties of its optical antipode when in clinical practice the drug is marketed as a racemate. Moreover, in the case of the 2-arylpropanoic acid NSAIDs, metabolic chiral inversion may take place (see Sections 1.6.3 and 2.3.3). Thus chiral methods of analysis need to be employed for drugs administered as racemates and for metabolite studies of prochiral compounds.

The discrimination of enantiomers requires the interaction of the chiral drug with another chiral agent, generating a diastereomeric (and hence physicochemically distinct) interaction. This diastereomeric "complex" may be reversible in the case of: (*i*) a chiral stationary phase in a high-performance liquid chromatographic (HPLC) or gas chromatographic (GC) column, (*ii*) a chiral component of a HPLC mobile phase or (*iii*) a chiral radioimmunoassay method relying

on the enantiodiscriminatory property of a particular antibody preparation. Alternatively, an irreversible (covalent) complex may be formed by chemical derivatization of the chiral drug with a chiral reagent followed by achiral chromatography of the generated diastereomers. The various methods of performing enantioselective assays as they pertain to clinical pharmacology are briefly discussed below.

Pseudoracemates

The application of pseudoracemates for enantiomeric analysis is one method which does not require use of a secondary chiral discriminatory agent. A pseudoracemate is a 1:1 mixture of enantiomers, one of which is the natural enantiomer whilst in the other one or more atoms have been isotopically labelled. The labelled enantiomer is not a true enantiomer (or mirror image reflection) of its nonlabelled optical antipode, but rather differs in physical properties, notably molecular weight. Following administration of a pseudoracemic drug, parent drug and metabolites in biological matrices are examined by conventional achiral analytical methods and the enantiomers are differentiated by mass spectroscopy (in the case of stable isotopes) or by radiochemical techniques. Caution is needed in the positioning of the labelled atom within the drug molecule which must be in a metabolically stable position. In addition, there should be no physiological isotope-effect (Hutt and Caldwell, 1988).

O'Reilly and coworkers (1980) used a [^{12}C]/[^{13}C] pseudoracemate of warfarin in man to elucidate the mechanism responsible for the augmented hypoprothrombinaemia of warfarin caused by the coadministration of the NSAID phenylbutazone. This example illustrates the importance of using enantioselective analytical methods when interpreting the disposition of a racemic drug and the effect thereon of a coadministered drug. Although unresolved warfarin plasma concentrations decreased during phenylbutazone treatment, an enhanced pharmacodynamic response to warfarin was observed. Enantioselective drug analysis revealed that phenylbutazone induced a significant increase in the plasma clearance of the less potent (in terms of hypoprothrombinaemic effect) (R)-enantiomer of warfarin while at the same time decreasing the clearance of its more potent optical antipode. Thus phenylbutazone augmented the clinical action of warfarin by inhibiting the metabolism of the more pharmacologically

potent (S)-warfarin, yet reduced combined (R)- plus (S)-warfarin plasma concentrations by greatly amplifying the plasma clearance of (R)-warfarin.

More recently, Mast *et al.* (1992) described the disposition, in humans, of the enantiomers of the 1,4-dihydropyridine calcium antagonist nitrendipine upon administration of: (*i*) the separate enantiomers of the drug and (*ii*) pseudoracemic ([$^{13}C_4$]-(R)-) nitrendipine. In addition to observing substantial enantioselectivity in the metabolism, bioavailability and disposition of nitrendipine upon administration of separate enantiomers of the drug, comparative studies with pseudoracemic drug showed that the (S)-enantiomer inhibited the first-pass metabolism of its optical antipode (Mast *et al.*, 1992). Moreover, in this study, the use of pseudoracemic nitrendipine enabled elucidation of the metabolic steps (proposed to be initial esterolysis followed by heterocyclic ring oxidation) responsible for the enantioselective first-pass biotransformation of the drug.

Enantioselective immunoassay

Enantioselective immunoassays exploit the inherent chirality of antibodies. Caution is needed in the production of such antisera, making sure that the stereochemical purity of the drug used to make the immunogen is sufficiently high. Even a relatively minor amount of optical impurity in the antigen stimulus may result in disproportionately greater cross-reactivity of the antibody preparation towards the undesired enantiomer (Cook, 1988). Cross-reactivity with metabolites may also result when the site of molecular modification by metabolism is sufficiently distal from the chiral centre (Cook, 1988). The technique has been adopted in enantioselective drug analyses of a variety of chiral drugs including chiral barbiturates, warfarin and methadone (Cook, 1988).

Chiral chromatography

Chiral chromatography has provided the most significant advances in the analysis of separate enantiomers when they are present together in a biological sample. Chromatographic resolution of optical isomers is performed by either derivatizing chiral drugs with chiral reagents to yield covalent diastereomeric compounds which are subsequently separated on achiral columns (by virtue of their physicochemically distinct properties) or alternatively, chiral

drugs may form transient diastereoisomeric interactions with either a chiral stationary phase or, in the case of HPLC, with a chiral component of the mobile phase.

The so-called "indirect" approach of chemically derivatizing a chiral drug to form a stable diastereomer has a number of advantages including the commercial availability of a wide range of chiral derivatizing agents (Srinivas and Igwemezie, 1992) and the ease with which most diastereomers may be separated on inexpensive and versatile achiral stationary phases. Moreover, chiral derivatizing agents may confer favourable detection properties on the drug of interest, for example by introducing a chromophore or fluorophore into the diastereomeric structure. Disadvantages of the indirect method include: (*i*) potential racemization of analyte or reagent during the derivatization reaction, (*ii*) time and expense incurred by performing the derivatization reaction, (*iii*) optical contamination of the chiral derivatizing agent, (*iv*) the requirement for the analyte to possess (or be readily generated) an appropriate functional "handle" to which is attached the derivatizing agent and (*v*) potential enantioselectivity in the derivatization reaction.

In the direct method of enantioselective drug analysis, many of the disadvantages outlined above for covalent diastereomeric generation are not of concern. Should derivatization be required for resolution of a chiral drug on a chiral stationary phase, this may be carried out with an achiral reagent. For example, the HPLC resolution of a range of 2-arylpropanoic acid NSAIDs has been achieved following reaction of the acid chlorides of ibuprofen, naproxen and fenoprofen with 3,5-dinitroaniline followed by chromatography of the corresponding enantiomeric benzoylamides on a commercially available terminal chiral-substituted naphthylurea stationary phase (Kakodkar and Zief, 1990). A large range of chiral stationary phases are available, some based on molecules such as β-cyclodextrin, bovine or human serum albumin and α_1-acid glycoprotein (see Section 2.3.2 for an interesting association between enantioselective protein binding and protein-based chiral stationary phase optical resolution) and other Pirkle-type phases which rely on π–π, dipole–dipole and other intermolecular diastereomeric interactions (Hermansson and Eriksson, 1986; Pirkle *et al.*, 1980; Wainer and Doyle, 1984). Some of the constraints on the use of such phases is their relative expense compared to achiral chromatography columns and problems with column life and peak

interference posed by analysis of drugs in biological matrices. Combination achiral/chiral HPLC systems have been used for direct analysis of chiral drugs in plasma and serum; for example, in the analysis of warfarin (McAleer *et al.*, 1992) and ketoprofen (Yagi *et al.*, 1990; see Section 4.1.2).

Chiral mobile phase additives for the direct analysis of enantiomers may be counter-ions such as quinine, cinchonine and camphorsulphonic acid enantiomers or alternatively, molecules including β-cyclodextrin and albumin have been used (Hutt and Caldwell, 1988; Lindner and Pettersson, 1985). However, this method appears to have limited application for chiral drug analysis in biological samples.

4.1.2 Enantioselective Analysis of Ketoprofen

Literature reports of methods for the enantiomeric assay of ketoprofen in biological samples are detailed in Table 4.1. With the exception of the report of Yagi and coworkers (1990), these enantioselective assays incorporate indirect chromatographic techniques via generation of diastereomeric amides. The single report of direct chromatographic analysis of ketoprofen enantiomers described a column-switching HPLC system with in-line deproteinization of plasma samples on a pretreatment column followed by resolution of drug on a chiral HPLC column (Yagi *et al.*, 1990).

Table 4.1 Reports of chromatographic methods for the enantiomeric assay of ketoprofen in biological samples.

Chiral Derivatizing Agent	*Chromatographic Type*	*Reference*
(R)-1-Phenylethylamine	Normal-phase HPLC	Sallustio *et al.*, 1986
(L)-Leucinamide	Reversed-phase HPLC	Bjorkman, 1987
(L)-Leucinamide	Reversed-phase HPLC	Foster and Jamali, 1987
(L)-Leucinamide	Reversed-phase HPLC	Palylyk and Jamali, 1991
(D)-Amphetamine	GC-MS	Jack *et al.*, 1992
	Chiral (stationary phase) HPLC	Yagi *et al.*, 1990

The method of Sallustio *et al.* (1986) involved extensive sample processing and required the availability of a twin HPLC system. Reversed-phase chromatography of extracted plasma samples (containing internal standard) was used to both quantify and fractionate unresolved ketoprofen in the original plasma aliquot. This was followed by normal-phase HPLC of appropriately extracted and derivatized eluates from the initial reversed-phase chromatographic step in order to calculate the relative fraction of each enantiomer in the sample of interest. Of the other methods described in the literature at the time of commencing the studies pertaining to this thesis, the analyses developed by Bjorkman (1987) and Foster and Jamali (1987) employed simpler sample processing than the method of Sallustio *et al.* (1986), with single-step reversed-phase chromatography allowing quantification of ketoprofen enantiomers in human plasma without prior chromatographic isolation of drug. However, chromatographic run times for single samples were approximately 20 min for each of these methods (Bjorkman, 1987; Foster and Jamali, 1987). Moreover, neither of these later methods checked for potential racemization during the derivatization reaction.

In summary, while the method of Sallustio *et al.* (1986) described resolution of derivatized (R)-1-phenylethylamides of (R)-and (S)-ketoprofen within approximately 4 min using normal-phase conditions, protracted sample processing and the requirement for either two HPLC systems (one reversed-phase, the other normal-phase) or frequent switching of phases on a single HPLC system was deemed to be less than ideal for multi-sample analyses. Since there was also a requirement for isolation of separate (R)- and (S)-ketoprofen HPLC eluates for the purpose of conducting the plasma protein binding studies (see Chapter 5), in addition to performing total (bound plus unbound) drug enantiomer analyses, the longer retention times of the methods of Bjorkman (1987) and Foster and Jamali (1987) posed difficulties for HPLC eluent fractionation of large numbers of samples within a practical time-frame. A new method was developed which involved simple extraction of unresolved ketoprofen together with internal standard ((S)-naproxen) from acidified plasma, followed by rapid normal-phase HPLC of the diastereomeric (S)-1-phenylethylamides of ketoprofen enantiomers. Single chromatographic run times of less than 8 min were achieved which enabled large numbers of samples to be analysed within a convenient time frame which was particularly useful when the

plasma protein binding of ketoprofen enantiomers was being assessed (see Section 5.2). The analytical method proved to be sensitive, specific and reproducible and the derivatization conditions did not lead to sample racemization.

4.2 Materials and Methods

Reagents and Chemicals

Racemic ketoprofen [(RS)-ketoprofen] was purchased from Sigma Chemical Company (St Louis, U.S.A.). The (R)- and (S)-enantiomers of ketoprofen were gifts of Dr Kathy Knights (Flinders University, Adelaide, South Australia) and Drs David Hamon and Ralph Massy-Westropp (University of Adelaide, Adelaide, South Australia) and (S)-naproxen (internal standard) was a gift of F. H. Faulding (Adelaide, Australia). (RS)-[1-^{14}C]-Ketoprofen (specific activity 8.70 μCi/μmol) was synthesized and authenticated by Ms Josie Newton under the supervision of Drs David Hamon and Ralph Massy-Westropp and was radiochemically purified by reversed-phase achiral HPLC prior to use (see Section 5.2). HPLC-Grade *n*-heptane, acetonitrile, sodium acetate, isopropyl alcohol and methanol were purchased from BDH Chemicals (Poole, England) as were analytical-grade sulphuric acid, *n*-hexane, ethyl acetate and dichloromethane for sample extraction. Thionyl chloride (redistilled successively, over quinoline and boiled linseed oil within 4 weeks of use) was purchased from May and Baker (Dagenham, England), sodium hydroxide from Ajax Chemicals (Sydney, Australia) and (S)-1-phenylethylamine was obtained from Sigma Chemical Company (St Louis, U.S.A.). (RS)-Fenoprofen calcium was obtained from Eli Lilly and Co. (Indianapolis, U.S.A.), (RS)-ibuprofen was a kind gift of Boots Co. (Nottingham, U.K.), mefenamic acid was obtained from Parke Davis (Sydney, Australia) and salicylic acid from F. H. Faulding (Adelaide, Australia).

Instrumentation and chromatographic conditions

The HPLC system consisted of a Model 510 pump, Wisp® autoinjector, Model 490 variable wavelength UV-absorbance detector and Model 840 Data Station, all from Waters Associates (Milford, U.S.A.). An SGE (Sydney, Australia) glass-lined HPLC column (250 mm x 4 mm internal diameter) containing 5 μm silica was operated at ambient temperature (22°C) through which the mobile phase (8% isopropyl alcohol in *n*-heptane) was pumped at 1.0 ml/min. This

solvent was filtered (0.22 μm) and degassed immediately prior to use and an in-line 2 μm filter (Waters Assoc.) was positioned ahead of the column. The column was monitored for UV-absorbance at a detection wavelength of 254 nm.

Sample preparation and derivatization

In a culture tube (100 mm x 16 mm) equipped with a PTFE-lined screw cap was added 1.00 ml of plasma, 0.05 ml of internal standard solution [(S)-naproxen, 200 μg/ml in methanol], 0.5 ml of 2.0 *M* sulphuric acid and 8 ml of extracting solvent (10% ethyl acetate in *n*-hexane). Each sample was mixed gently for 10 min on a rotary mixer (30 rpm) and then centrifuged for 10 min (1500 *g*). The organic layer was transferred to a fresh culture tube and evaporated to dryness at 45°C under a stream of purified nitrogen (TurboVap™, Zymark, Massachusetts, USA). The dried residue was reconstituted with 0.1 ml of 1.5% thionyl chloride in *n*-hexane (freshly prepared), vortex-mixed and the tube firmly capped and heated for 1 h at 75°C in a dry heat bath.

The sample was subsequently allowed to cool to room temperature before adding 0.5 ml of 2% (S)-1-phenylethylamine in dichloromethane (freshly prepared), the contents vortex-mixed and the tube recapped and left to stand at room temperature for a further 15 min. Final extraction of the lipophilic amides was accomplished by the addition of 0.5 ml of 2.0 *M* sulphuric acid and 5 ml of *n*-hexane. Mixing, centrifugation and evaporation of the organic layer were as described above for the initial extraction step. The dried residue was reconstituted with 0.25 ml of mobile phase and 0.2 ml was injected onto the HPLC column.

Calibration, precision and accuracy

Racemic ketoprofen plasma standards were prepared by adding 1.00 ml of drug-free plasma to culture tubes containing dried methanolic extracts of ketoprofen such that the final concentration range of 0.156 μg/ml to 10.00 μg/ml of each enantiomer was achieved. These standards were taken through the sample preparation and derivatization methods described above. Separate calibration curves for each ketoprofen enantiomer were constructed as the peak-area ratios of the diastereomers to the internal standard diastereomer and least-squares linear regression analysis was performed to determine slopes, *y*-intercepts and regression

coefficients. The concentration-normalised peak-area ratios for each enantiomeric ketoprofen standard over the concentration range was also calculated for each set of standards. The accuracy and precision of the method were assessed by preparing methanolic solutions of racemic ketoprofen from weighings independent of those used for preparing the calibration standards. Addition of drug-free plasma to dried methanolic extracts yielded ketoprofen enantiomeric spike concentrations in plasma of 0.200 µg/ml and 9.00 µg/ml. Aliquots (1.00 ml) of these quality control plasma samples were analysed to determine intra-day accuracy and precision, and inter-day accuracy and precision of the assay (over an 8 week period). Routinely, quality control specimens (containing 0.200 µg/ml and 9.00 µg/ml of each enantiomer as (RS)-ketoprofen) were included with each batch of unknown samples analyzed. The results from assay runs were rejected if the measured enantiomeric concentrations of the lower and upper quality control plasma samples differed from the spike concentrations by more than 15% and 10%, respectively.

Extraction efficiency

In order to optimize and quantify the initial extraction of unresolved ketoprofen enantiomers and internal standard from plasma a reversed-phase nonstereoselective HPLC method was used (see Section 5.2). Briefly, this achiral method involved simple extraction of ketoprofen and internal standard from acidified plasma and chromatography on a radially compressed (model RCM-100) phenyl cartridge (Rad-Pak®, 4 µm particle size, 100 mm x 8 mm internal diameter, Waters Assoc.) through which the mobile phase (50% acetonitrile in 10 m*M* acetate buffer, pH3) was pumped at 2.0 ml/min (UV-wavelength detection at 260 nm). Instrumentation was identical to that described above for the enantioselective ketoprofen assay.

The peak areas after injection of unresolved ketoprofen and internal standard extracted from plasma were compared to those generated from direct injections of aqueous solutions of these compounds. Extraction efficiency was assessed at the upper and lower ends of the calibration range (18.0 µg/ml and 0.400 µg/ml of unresolved racemic ketoprofen) and of the internal standard.

Derivatization efficiency

A methanolic aliquot of radiochemically pure ketoprofen [(RS)-[1-^{14}C]-ketoprofen, specific activity = 8.70 μCi/μmol] was added to drug-free plasma to achieve an enantiomeric concentration of 1.25 μg/ml and taken through the enantioselective assay extraction and derivatization steps. The HPLC eluates corresponding to the (R)-[1-^{14}C]- and (S)-[1-^{14}C]-ketoprofen diastereomeric amide peaks were collected in glass tubes, evaporated to dryness under nitrogen (45°C), reconstituted and counted in a xylene-based liquid scintillant (PCS II, Amersham, Buckinghamshire, England). Counting was carried out in a Nuclear-Chicago (U.S.A) liquid scintillation system (model Unilux III) and quench correction was performed with the internal standard method ([^{14}C]-toluene, Amersham). These counts were compared with counts from pure radiolabelled (RS)-ketoprofen. The combined extraction and derivatization efficiency for ketoprofen could thus be determined together with a measure of potential chiral discrimination during the derivatization reaction between enantiomeric ketoprofen acyl-chlorides and (S)-1-phenylethylamine.

Assigning absolute configuration

Each authentic pure enantiomer of ketoprofen (unlabelled) was taken through the complete sample preparation method to establish its retention time. Potential racemization (during sample preparation) was also checked by this method.

4.3 Results and Discussion

Chromatograms resulting from the analysis of: (*i*) drug-free plasma, (*ii*) drug-free plasma spiked with (RS)-ketoprofen and internal standard and (*iii*) plasma containing an unequal concentration of ketoprofen enantiomers together with internal standard are depicted in Figure 4.1. No interfering endogenous plasma peaks were observed. Under the chromatographic conditions described for the enantioselective assay, the retention times for the (R)- and (S)-ketoprofen diastereomeric amides were 5.2 and 7.2 min, respectively. (S)-Naproxen-(S)-1-phenylethylamide conveniently eluted between the ketoprofen peaks at 6.1 min.

Calibration curves generated over the enantiomeric concentration range from 0.156 μg/ml to 10.00 μg/ml (supplied as racemic drug) were linear for both enantiomers of ketoprofen. Linear least-squares regression analysis for twelve calibration curves is depicted in Table 4.2. In every case the correlation coefficient was > 0.999.

The intra-day accuracy and precision of the assay was assessed by analysing 6 separate aliquots of two independently prepared quality control plasma samples covering the upper and lower limits of the ketoprofen enantiomeric concentration range. For the first quality control sample containing 9.00 μg/ml of each enantiomer, the mean concentration of (R)-ketoprofen and (S)-ketoprofen were determined to be 9.25 μg/ml (CV = 3.81%) and 9.08 μg/ml (CV = 2.96%), respectively. The second sample contained 0.200 μg/ml of (R)-ketoprofen and (S)-ketoprofen. The concentrations determined were 0.217 μg/ml (CV = 7.92%) and 0.193 μg/ml (CV = 8.90%), respectively.

The inter-day accuracy and precision were determined by analysing the quality control plasma samples containing 9.00 μg/ml and 0.200 μg/ml of each enantiomer (added as the racemate) over an eight week period. The mean (n = 12) concentrations of (R)- and (S)-ketoprofen for the lower plasma concentration samples were 0.191 μg/ml (CV = 4.70%) and 0.196 μg/ml (CV = 4.21%), respectively. The accuracy (and precision) values (n = 12) for the 9.00 μg/ml plasma samples were 9.24 μg/ml (CV = 3.90%) and 9.36 μg/ml (CV = 5.00%) for the (R)- and (S)-enantiomers of ketoprofen, respectively.

The mean (± SD) extraction efficiency of 10% ethyl acetate in *n*-hexane/sulphuric acid (*vide supra*) was assessed (n = 6) for 18.0 μg/ml of racemic ketoprofen in plasma and found to be 83.9 (± 1.4)% relative to an unextracted aqueous aliquot of ketoprofen utilizing the nonstereoselective assay described above. The efficiency of extraction (n = 6) for 0.400 μg/ml of (RS)-ketoprofen was 86.5 (± 3.3)%. The respective value (n = 12) for the internal standard [(S)-naproxen] was 95.5 (± 2.9)%.

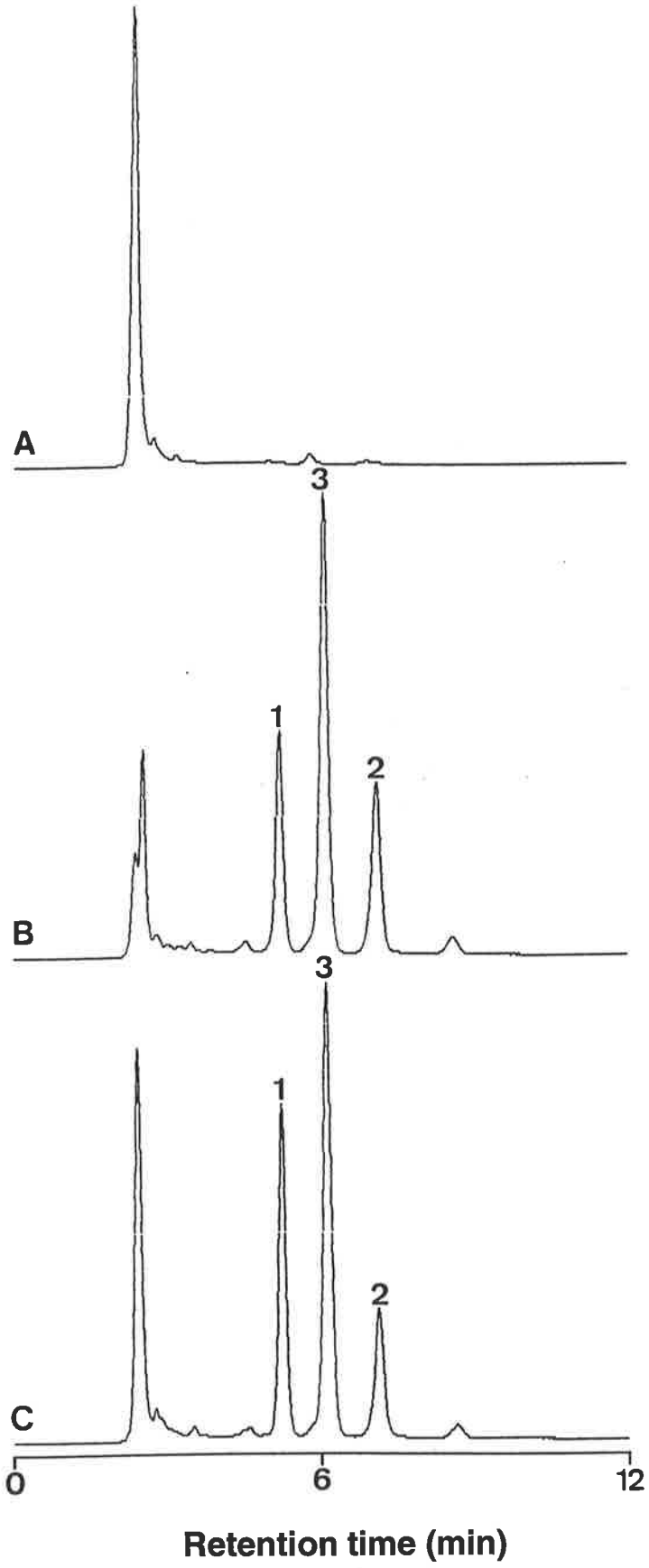


Figure 4.1 Chromatograms of derivatized samples. (**A**) Extract of drug-free plasma (1.00 ml) without internal standard. (**B**) Drug-free plasma sample spiked with (RS)-ketoprofen (0.625 µg/ml of each enantiomer) and internal standard. (**C**) Plasma containing 0.915 µg/ml of (R)- and 0.486 µg/ml of (S)-ketoprofen together with internal standard. Peaks (**1**) and (**2**) represent the diastereomeric (S)-1-phenylethylamides of (R)-ketoprofen and (S)-ketoprofen, respectively; and peak (**3**) the (S)-1-phenylethylamide of (S)-naproxen (the internal standard).

Table 4.2 Linear least-squares regression analysis for twelve calibration curves for each ketoprofen enantiomer constructed independently over an 8 week period. Data are presented as mean (± SD) values.

Regression Parameter	(R)-Ketoprofen	(S)-Ketoprofen
Slope	2.87 (± 0.19)	2.94 (± 0.21)
y-Intercept	0.0120 (± 0.00054)	0.0169 (± 0.00062)
Regression Coefficient	>0.999	>0.999

Assessment of the combined extraction and derivatization methodologies of the enantioselective assay entailed analysis of plasma samples containing 1.25 µg/ml of each enantiomer of ketoprofen (supplied as purified (RS)-[1-^{14}C]-ketoprofen) over a 2 week period (n = 8). The mean (± SD) percentage yield for unresolved ketoprofen was 79.6 (± 6.1)% and the ratio of (R)-ketoprofen to (S)-ketoprofen diastereomeric amide counts was 1.002 (± 0.021). Thus, derivatization to the diastereomeric amides with (S)-1-phenylethylamine was nonstereoselective and largely quantitative.

Validation of the method in terms of potential racemization of ketoprofen enantiomers during sample processing was examined by analysis of plasma samples containing 5.00 µg/ml of pure (R)-ketoprofen and pure (S)-ketoprofen over a 2 week period (n = 8 for each). The concentration of the contaminant optical antipode remained less than 2.5% in each case providing evidence that no racemization was taking place during sample extraction and derivatization.

It is important to establish the specificity of any analytical method which is designed for measurement of drug concentrations in biological matrices, since the possibility exists for subjects to be coadministered other drugs. Given the acidic environment in which extraction of xenobiotics takes place with this assay, a range of acidic drugs was assessed in terms of their retention times relative to the elution profiles of ketoprofen and internal standard

diastereomers. The retention times for the diastereomeric amides of the (R)- and (S)-enantiomers of ibuprofen were 3.3 and 4.1 min and for fenoprofen, 3.7 and 4.6 min, respectively. Mefenamic acid eluted as its respective amide at 3.0 min while salicylic acid had a retention time of 3.8 min. In addition, *ex vivo* plasma samples obtained from patients with rheumatoid arthritis who had been prescribed a variety of xenobiotics not including nonsteroidal anti-inflammatory agents were subjected to this enantioselective ketoprofen assay as part of the clinical study detailed in Chapter 8. These coadministered drugs represented a broad range of therapeutic agents none of which (including putative metabolites) interfered with either of the ketoprofen peaks or the internal standard peak (see Table 8.1 in Section 8.2 for a list of these drugs). In summary, a rapid, sensitive and specific HPLC assay for the determination of ketoprofen enantiomers in plasma has been described. The method performed with adequate accuracy and precision and was readily adapted for analysis of ketoprofen in urine samples obtained from patients dosed with racemic drug (see Section 8.2). In combination with radiochemical-aided detection (see Section 5.2) the method was also applied to studies of the plasma protein binding of (R)- and (S)-ketoprofen. In addition, the assay was used to indirectly quantify (as hydrolyzed enantiomeric aglycones) the acyl-glucuronide metabolites of ketoprofen (discussed in Chapter 6).

Chapter 5

Plasma Protein Binding of Ketoprofen Enantiomers in Man: Method Development and its Application

5.1 Introduction

NSAIDs, including ketoprofen, are characterized by their extensive reversible binding to human plasma proteins of which albumin (at least for acidic NSAIDs) is the principal binding protein (Lin *et al.*, 1987; Verbeeck *et al.*, 1983; Wanwimolruk *et al.*, 1982). These drugs are also characterized by extensive hepatic metabolism with a low extraction ratio (Lin *et al.*, 1987). This implies that the unbound fraction in plasma of such drugs will be a determinant of the clearance of total (bound plus unbound) drug and together with the unbound fraction in tissues, will influence drug distribution throughout the body (Wilkinson and Shand, 1975). Similarly, it is generally considered that only unbound drug is free to distribute and engage with receptors to elicit pharmacological and toxicological effects. Moreover, protein binding of drugs may alter as a result of disease states or from displacement by competing ligands for mutual binding sites and in the case of highly bound drugs significant elevations in the unbound fraction may result. In these cases, knowledge of the unbound fraction is important in interpreting mechanisms for changes to total clearance or total drug concentrations.

Data from nonstereoselective protein binding studies have shown that ketoprofen is approximately 99% bound at therapeutic concentrations of unresolved drug (Netter *et al.*, 1987; Williams *et al.*, 1981). However, there are no clinically relevant reports of the protein binding of individual enantiomers of ketoprofen. Given the ability of plasma proteins to discriminate between optical isomers (see Section 1.6.2), the implication of the high degree of binding of unresolved ketoprofen is that even a minor difference between enantiomers in the fraction bound may represent a considerable difference between their respective unbound fractions in plasma. Moreover, in light of the enantioselective protein binding studies with chiral 2-arylpropanoic acid congeners, 2-phenylpropanoic acid (Jones *et el.*, 1986), ibuprofen

(Evans *et al.*, 1989) and flurbiprofen (Knadler *et al.*, 1989), individual enantiomers may compete with each other for plasma protein binding sites. This is particularly important since most chiral NSAIDs are used clinically as racemates.

A method was developed for determining the unbound fraction of ketoprofen enantiomers in human plasma. Radiolabelled (RS)-ketoprofen was synthesized and purified radiochemically by achiral HPLC and subsequently used in conjunction with an enantioselective HPLC method (detailed in Section 4.2) to quantify the unbound fraction of (R)- and (S)-ketoprofen in plasma. The method was used to investigate aspects of ketoprofen enantiomer binding (in plasma harvested from healthy drug-free volunteers) including: (*i*) the linearity of the unbound fraction as a function of total (bound plus unbound) drug concentration, (*ii*) the influence of one enantiomer on the unbound fraction of its optical antipode, and (*iii*) the effect of the acyl-glucuronide metabolites of ketoprofen on the unbound fraction of each enantiomeric aglycone. Later (Chapter 8), the method was applied to enantioselective pharmacokinetic and pharmacodynamic studies of ketoprofen in patients with rheumatoid arthritis and varying degrees of impairment of renal function, most of whom were taking medication in addition to ketoprofen. Clearly, factors such as concurrently administered drugs and pathophysiological status may modulate the binding of such drugs to plasma protein (Day *et al.*, 1987; Lin *et al.*, 1987; Reidenberg and Drayer, 1984) and these are considered in Chapter 8.

5.2 Materials and Methods

Chemicals

Sources of racemic and enantiomeric ketoprofen pure substances together with suppliers of chromatographic chemicals and equipment used during this method development are detailed in Section 4.2. (RS)-[1-^{14}C]-Ketoprofen (8.70 μCi/μmol) was radiochemically purified by HPLC as described below. Pre-weighed pH-buffered vials of type VII-A purified β-glucuronidase were purchased from Sigma Chemical Company (St Louis, U.S.A.). Analytical-grade sodium chloride, sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Ajax Chemicals (Sydney, Australia) and human serum albumin, fraction V,

from Calbiochem (San Diego, U.S.A.). Biosynthetic 1-*O*-β-(R/S:44.6/55.4)-ketoprofen acyl-glucuronide was purified from human urine by HPLC as described below.

Radiochemical purification of (RS)-[1-^{14}C]-ketoprofen

Individual batches of radiolabelled ketoprofen were purified by reversed-phase HPLC as near as practical to the time such material would be required for the plasma protein binding investigations (within 48 h of use). The HPLC system consisted of a Model 510 pump, Wisp^{R} autoinjector, Model 490 variable wavelength UV-absorbance detector and Model 840 Data Station, all from Waters Associates (Milford, U.S.A.). A Gilson Model 202 fraction collector (Villiers le Bel, France) was configured with the system. A radially-compressed phenyl cartridge (Rad-Pak®, 4 μm, 100 mm x 8 mm internal diameter, Waters Assoc.) was eluted with 40% acetonitrile in 10.0 m*M* acetate buffer (pH3.0) at a flow-rate of 2.0 ml/min and the eluent was monitored for UV-absorbance at 260 nm.

Aliquots (100 μl) of a 0.01% w/v methanolic solution of (RS)-[1-^{14}C]-ketoprofen were injected into the HPLC system and the eluate fraction corresponding to unresolved ketoprofen was collected (retention time *circa* 4 min) in a glass tube. The volume of the eluate was reduced by approximately 50% under a stream of purified nitrogen (45°C) before the addition of 2.0 *M* sulphuric acid (0.5 ml) and 10% ethyl acetate in *n*-hexane (5 ml). The contents of the tube were vortex-mixed (20 sec) and centrifuged (2000 *g* for 10 min) and the top layer transferred to a fresh glass tube and evaporated to dryness under nitrogen. The residue was reconstituted with methanol and combined with similar reversed-phase HPLC-purified aliquots. The final concentration of purified (RS)-[1-^{14}C]-ketoprofen (*circa* 50 μg/ml in methanol) was determined by reinjecting an appropriate volume of the solution and comparing the ketoprofen peak area against a range of nonradiolabelled ketoprofen standards. An appropriate volume of the methanolic solution of HPLC-purified radiolabelled ketoprofen was transferred to a fresh tube and evaporated to dryness under nitrogen prior to reconstitution (care was taken to avoid frothing) with 4.0% human serum albumin in isotonic phosphate buffer (0.067 *M*, pH7.4). The final concentration of purified (RS)-[1-^{14}C]-ketoprofen in 4.0% albumin was 2.00 μg/20 μl and represented the means by which (RS)-[1-^{14}C]-ketoprofen was spiked (20.0 μl) into plasma (1.00 ml) as discussed below.

Purification of biosynthetic ketoprofen acyl-glucuronides

The reversed-phase HPLC system described above for radiochemical purification of labelled ketoprofen was adopted as a means of purifying the chief metabolite (acyl-glucuronoconjugate) of ketoprofen from pH-stabilized urine. A volunteer ingested 200 mg of (RS)-ketoprofen (Orudis®, May and Baker, Melbourne, Australia) and immediately pH-adjusted (orthophosphoric acid, pH3.0) his post-dose urine which was voided at frequent intervals. Urine collected between 2 and 3.5 h after ketoprofen administration (original pH4.8) contained approximately 400 µg/ml (following alkaline hydrolysis of an aliquot and determination of the aglycone concentration) of glucuronide expressed as unresolved ketoprofen equivalents. The remainder of this urine sample was stored at -20°C and subsequently used for the isolation of the acyl-glucuronoconjugates.

A filtered (0.8 µm) 200 µl aliquot of this urine was injected directly into the reversed-phase HPLC system. The (R)- and (S)-ketoprofen acyl-glucuronides eluted as a single peak at 2.8 min and the corresponding eluate fraction was collected in a glass tube. Putative peak identity was based on the observation of the disappearance of this eluate peak with a reciprocal appearance of a peak of identical retention time to the agylcone, following alkali hydrolysis. The eluate volume was reduced by *circa* 50% under nitrogen (to remove acetonitrile from the mobile phase eluate) followed by the addition of 8 ml of ethyl acetate. The tube contents was vortex-mixed and centrifuged, and the upper layer transferred to a fresh glass tube, placed in a waterbath (40°C) and dried under a nitrogen stream. The dried residue was reconstituted with methanol. This process was repeated until sufficient glucuronide was harvested. The ratio of (R)- to (S)-ketoprofen acyl-glucuronide was determined by the enantioselective normal-phase HPLC assay (see Chapter 4) following complete hydrolysis of the glucuronides (*vide infra*). Structural authentication of the purified acyl-glucuronides of ketoprofen was undertaken by ^{1}H-NMR and mass spectroscopy techniques and is detailed in Section 6.2.

Plasma protein binding experiments

The *in vitro* plasma protein binding of ketoprofen enantiomers was determined in plasma from each of 6 healthy volunteers, none of whom were taking any medication. The mean age and serum albumin concentration of these subjects (2 female) were 39 yr (range: 25-54 yr) and 38 g/l (range: 35-44 g/l), respectively. Plasma was harvested by centrifugation (2000 *g*, 10 min) of heparinized blood sampled via venepuncture of an arm vein and this plasma was stored frozen (-20°C) prior to use.

Subsequently, plasma was warmed to 37°C in a water-bath and pH adjusted (pH7.4) with 2.5% orthophosphoric acid; typically, 5.0 µl of acid was required per millilitre of plasma. Aliquots (1.00 ml) of temperature- and pH-adjusted plasma from each subject were added to glass tubes containing known amounts of (R)-ketoprofen alone, (S)-ketoprofen alone, or (RS)-ketoprofen. Pure enantiomers or racemic ketoprofen (all three as nonradiolabelled drug) had been added to these tubes as varying volumes of methanolic solutions (100 µg/ml) and evaporated to dryness under nitrogen prior to the addition of plasma. A 20 µl aliquot of HPLC-purified (RS)-[1-^{14}C]-ketoprofen (2.0 µg in 20 µl of 4.0% human serum albumin, *vide supra*) was added to each millilitre of nonradiolabelled ketoprofen-spiked plasma.

The final enantiomeric concentration ranges in plasma were: 1.00 to 19.0 µg/ml of (R)-ketoprofen (with a constant 1.00 µg/ml of (S)-[1-^{14}C]-ketoprofen throughout); 1.00 to 19.0 µg/ml of (S)-ketoprofen (with a constant 1.00 µg/ml spike of (R)-[1-^{14}C]-ketoprofen throughout); and 1.00 to 19.0 µg/ml of both enantiomers present together. Finally, each spiked plasma sample was carefully mixed to avoid frothing and returned to the water bath (set at 37°C) oscillating at 20 cycles per minute, for 30 min prior to ultra-filtration.

Determination of the enantiomeric percentage unbound in plasma

One millilitre of ketoprofen spiked plasma was instilled in the sample reservoir of a Centrifree ® Micropartition System (Amicon Division, Danvers, U.S.A.) and centrifuged at 2000 *g* for 30 min (37°C) in a Heraeus Suprafuge 22 with a HFA 20.16 fixed-angle rotor (Osterode, Germany). Part of the ultra-filtrate (150 µl) was added to 10 ml of liquid scintillant (PCS II,

Amersham, Sydney, Australia) and counted (10 min) (Nuclear-Chicago, Unilux III, U.S.A.). Quench correction was performed using the channels-ratio method to arrive at a dpm value per 150 µl. Arithmetic division of the ultra-filtrate dpm (containing unbound species) by the corresponding dpm value (volume-normalised) for pre-filtered plasma (*i.e.* dpm/150 µl of plasma containing bound plus unbound species) and multiplication by 100 gave rise to the percentage unbound in plasma of unresolved ketoprofen [$fu_{(\text{unresolved})}$ (%)].

The remaining ultra-filtrate (approximately 175 µl) was subjected to the enantioselective normal-phase HPLC assay (see Section 4.2 and below) for determination of the enantiomeric distribution of dpm. To this ultra-filtrate portion in a glass tube was added 2.0 *M* sulphuric acid (0.5 ml), 100 µl of racemic nonradiolabelled ketoprofen (25 µg/ml in methanol) [to serve as cold carrier and enable visualization of ketoprofen diastereomer peaks] and 10% ethyl acetate in *n*-hexane (8 ml). The contents of the tube were vortex-mixed and centrifuged, following which the top layer was transferred to a fresh glass culture tube and evaporated to dryness under nitrogen. Freshly prepared 1.5% thionyl chloride in *n*-hexane (100 µl) was added to the dried contents, the tube was firmly capped (PTFE-lined cap), vortex-mixed and subsequently heated for 1 h in a dry heat bath (75°C). The tube contents were then allowed to cool to room temperature, freshly prepared 2.0% (S)-1-phenylethylamine in dry dichloromethane (500 µl) was added. The tube was vortex-mixed and left to stand for 15 min at room temperature. Finally, unreacted amine was removed by acidification of the tube contents with 2.0 *M* sulphuric acid (0.5 ml) and extraction of the diastereomeric amides of ketoprofen with 5 ml of *n*-hexane. Following drying of the organic layer under nitrogen, the residue was reconstituted in 250 µl of mobile phase (8% isopropyl alcohol in *n*-heptane) and 200 µl injected onto the column which contained 5 µm silica (250 x 4 mm internal diameter, SGE, Sydney, Australia). The mobile phase was pumped at 1.0 ml/min and the diastereomeric (S)-1-phenylethylamides of (R)- and (S)-ketoprofen were visualized as peaks eluting at *circa* 5.5 min and 7.3 min, respectively. The HPLC eluate fractions corresponding to the (R)-[1-^{14}C]- and (S)-[1-^{14}C]-ketoprofen diastereomers were collected in separate glass tubes and evaporated to dryness under nitrogen. Following reconstitution of the dried residues with 10 ml of liquid scintillant, each of the eluted diastereomers was counted (40 min) and corrected for quenching to yield dpm values for (R)-ketoprofen ($dpm_{(R)}$) and (S)-ketoprofen ($dpm_{(S)}$) in

the plasma ultra-filtrate. The percentage unbound in plasma for (R)-ketoprofen [$fu_{(R)}$ (%)] and (S)-ketoprofen [$fu_{(S)}$ (%)] was calculated according to Equations 5.1 and 5.2, respectively:

$$fu_{(R)}\,(\%) = fu_{(\text{unresolved})}\,(\%) \times \frac{2 \times \text{dpm}_{(R)}}{\text{dpm}_{(R)} + \text{dpm}_{(S)}} \qquad (5.1)$$

$$fu_{(S)}\,(\%) = fu_{(\text{unresolved})}\,(\%) \times \frac{2 \times \text{dpm}_{(S)}}{\text{dpm}_{(R)} + \text{dpm}_{(S)}} \qquad (5.2)$$

Influence of ketoprofen glucuronides on the plasma protein binding of ketoprofen enantiomers

An aliquot (100 µl) of HPLC-purified and authenticated (see Section 6.2) 1-*O*-β-(R/S:44.6/55.4)-ketoprofen acyl-glucuronide (100 µg/ml in methanol) was added to a glass tube and evaporated to dryness under nitrogen. The dried glucuronoconjugate was solubilized with 4.0% human serum albumin (20.0 µl) containing 2.00 µg of purified (RS)-[1-^{14}C]-ketoprofen prior to the addition of 1.00 ml of drug-free plasma (37°C, pH7.4) obtained separately from each of four healthy volunteers (a sub-group of the original six volunteers, see Table 5.1 for details). The tube contents were processed as described above to determine the percentage unbound of (R)- and (S)-ketoprofen in the presence of 10·0 µg/ml of the glucuronoconjugate in plasma. Prior to spiking into plasma, an aliquot of the purified acyl glucuronide was hydrolysed with both β-glucuronidase (100 units/ml, 37°C, 60 min) and alkali (1.0 *M* sodium hydroxide, 22°C, 15 min) on separate occasions. This was to ascertain the degree of intramolecular acyl rearrangement (see Section 3.3) that had taken place during the isolation and purification procedures and to confirm that no racemization of the aglycone occurred during alkaline hydrolysis. No attempt was made to quench the reactivity of these purified metabolites upon their addition to plasma.

Statistical analysis

Data are presented as the arithmetic mean ± SD. Comparison of the percentage unbound in plasma of each enantiomer and the influence of the glucuronoconjugates, the presence of the optical antipode and increasing plasma ketoprofen concentration on percentage unbound of

each enantiomer were evaluated using analysis of variance. Multiple comparison procedures were performed using Fisher's least significant difference (LSD) test.

5.3 Results and Discussion

Validation of methods

The reproducibility of the method for determining the *in vitro* plasma protein binding of (R)- and (S)-ketoprofen was assessed by spiking drug-free plasma from one subject with 2.00 μg/ml of (RS)-[1-^{14}C]-ketoprofen. The mean (± SD) percentage unbound for six replicate determinations of unbound (R)-ketoprofen was 0.862 (± 0.085)%. The corresponding value for unbound (S)-ketoprofen was 0.792 (± 0.083)%.

There was negligible sorption of ketoprofen enantiomers onto the Centrifree® ultra-filtration membrane. Less than 3% of ketoprofen was lost when a protein-free solution of 0.020 μg/ml of (RS)-[1-^{14}C]-ketoprofen in isotonic phosphate buffered saline (pH7.4) was ultra-filtered.

The use of radiolabelled ligand in determining the degree of plasma protein binding of highly-bound drugs requires a source of radiochemically pure drug. Relatively minor amounts of poorly-bound radiochemical impurities can lead to spuriously elevated estimates of the unbound fraction (Bjornsson *et al.*, 1981; Evans *et al.*, 1989). A reversed-phase HPLC method was used for radiochemical purification of synthetic material [Figure 5.1(a)]. To check for the presence of radiochemical impurities in this material, a 1.00 ml sample of drug-free plasma was spiked with (20.0 μg/ml) of HPLC-purified (RS)-[1-^{14}C]-ketoprofen. The sample was ultra-filtered after temperature and pH adjustment using the conditions described above. The ultra-filtrate (*circa* 300 μl) was derivatized and subjected to normal-phase enantioselective HPLC analysis. Successive 15 sec eluate fractions were collected over 10 min post-injection and liquid scintillation counting was performed on each fraction. The radiochromatogram constructed from these eluates [Figure 5.1(b)] clearly shows the presence of radiolabelled (R)- and (S)-ketoprofen eluted as their respective (S)-1-phenylethylamide diastereomers. No other peaks (radiometric signals above background) were observed confirming the radiochemical purity of the radiolabelled ketoprofen spiked into plasma.

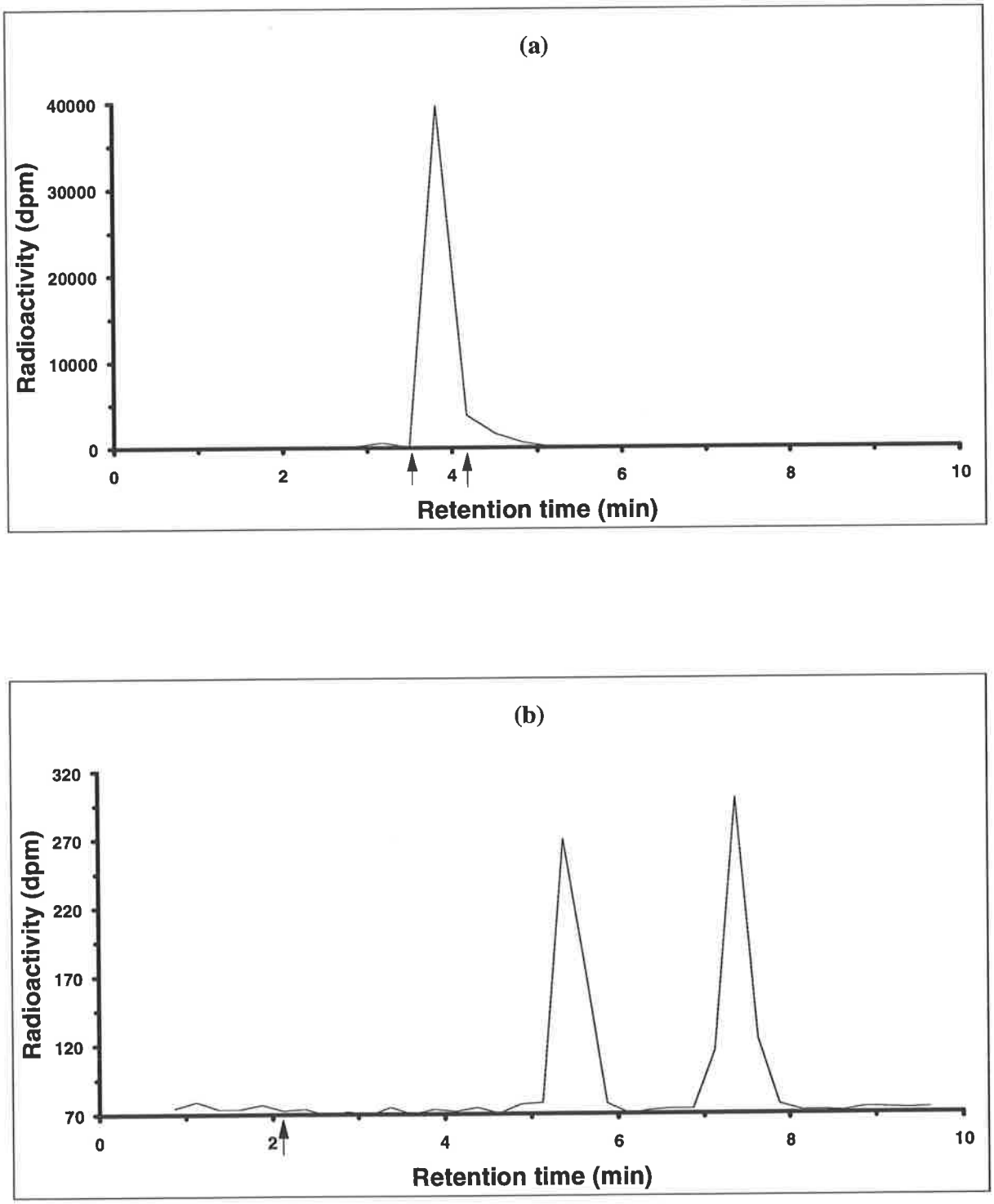


Figure 5.1 Radiochromatograms of: (**a**) reversed-phase HPLC purification of (RS)-[1-^{14}C]-ketoprofen (10 μg) from synthesized material (arrows indicate elution of purified ketoprofen), and (**b**) normal-phase enantioselective HPLC elution of derivatized unbound (R)- and (S)-ketoprofen obtained by ultra-filtration of plasma (1.0 ml) containing 20.0 μg/ml of purified (RS)-[1-^{14}C]-ketoprofen (arrow indicates column void time). Twenty second and 15 sec eluate fractions were collected and counted to construct panels (**a**) and (**b**), respectively.

Equations 5.1 and 5.2 used to calculate the percentage unbound for each enantiomer assume that derivatization and subsequent collection of the normal-phase HPLC eluates of the (S)-1-phenylethylamide diastereomers of ketoprofen was nonstereoselective. This was checked routinely by spiking drug-free plasma with 2.0 μg/ml of (RS)-[1-^{14}C]-ketoprofen, and extracting and derivatizing the sample without ultra-filtration. The ketoprofen diastereomer peaks were collected and liquid scintillation counting performed thereon. The mean (± SD) ratio of dpm of (R)-ketoprofen to (S)-ketoprofen (as their respective diastereomeric amides) from 8 such determinations conducted over the course of the binding experiments was 0.9963 (± 0.0098). This confirmed that the derivatization and fractionation conditions gave rise to equal quantities of each diastereomer.

The rationale for using *in vitro* methods to determine ketoprofen enantiomer plasma binding rather than *ex vivo* methods (binding of enantiomers in post-dose plasma) was two-fold. Firstly, it was desired to examine enantiomer binding when the enantiomers were present both alone and together in the one plasma sample to investigate possible interactions between the enantiomers for plasma protein binding sites. Previous studies (Foster *et al.,* 1988a,b; Sallustio *et al.*, 1988b) have demonstrated marked similarity between total (bound plus unbound) plasma concentrations of ketoprofen enantiomers upon racemic drug dosing to humans (pure ketoprofen enantiomers are not currently available for administration to humans). Hence, with *ex vivo* studies it would not be possible to examine enantiomer binding when the enantiomer of interest was present alone in the sample. Secondly, the facile hydrolysis of the glucuronides of ketoprofen under the physiological temperature and pH conditions employed in the binding experiments necessitates the use of metabolite-free plasma. The labile nature of acyl-glucuronides under physiological conditions has been long recognised (Faed, 1984; Hasegawa *et al.*, 1982; Upton *et al.*, 1980; see Section 3.3) and is addressed experimentally for ketoprofen conjugates in Chapter 6. The presence of ketoprofen glucuronides in *ex vivo* plasma has been confirmed in previous studies (Foster *et al.*, 1988a; Williams *et al.*, 1981). Iwakawa and coworkers (1990) in a recent study recognised the potential sample-handling problems posed by the glucuronides of carprofen during examination of the stereoselective binding of carprofen enantiomers and their conjugates to human serum albumin.

Binding of ketoprofen enantiomers to human plasma protein

The mean (± SD bars) percentage unbound in plasma of (R)-ketoprofen as a function of total (bound plus unbound) plasma concentration of the (R)-enantiomer is depicted for the six healthy volunteers in Figure 5.2. Separate *in vitro* experiments were undertaken to examine the influence of total (R)-ketoprofen concentration with this enantiomer spiked into plasma as either racemic drug or as optically pure drug. The corresponding data for the percentage unbound in plasma of (S)-ketoprofen are given in Figure 5.3.

The data in Figures 5.2 and 5.3 were assessed by analysis of variance to determine: (*i*) whether the plasma protein binding of ketoprofen was enantioselective, (*ii*) the linearity of the binding over the plasma concentration range examined and (*iii*) whether there was displacement of ketoprofen enantiomers by their corresponding optical antipodes from plasma binding sites. Firstly, with respect to the degree of binding enantioselectivity, comparisons between the percentage unbound for (R)- and (S)-ketoprofen (present as racemic drug in plasma) showed there to be no difference ($P>0.05$) between enantiomers. Secondly, the linearity of the plasma binding of each enantiomer over the concentration range of 1.00-19.0 μg/ml was examined when (R)- and (S)-ketoprofen were each present alone in plasma. There were no differences ($P>0.05$) between percentage unbound values across the concentration range for each enantiomer. Thus the individual binding of (R)- and (S)-ketoprofen was linear up to an enantiomeric plasma concentration of 19.0 μg/ml. Moreover, the linearity of binding was also evaluated for those data obtained for racemic ketoprofen over the concentration range of 1.00 to 19.0 μg/ml of each enantiomer (2.00 to 38.0 μg/ml of racemate). In this case, there were no differences ($P>0.05$) between the percentage unbound values for (R)-ketoprofen across this concentration range. For (S)-ketoprofen the percentage unbound value at a supra-therapeutic (Foster *et al.*, 1988a,b; Sallustio *et al.*, 1988b) enantiomeric concentration of 19.0 μg/ml was different ($P<0.05$) from the corresponding values for concentrations of 9.00 μg/ml and below, although this was a small difference. Finally, with respect to potential displacement of each enantiomer by its optical antipode, there was no difference in percentage unbound values between each enantiomer present alone and when present as racemic drug except for the (R)-enantiomer at a concentration of 19.0 μg/ml ($P<0.05$).

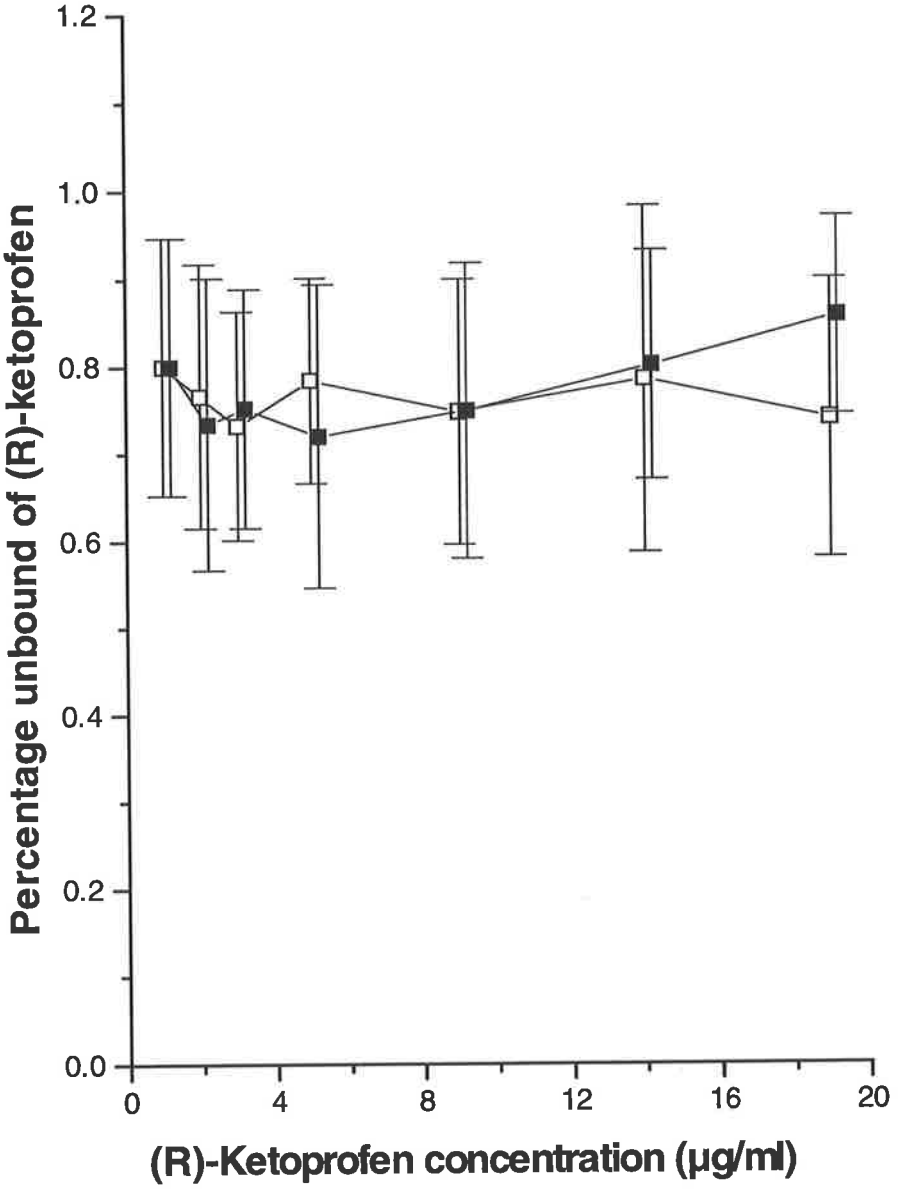


Figure 5.2 Percentage unbound of (R)-ketoprofen as a function of plasma (R)-ketoprofen concentration both alone (❑) and in the presence (■) of an equal concentration of (S)-ketoprofen. Data are presented as the arithmetic mean (with vertical bars representing ± SD) from 6 healthy volunteers. For the purpose of graphic clarity the data have been horizontally displaced.

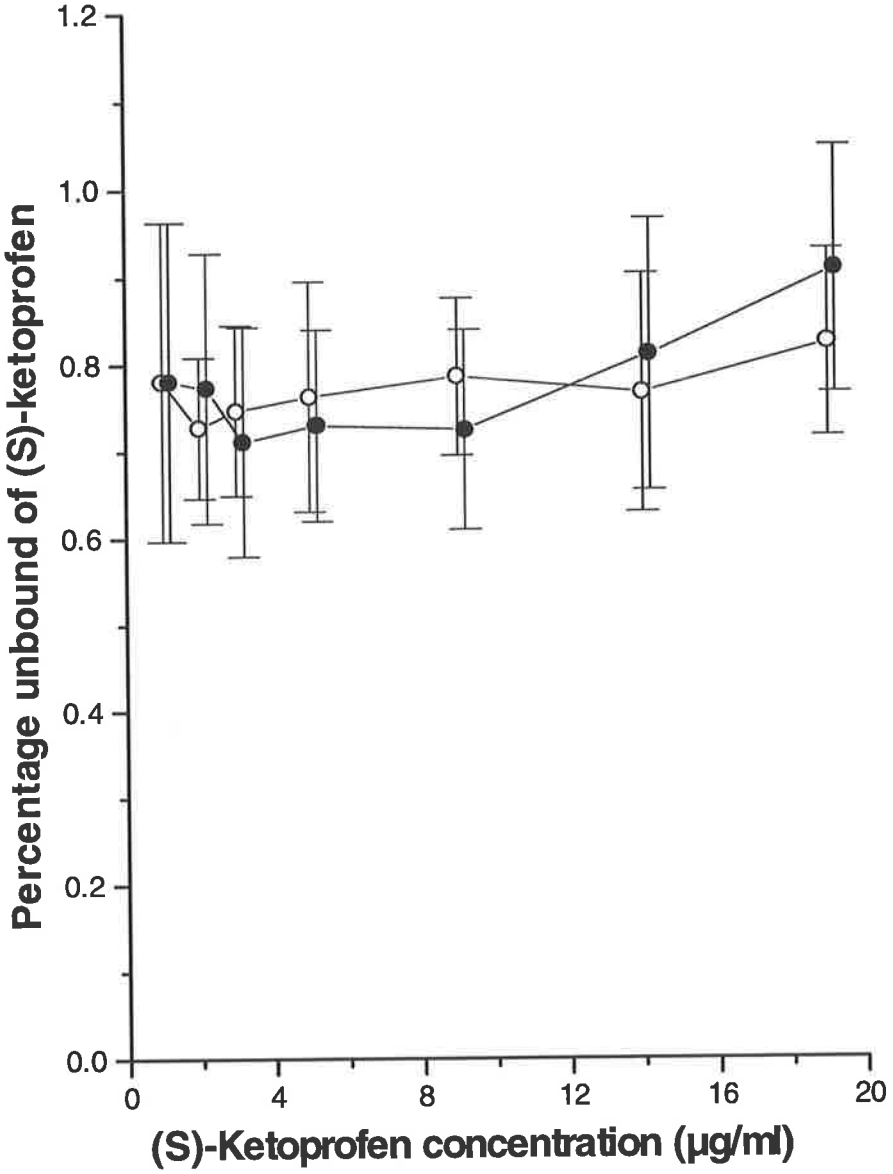


Figure 5.3 Percentage unbound of (S)-ketoprofen as a function of plasma (S)-ketoprofen concentration both alone (○) and in the presence (●) of an equal concentration of (R)-ketoprofen. Data are presented as the arithmetic mean (with ± SD bars) from 6 healthy volunteers. For the purpose of graphic clarity the data have been horizontally displaced.

Previous studies using nonstereoselective methods have reported percentage unbound values for unresolved ketoprofen of approximately 1% (Netter *et al.*, 1987; Williams *et al.*, 1981). Both studies subjected *ex vivo* plasma samples to equilibrium dialysis which might explain the marginally higher values (with respect to unresolved drug) than obtained in the present study. In addition, the previous studies (Netter *et al.*, 1987; Williams *et al.*, 1981) found the plasma binding of unresolved ketoprofen to be independent of therapeutic drug concentration. Rendic *et al.* (1980) examined the separate binding of ketoprofen enantiomers to human serum albumin using gel filtration. At a ligand to albumin molar ratio of unity (1.44×10^{-5} *M* of each) the percentage unbound for (S)-ketoprofen was 13.5% and 9.00% for (R)-ketoprofen. When the concentration of both ligand and albumin was increased by 10-fold, the percentage unbound of (S)- and (R)-ketoprofen was 2.6 and 1.9%, respectively. These workers suggested that the stereoselective nature of ketoprofen binding to serum albumin was strongly influenced by experimental conditions, particularly protein concentration. Perhaps differing experimental conditions impart conformational changes to protein structure which, in turn, alters individual enantiomer binding properties. Thus, given the very high ligand to albumin molar ratios examined by these workers (Rendic *et al.*, 1980) and the fact that binding to albumin alone at room temperature was investigated, it is difficult to put these results into context with the data generated under physiological conditions in the present study. Moreover, the study of Rendic *et al.* (1980) did not examine the influence of each enantiomer on the binding of its optical antipode.

Noctor *et al.* (1991) examined the relative retention times of ketoprofen enantiomers on a human serum albumin-based HPLC chiral stationary phase. These workers have suggested that this immobilized protein broadly retains the binding properties of native human serum albumin. While Noctor and coworkers (1991) were able to resolve ketoprofen enantiomers with a fatty acid-free mobile phase, the addition of octanoic acid to the column eluent led to a complete loss of chiral recognition for these enantiomers. Such observations are consistent with the lack of stereoselective binding of ketoprofen to human plasma observed here under physiological conditions when endogenous fatty acids are present. Iwakawa *et al.* (1990) demonstrated a reversal of the degree of binding of carprofen enantiomers to fraction V versus fatty acid-free human serum albumin. They postulated that fatty acids induced conformational

changes to albumin structure which, in turn, led to the observed stereoselective change in (R)-carprofen binding.

At clinically relevant drug concentrations, there was no evidence of nonlinearity in the plasma protein binding of ketoprofen enantiomers. Similar observations have been made for the plasma protein binding of flurbiprofen enantiomers (Knadler *et al.*, 1989) where the percentage unbound of each enantiomer was independent of concentration up to 26 μg/ml. Studies with ibuprofen in human plasma (Evans *et al.*, 1989) and 2-phenylpropanoic acid in rabbit albumin and plasma (Jones *et al.*, 1986) have demonstrated competition between enantiomers for binding sites. Competition between the respective enantiomers of ibuprofen (Lee *et al.*, 1985) and fenoprofen (Hayball and Meffin, 1987), while not individually studied, was postulated to explain pharmacokinetic differences between enantiomers administered alone and enantiomers administered as racemic drug. Significantly higher ligand to protein molar ratios were achieved with these drugs than would be expected with ketoprofen dosing in humans. Recently, Muller *et al.* (1992) reported that the *in vitro* binding of etodolac enantiomers to fatty acid-free human serum albumin was concentration dependent with the enantioselective binding at low drug:protein ratios [(R)-etodolac was more avidly bound] being reversed at higher ratios.

The binding studies with ibuprofen (Evans *et al.*, 1989) in humans and 2-phenylpropanoic acid (Jones *et al.*, 1986) in rabbits demonstrated higher percentage unbound values for the (S)-enantiomers compared to their respective optical antipodes. In contrast, studies in humans with carprofen (Iwakawa *et al.*, 1990) and flurbiprofen (Knadler *et al.*, 1989) demonstrated stereoselectivity in the opposite direction with the percentage unbound of the (R)-enantiomer exceeding the corresponding (S)-enantiomer. The binding of pirprofen to human serum albumin (fraction V, with fatty acids) has been reported to be nonstereoselective (Otagiri *et al.*, 1989) while pirprofen binding to fatty acid-free human serum albumin was slightly higher for (S)-pirprofen (Oravcova *et al.*, 1991). The present studies with ketoprofen demonstrate a lack of stereoselectivity with respect to plasma protein binding within the therapeutic range of the drug in healthy volunteers. In a cohort of elderly rheumatoid arthritic patients receiving a variety of concurrent medication, the binding of ketoprofen was enantioselective with the percentage unbound of (S)-ketoprofen exceeding that of its optical antipode (see Chapter 8).

Thus, it appears that no generalizations can be made concerning the binding properties of the enantiomers of 2-arylpropanoic acid NSAIDs, particularly in light of potential influences from experimental conditions such as ligand to albumin molar ratio (*vide supra*) and the fatty acid content of albumin (Birkett *et al.*, 1977; Jones *et al.*, 1986; *vide supra*).

Ketoprofen protein binding in the presence of its acyl-glucuronides

The influence of the major metabolite of ketoprofen in humans (ketoprofen glucuronide) on the plasma protein binding of (R)- and (S)-ketoprofen was assessed in 4 subjects (Table 5.1). The biosynthetic metabolite was characterized by complete alkaline hydrolysis and subsequent enantioselective analysis of the aglycone to be 1-*O*-β-(R/S:44.6/55.4)-ketoprofen acyl-glucuronide. Hydrolysis of the HPLC-purified glucuronoconjugate was also complete when treated with β-glucuronidase. This confirmed that no regioisomerism of the metabolite had taken place during its isolation and purification up to the point when it was spiked into plasma to assess its influence on ketoprofen binding (see Section 6.2). The binding of each enantiomer (total plasma concentration of 1.00 μg/ml) was unaffected (P>0·05) by 10.0 μg/ml of glucuronoconjugate (expressed as unresolved ketoprofen aglycone equivalents). Studies with carprofen (Iwakawa *et al.*, 1990) have suggested differences in the binding region between carprofen enantiomers and the acyl-glucuronides.

Table 5.1 Percentage unbound of (R)- and (S)-ketoprofen in the plasma of 4 subjects spiked with 2.0 μg/ml of (RS)-[1-^{14}C]-ketoprofen alone or in the presence of biosynthetic 1-*O*-β-(R/S:44.6/55.4)-ketoprofen acyl-glucuronide (10.0 μg/ml).

				Ketoprofen alone		*Ketoprofen plus glucuronides*	
Subject	*Gender*	*Age* (yr)	*Serum albumin* (g/l)	$fu_{(R)}$ (%)	$fu_{(S)}$ (%)	$fu_{(R)}$ (%)	$fu_{(S)}$ (%)
1	M	54	39	0.749	0.911	0.774	0.905
2	M	29	44	0.858	0.604	0.756	0.730
3	M	51	37	0.807	0.948	0.882	0.915
4	F	25	35	0.841	0.858	0.878	0.948
Mean				0.814	0.829	0.823	0.875
SD				0.048	0.154	0.067	0.098

It should be noted that both rearrangement and deconjugation of acyl-linked glucuronides of ketoprofen will have occurred to some degree under the physiological conditions (pH7.4, 37° C) used for assessing the protein binding of the enantiomeric aglycones (see Chapter 6). However, significant concentrations of both the (R)- and (S)-ketoprofen acyl-linked conjugates should be present at the completion of the binding experiments. Less than 1 h elapsed between addition of the glucuronides to plasma and the completion of the ultra-filtration process, which compares with apparent first-order net hydrolysis half-lives for (R)- and (S)-ketoprofen conjugates of *circa* 1.4 and 3.5 h, respectively (see Section 6.2 for details of these experiments).

In summary, at clinically relevant concentrations in plasma from healthy drug-free subjects, the reversible plasma protein binding of ketoprofen is nonstereoselective, the binding of ketoprofen enantiomers is not concentration-dependent nor does the presence of one enantiomer modify the binding of its optical antipode. Moreover, the plasma protein binding of ketoprofen enantiomers is not influenced by the presence of the acyl-linked glucuronide metabolites of ketoprofen.

Chapter 6

Stereoselective Interactions of Ketoprofen Glucuronides with Human Plasma Protein and Serum Albumin

6.1 Introduction

The importance of acyl-glucuronidation as a biotransformation process for ketoprofen in man is detailed in Section 2.3.3 and the existence of a cycle of reversible glucuronide conjugation for acyl-glucuronides and their respective aglycones (the so-called "futile cycle") has been discussed in Sections 2.3.4 and 3.5. The futile cycle mechanism has provided an explanation for the paradoxically reduced clearances of the predominantly nonrenally cleared (as parent drug) NSAIDs ketoprofen (Advenier *et al.*, 1983; Stafanger *et al*, 1981), naproxen (Van den Ouwenland *et al.*, 1988), ximoprofen (Taylor *et al.*, 1991) and benoxaprofen (Aronoff *et al.*, 1982) in patients with renal dysfunction or in elderly patients in whom renal function would be expected to be diminished. In such a cycle, net clearance is due to glucuronoconjugation and competition between elimination of glucuronide by renal and hydrolytic clearances. This predicts that inhibition of the renal elimination of the acyl-glucuronide will lead to a reduction in net drug clearance due to accumulation and deconjugation of the physiologically labile glucuronide metabolite (Faed, 1984).

In addition to direct hydrolysis of the biosynthetic 1-*O*-β-acyl-glucuronide to the corresponding aglycone, intramolecular rearrangement via acyl migration of the drug moiety to other positions on the glucuronic acid ring and subsequent hydrolysis of these isomers are potential sources of deconjugated drug (see Section 3.3). Moreover, a quantitatively minor competing pathway is covalent binding via the acyl-glucuronide forming a drug-protein adduct (see Section 3.4). Each of these processes involves potential regeneration of pharmacologically active aglycone. Whereas the hydrolysis of covalent drug-protein adducts has been a quantitatively minor contributor to regenerated aglycone, the hydrolysis of positional isomers of glucuronides has been shown to be a significant pathway for acyl-linked

conjugates of drugs including fenoprofen (Volland *et al.*, 1991), oxaprozin (Ruelius *et al.*, 1986) and diflunisal (Watt and Dickinson, 1990).

The aim of the present study was to examine the mechanisms involved in *in vitro* deconjugation of acyl-glucuronides using ketoprofen glucuronides as model metabolites. Significant concentrations of ketoprofen glucuronides have been detected in the plasma of elderly patients with reduced renal function (Foster *et al.*, 1988a) and in young healthy subjects coadministered probenecid (Upton *et al.*, 1982). Specifically, the relationship between the reversible binding of these conjugates to human plasma or human serum albumin and net hydrolysis [i.e. hydrolysis of biosynthetic glucuronide and the positional isomers, together with (possibly) hydrolysis of drug-protein adduct] has been studied. A further aim was to investigate factors which might influence these processes. Moreover, since ketoprofen (in common with most NSAIDs possessing a chiral carbon atom) is marketed for clinical use as a racemate, the stereoselectivity of glucuronide net hydrolysis and reversible binding has been examined. Given the quantitative importance of the rearrangement reaction for acyl-linked conjugates *in vitro* (see Section 3.3), additional experiments were carried out to investigate this process for ketoprofen glucuronides in various incubation media at physiological pH and temperature.

6.2 Materials and Methods

Purification and characterisation of ketoprofen glucuronides

The purification procedure for biosynthetic ketoprofen glucuronides has been described in detail in Section 5.2. Characterisation of the purified (R)- and (S)-ketoprofen glucuronides was carried out by both chemical and physical means. The extent of hydrolysis of the glucuronides by purified β-glucuronidase (type VII-A, Sigma Chemical Company, St Louis, U.S.A.: 100 units/ml, 37°C, 60 min) was identical to the extent obtained by complete alkaline hydrolysis (1.0 *M* sodium hydroxide, 22°C, 15 min). This suggested exclusive attachment of ketoprofen to the C-1 position of the sugar moiety with a β-linkage; and consequently, a lack of regioisomeric compounds in the purified material. In addition, enantioselective analysis of the aglycone (see Section 4.2) confirmed the proportion of (R):(S)-ketoprofen to be 44.6:55.4

following hydrolysis of the purified biosynthetic metabolites by either chemical or enzymatic means.

Confirmation of structure as 1-*O*-β-(R,S)-ketoprofen-(D)-glucopyranosiduronate (glucuronide) was obtained by high-field (300 MHz) ^{1}H-NMR spectroscopy. Two overlapping doublets at 5.4 δ (relative to tetramethyl silane [TMS]) of unequal heights (corresponding to the enantiomeric ratio of aglycone) with coupling constants (5.7 and 6.3 Hz) the same for each and consistent with trans-diaxial hydrogen atoms (anomeric protons) confirmed acyl attachment at C-1 of (D)-glucuronic acid in the β-configuration. Hydroxyl protons were not seen, presumably replaced by deuterium from the solvent (CD_3OD). Two overlapping doublets, again of unequal height, at 1.45 δ with coupling constants of 6.3 and 6.6 Hz corresponded to the α-methyl protons of the two diastereomers split by the corresponding alpha protons.

Fast-atom bombardment mass spectroscopy of glucuronides dispersed in thioglycerol showed fragments consistent with a glucuronide conjugate of ketoprofen. Major ions at m/z 453 and 276 corresponded to sodium adducts of both the molecular ion and fragment aglycone ion. In addition, fragment ions were recorded at m/z 253 (aglycone ion), 209 and 105.

Synthesis of ketoprofen ethyl-ester

Racemic ketoprofen (200 mg, 0.79 mmol) in 5 ml of dichloromethane and 0.5 ml of thionyl chloride (previously distilled over quinoline and boiled linseed oil, successively) was refluxed for 2 h, and the solvent and excess thionyl chloride removed under a stream of purified nitrogen. The residue was dissolved in 2 ml of dry toluene and evaporated to dryness. This azeotroping process was repeated. The resulting crude racemic ketoprofen acid chloride was dissolved in 5 ml of dichloromethane and added dropwise with stirring (4 h) over an ice-bath to 1 ml of anhydrous ethanol containing 20 µl of dry pyridine. The solution was filtered, evaporated to dryness and redissolved in 5 ml of dichloromethane. This solution was washed thrice with 10 ml of phosphate buffer (0.1 *M*, pH6). The organic layer was dried (magnesium sulphate) and the solvent removed under a stream of nitrogen to yield ketoprofen ethyl-ester as an oil (167 mg, 75%). A portion of this material was subjected to complete alkaline hydrolysis

(1.0 *M* sodium hydroxide, 50°C, 2 h) and yielded an acid with physical properties identical with those of authentic racemic ketoprofen.

Hydrolysis experiments

The rates of net hydrolysis of the glucuronide or ethyl-esters of (R)- and (S)-ketoprofen were determined in a number of different matrices. The concentrations of the diastereomeric glucuronides or enantiomeric ethyl-esters were determined by a difference method. The concentration of each hydrolysed enantiomeric aglycone, after a given incubation time, was subtracted from the initial time zero concentration of ester (expressed as ketoprofen equivalents) and the difference subsequently normalised as a percentage of the initial ester concentration.

For the net hydrolysis of the glucuronoconjugates of ketoprofen, the HPLC-purified diastereomeric glucuronides were added to various media to achieve a concentration of approximately 15 μg/ml (*circa* 8.3 μg/ml and 6.7 μg/ml of (S)- and (R)-ketoprofen glucuronide, respectively) expressed as ketoprofen equivalents. The media comprised: (*i*) fraction V human serum albumin (HSA) (Calbiochem, San Diego, CA; lot 902736, 99.5% pure by electrophoresis) at concentrations of 40 g/l, 4.0 g/l and 0.40 g/l in isotonic phosphate buffered (0.067 *M*, pH7.4) saline (PBS), (*ii*) essentially fatty acid-free fraction V HSA (Sigma Chem. Co.; lot 118F9311) 4.0 g/l in PBS and, (*iii*) plasma obtained via arm vein venepuncture from four healthy volunteers. The media were adjusted to a final pH of 7.4 with orthophosphoric acid (2.5%) and a temperature of 37°C (oscillating waterbath, 20 cpm). In addition, the hydrolysis of ketoprofen glucuronides was determined in protein-free PBS at pH7.4 and pH9.0. The influence of warfarin sodium (100 μ*M*; Sigma Chem. Co.) and diazepam (100 μ*M*; Roche, Sydney, Aust.) on the rates of hydrolysis of the diastereomeric glucuronides was examined in HSA solutions by pre-incubating with warfarin or diazepam for 15 min prior to addition to ketoprofen glucuronides. The experiments were performed throughout by adding fresh temperature- and pH-adjusted medium (10.0 ml) to a glass culture tube containing the glucuronides (from a methanolic solution evaporated to dryness under nitrogen immediately prior to addition of medium). This solution was mixed and rapidly aliquoted (1.00 ml) into individual glass tubes which were closed with PTFE-lined screw caps

and returned to the waterbath. One of the aliquots of glucuronides was hydrolysed to completion (1.0 *M* sodium hydroxide, 100 µl, 22°C, 15 min) to determine the initial (time zero) (R)- and (S)-ketoprofen glucuronide concentration. Thereafter, timed samples were removed from the waterbath and the hydrolysis reaction quenched (pH decreased to 3.0; Faed, 1984; see Section 3.3) with 0.025 ml of 2.0 *M* sulphuric acid. Incubations of glucuronides were carried out for up to 6 h in the case of plasma and 40 g/l HSA, up to 8 h for 4.0 g/l HSA and up to 24 h for 0.4 g/l HSA and protein-free PBS solutions. The quench conditions were verified experimentally for ketoprofen glucuronides. The liberated aglycones were immediately extracted into hexane/ethyl acetate prior to enantioselective analysis as described in Section 4.2. The influence of physostigmine (8.0 m*M*; Sigma Chem. Co.) and sodium fluoride (120 m*M*; Ajax Chemicals, Sydney, Aust.) on the rates of hydrolysis of the acyl-linked glucuronoconjugates of ketoprofen in plasma were also examined.

Hydrolysis experiments with the ethyl-esters of ketoprofen were essentially the same as described for the glucuronides, with the following exceptions. The synthesized material spiked into media (15 µg/ml) was racemic and complete hydrolysis (time zero aliquot) was effected by 100 µl of 1.0 *M* sodium hydroxide at 50°C for 2 h. Fraction V HSA (40 g/l), human plasma from a single volunteer and protein-free PBS (all at 37°C and pH7.4) were the media tested. The influence of physostigmine (8.0 m*M*) and sodium fluoride (120 m*M*) on the rates of hydrolysis of the enantiomeric ethyl-esters was examined. Incubations of these esters were carried out for up to 6 h. Again, the difference method was used to quantify the ethyl-esters of (R)- and (S)-ketoprofen.

Rearrangement of ketoprofen glucuronides

The degradation of the biosynthetic glucuronides of (R)- and (S)-ketoprofen (present together at 15 µg/ml of (R)/(S):44/56) to respective rearrangement isomers was monitored in PBS at 37 °C and pH7.4 with and without 40 g/l of fraction V HSA by the so-called "fractionated" hydrolysis method (Spahn-Langguth and Benet, 1992). These experiments were essentially the same as described above for assessing the net hydrolysis (deconjugation) of conjugates, with additional parallel samples cleaved with purified type VII-A β-glucuronidase (50 units/ml, pH *circa* 7.4, 37°C, 15 min). Conjugates which were resistant to this optimised β-glucuronidase

treatment were assumed to be rearrangement isomers, and together with the β-glucuronidase-sensitive conjugates (the original biosynthetic glucuronides), were quantified indirectly by the difference method (*vide supra*) after accounting for acyl-linked metabolite hydrolysis due to the respective incubation media.

Protein binding of ketoprofen glucuronides

The *in vitro* reversible protein binding of (R)- and (S)-ketoprofen glucuronide was determined in human plasma and in various concentrations of HSA (both fraction V and fatty acid-free) with and without warfarin (100 μ*M*) or diazepam (100 μ*M*). Binding was determined at physiological pH and temperature. The unbound species were obtained by ultra-filtration (Centrifree®, Amicon Division, Danvers, U.S.A.) of 1.0 ml aliquots in a pre-warmed (37°C) centrifuge (2000 *g*, 10 min; Heraeus Suprafuge 22 with HFA20.16 fixed-angle rotor, Osterode, Germany). Less than 15 min elapsed between the addition of glucuronides to plasma or HSA and the acid-quenching of the corresponding ultra-filtrate. The concentrations of unbound diastereomeric glucuronides were determined by the difference method (*vide supra*). Typically, the aglycone concentration in the non-hydrolysed (acid-quenched) ultra-filtrate was less than 10% of that in the alkaline hydrolysed ultra-filtrate. There was no detectable sorption of glucuronides onto the ultra-filtration membrane.

In the experiments conducted with human plasma, the protein binding of (R)- and (S)-ketoprofen glucuronide (present together as 44.6% (R)- and 55.4% (S)-) was examined over the plasma concentration range of approximately 2.0-40 μg/ml (expressed as unresolved ketoprofen equivalents). Plasma (harvested within 1 h of use) was obtained from each of four healthy volunteers, none of whom was taking any medication (2 female, age range: 27-54 yr, serum albumin concentration range: 37-42 g/l; see Table 6.1).

The binding of ketoprofen glucuronides, at a constant concentration of 15 μg/ml (8.3 and 6.7 μg/ml of (S)- and (R)-ketoprofen glucuronide, respectively) was investigated in the HSA solutions. When the influence of warfarin or diazepam on the binding of the glucuronides was investigated, HSA solutions were preincubated (15 min) with warfarin or diazepam prior to addition of the glucuronides.

Analytical methods

In order to quantify the glucuronide or ethyl-esters of (R)- and (S)-ketoprofen, samples (hydrolysis incubation media or ultra-filtrate) were alkali-hydrolysed (at time zero only, in the case of hydrolysis experiments) and corresponding matched or timed samples protected against hydrolysis (acid-quenched with 0.025 ml of 2.0 *M* sulphuric acid). The difference in the aglycone concentrations between the alkali-treated samples and those of acid-protected samples was taken as a measure of (R)- or (S)-ketoprofen: glucuronide or ethyl-ester. The degradation of the biosynthetic glucuronides and the appearance of their isomerization products were assessed by cleavage of timed samples with β-glucuronidase. The fractionated difference method was used in these experiments with aglycone arising from β-glucuronidase treatment assumed to represent the original 1-*O*-β-acyl-glucuronide metabolite. The enantioselective HPLC analytical method employed for ketoprofen has been described in detail elsewhere (see Section 4.2). Calibration was carried out by spiking drug-free incubation media (hydrolysis experiments) or compound sodium lactate injection B.P. or PBS (protein binding ultra-filtrate from plasma or HSA, respectively) with racemic ketoprofen in the concentration range 0.31-20.0 μg/ml.

Data analysis

Half-lives ($t_{½}$) for the net hydrolysis of glucuronide or ethyl-esters of ketoprofen were calculated for the apparent first-order processes in each of the media after performing least-squares linear regression analysis (from time zero) of the logarithmic transform of percentage remaining ester as a function of linear time. The correlation coefficient (*r* value) was calculated for the incubation experiments. The percentage unbound values of the glucuronides (*%Unbound*) were calculated as the ultra-filtrate (unbound) concentration of (R)- or (S)-ketoprofen glucuronide divided by the total (bound plus unbound) concentration of the respective glucuronide. Analysis of variance or the Student's *t* test was used, as appropriate, to assess the statistical significance of differences between groups. Differences were considered significant at $P<0.05$.

6.3 Results

Hydrolysis of glucuronides to aglycones

The apparent first-order net hydrolysis $t_{½}$ of (R)-ketoprofen glucuronide was less than 50% of the corresponding value for (S)-ketoprofen in human plasma at physiological pH and temperature (Table 6.1 and Figure 6.1, upper panels). This rapid, stereoselective deconjugation reaction was unaffected by the presence of plasma esterase inhibitors (8.0 m*M* physostigmine or 120 m*M* sodium fluoride; data not shown). The $t_{½}$ values for the glucuronides following incubation in a physiological concentration of HSA (40 g/l in PBS; Table 6.2) were similar to those recorded in plasma. Whilst maintaining a constant initial concentration of glucuronides (15 µg/ml) and decreasing the incubate HSA concentration by successive orders of magnitude, $t_{½,(R)\text{-gluc}}$ and $t_{½,(S)\text{-gluc}}$ increased to a far lesser extent than the corresponding fall in HSA concentration (Table 6.2). The marked stereoselective nature of the glucuronoconjugate net hydrolysis was maintained in these less concentrated HSA solutions. Net hydrolysis of ketoprofen glucuronides in protein-free PBS was minimal at physiological pH and temperature. Rapid and largely non-stereoselective hydrolysis of the conjugates was observed when the pH of this solution increased to pH9.0 (Table 6.2).

Table 6.1 *In vitro* net hydrolysis and reversible protein binding of (R)- and (S)-ketoprofen glucuronide in human plasma harvested from four healthy volunteers.

Subject (gender)	*Age* (yr)	*Serum Albumin* (g/l)	$t_{1/2,(R)\text{-gluc}}$ (hr)	$t_{1/2,(S)\text{-gluc}}$ (hr)	$\%Unbound_{(R)\text{-gluc}}$[a]	$\%Unbound_{(S)\text{-gluc}}$[a]
I (f)	28	38	1.77	4.71	11.1	8.59
II (m)	54	39	1.11	2.93	14.4	9.76
III (f)	27	37	1.18	2.99	12.6	9.37
IV (m)	26	42	1.42	3.22	12.3	8.77
Mean			1.37*	3.46	12.6**	9.12
SD			0.30	0.84	1.4	0.54

[a] Analysis of variance indicated that there was no difference ($P>0.05$) in $\%Unbound_{(R)\text{-gluc}}$ and $\%Unbound_{(S)\text{-gluc}}$ over the concentration range examined (approximately 1-20 µg/ml of ketoprofen equivalents) and therefore the values for each subject at the various concentrations were averaged.

* Statistically different ($P<0.005$; unpaired t test) from the corresponding value for (S)-ketoprofen glucuronide.

** Statistically different ($P<0.005$; analysis of variance) from the corresponding value for (S)-ketoprofen glucuronide.

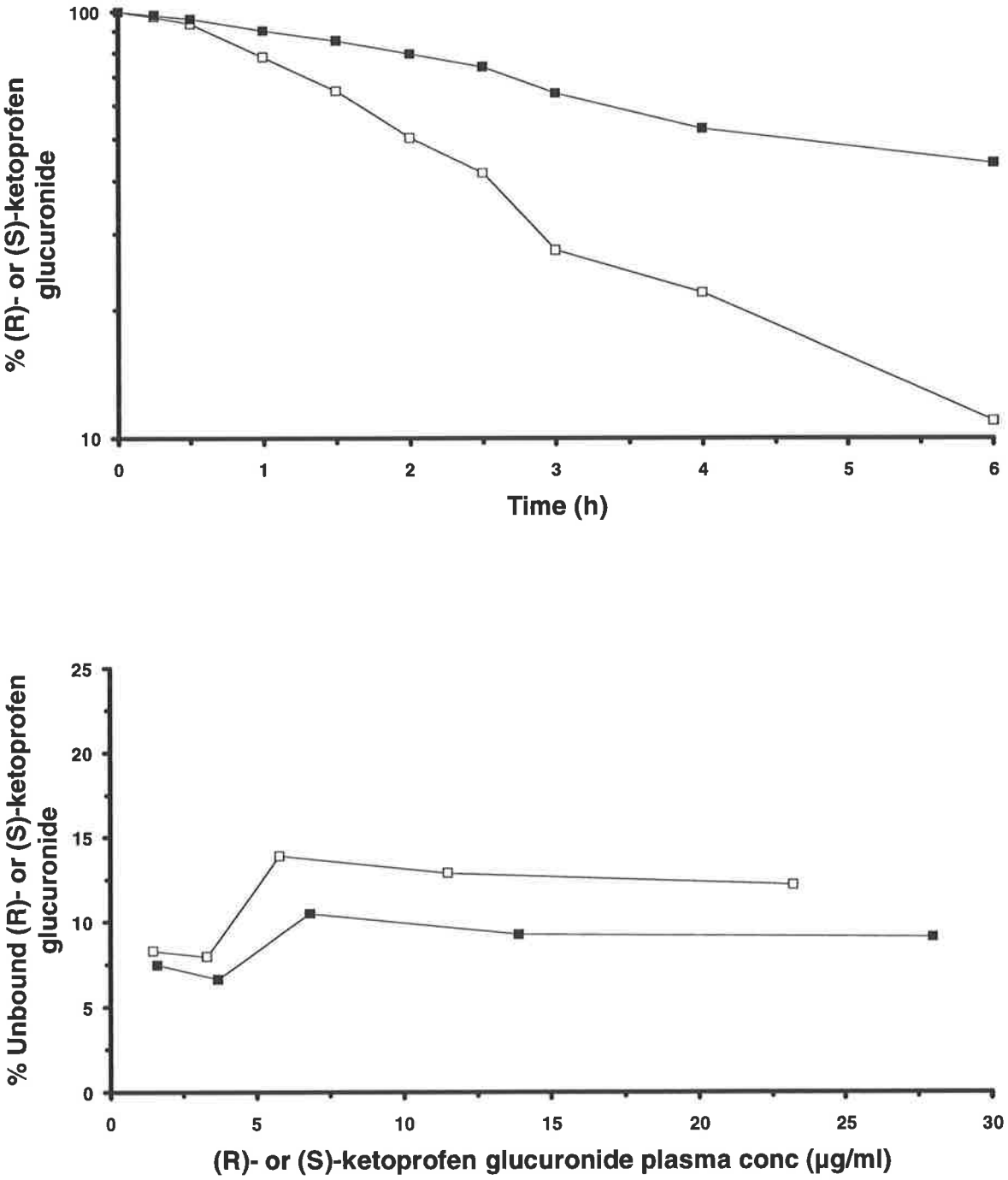


Figure 6.1 Net hydrolysis and protein binding (expressed as ketoprofen equivalents) at physiological pH and temperature of (R)-ketoprofen acyl-glucuronide (❑) and (S)-ketoprofen acyl-glucuronide (■) in plasma from subject **I**. The upper panel is a semi-logarithmic plot of percentage glucuronides remaining after incubation of 15 µg/ml of combined (R)- and (S)-acyl-glucuronides and the lower panel depicts the protein binding of the glucuronides.

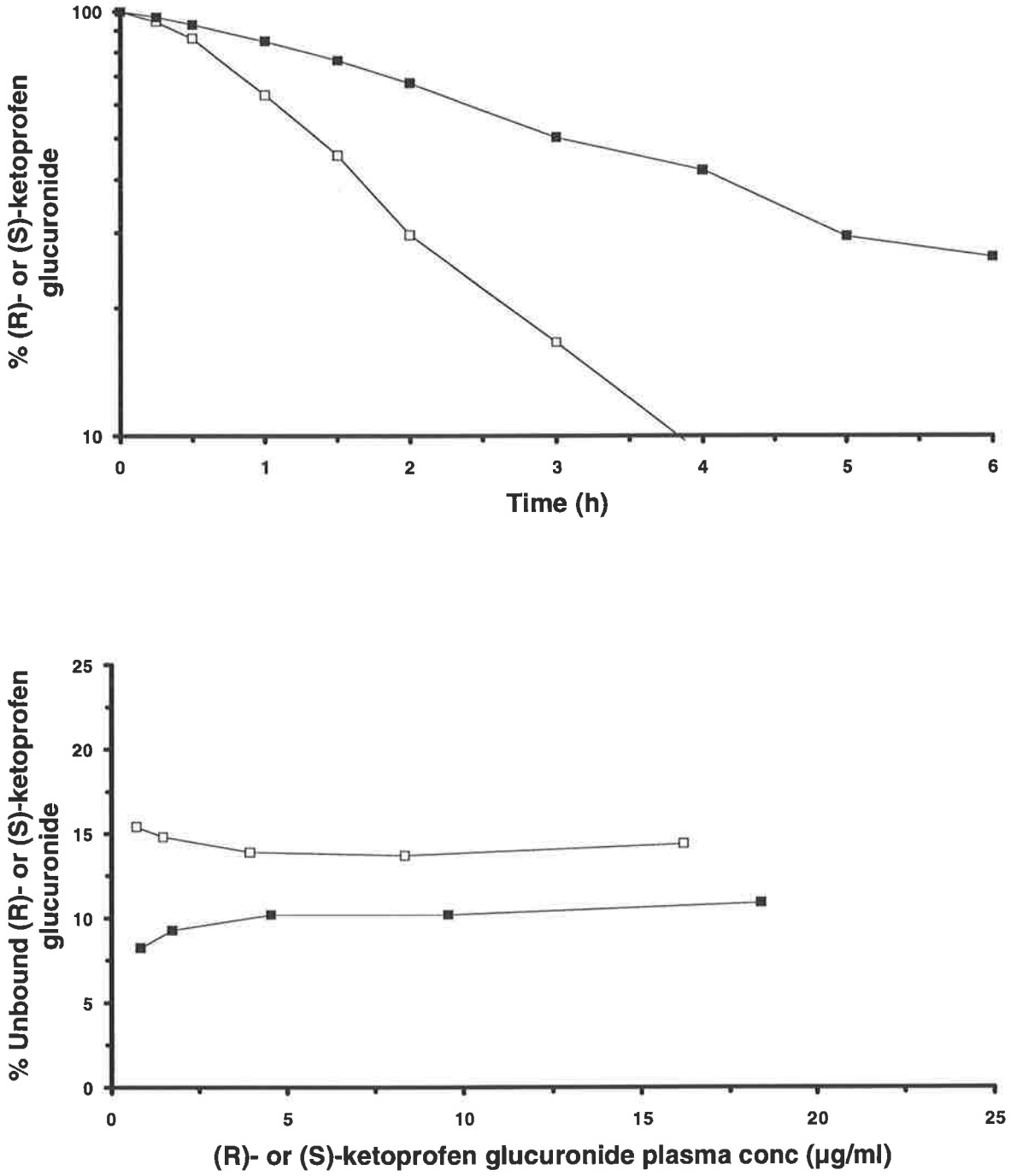


Figure 6.1 (continued) Net hydrolysis and protein binding (expressed as ketoprofen equivalents) at physiological pH and temperature of (R)-ketoprofen acyl-glucuronide (❑) and (S)-ketoprofen acyl-glucuronide (■) in plasma from subject **II**. The upper panel is a semi-logarithmic plot of percentage glucuronides remaining after incubation of 15 µg/ml of combined (R)- and (S)-acyl-glucuronides and the lower panel depicts the protein binding of the glucuronides.

Table 6.2 Net hydrolysis and reversible protein binding of (R)- and (S)-ketoprofen glucuronide (15 μg/ml of both metabolites; 6.7 μg/ml of (R)- and 8.3 μg/ml of (S)-ketoprofen glucuronide) upon incubation in fraction V human serum albumin (HSA), and net hydrolysis in protein-free isotonic phosphate buffered saline (PBS).

	HSA concentration in PBS (37°C, pH 7.4)			*Protein-free PBS* (37°C)	
	40 g/l	4.0 g/l	0.4 g/l	pH7.4	pH9.0
$t_{1/2,(R)\text{-gluc}}$ (hr)	1.68	2.70	5.47	>24	3.91
Correlation coefficient[a]	0.986	0.998	0.996	0.998	0.998
$\%Unbound_{(R)\text{-gluc}}$[b]	16.9*	68.0*	94.5	-	-
SD	0.9	1.5	2.6		
$t_{1/2,(S)\text{-gluc}}$ (hr)	6.75	7.34	17.5	>24	3.48
Correlation coefficient[a]	0.993	0.997	0.999	0.995	0.996
$\%Unbound_{(S)\text{-gluc}}$[b]	11.7	60.4	93.4	-	-
SD	0.5	1.7	2.7		

[a] Correlation coefficient (*r* value) calculated for the linear regression of the logarithmic transformation of percentage glucuronide remaining as a function of time.

[b] Mean and standard deviation of replicate determinations of HSA binding of each glucuronide (n = 6 for 40g/l and 4.0g/l; n = 3 for 0.4g/l) .

* Statistically different ($P<0.0001$; unpaired *t* test) from the corresponding value for (S)-ketoprofen glucuronide.

Results from the co-incubation of either warfarin sodium (100 μ*M*) or diazepam (100 μ*M*) with ketoprofen glucuronides in either fraction V or fatty acid-free HSA (4.0 g/l) are given in Table 6.3. Warfarin appeared to marginally increase $t_{½,(S)\text{-gluc}}$ in both HSA-type solutions and $t_{½(R)\text{-gluc}}$ in fatty acid-free HSA. Coincubations of glucuronides with diazepam gave rise to slight changes in $t_{½}$ values from control values, notably, an increase in $t_{½,(R)\text{-gluc}}$ in both HSA-type solutions, an increase in $t_{½,(S)\text{-gluc}}$ in fatty acid-free HSA and a decrease in $t_{½,(S)\text{-gluc}}$ in fraction V HSA.

Table 6.3 Influence of warfarin (100 μ*M*) and diazepam (100 μ*M*) on the net hydrolysis and reversible protein binding (37°C, pH7.4) of (R)- and (S)-ketoprofen glucuronide (15 μg/ml of both metabolites; 6.7 μg/ml of (R)- and 8.3 μg/ml of (S)-ketoprofen glucuronide) in fraction V or fatty acid-free HSA (4.0 g/l).

	Fraction V HSA			*Fatty acid-free HSA*		
	Control	*Warfarin*	*Diazepam*	*Control*	*Warfarin*	*Diazepam*
$t_{1/2,(R)\text{-gluc}}$ (hr)	2.70	2.82	3.04	2.86	3.19	3.00
%Unbound$_{(R)\text{-gluc}}$[a]	68.0	69.8	70.4*	55.8	57.7**	61.0***
SD	1.5	1.4	1.9	1.3	1.1	0.7
$t_{1/2,(S)\text{-gluc}}$ (hr)	7.34	9.13	6.21	6.35	8.74	7.64
%Unbound$_{(S)\text{-gluc}}$[a]	60.4	67.2***	61.6	52.5	58.0***	56.4***
SD	1.7	1.3	2.1	1.2	1.1	0.7

[a] Percentage unbound of (R)- and (S)-ketoprofen glucuronide expressed as the mean value and standard deviation from 6 replicate determinations.

Statistically different (*P<0.05, **P<0.01 and ***P<0.001: analysis of variance) from the corresponding value for the control sample (no warfarin or diazepam).

Protein binding of the glucuronides

The reversible binding of (R)- and (S)-ketoprofen glucuronide (present simultaneously) to human plasma protein was independent of glucuronide concentration (P>0.05; analysis of variance) over the range of 1.0-20 μg/ml (depicted for 2 representative subjects, Figure 6.1, lower panels). The mean (n = 4) *%Unbound* of (R)-ketoprofen glucuronide was approximately 1.4-times that of the corresponding value for its diastereomer (P<0.005; Table 6.1). When a constant amount of ketoprofen glucuronides was added to HSA solutions of varying strength, the stereoselective nature of the binding observed at 40 g/l and 4.0 g/l was abolished in the most dilute HSA solution (0.4 g/l); the *%Unbound* values for each diastereomer were approximately 95% in this solution (Table 6.2). Warfarin sodium (100 μ*M*) displaced (S)-ketoprofen glucuronide in both fraction V and fatty acid-free HSA (4.0 g/l,

$P<0.001$) and displaced (R)-ketoprofen glucuronide in fatty acid-free HSA only ($P<0.01$); diazepam (100 μ*M*) displaced (R)-ketoprofen glucuronide in both types of HSA ($P<0.05$ for fraction V and $P<0.001$ for fatty acid-free) and (S)-ketoprofen glucuronide in fatty acid-free HSA only ($P<0.001$). The magnitude of these changes were small in each case (Table 6.3).

Hydrolysis of the ethyl-esters of ketoprofen

The hydrolysis of the ethyl-esters of (R)- and (S)-ketoprofen in human plasma from a single volunteer and the influence thereon of physostigmine (8.0 m*M*) or sodium fluoride (120 m*M*) are depicted in Figure 6.2. In the absence of esterase inhibitors the apparent first-order hydrolysis $t_{½}$ values for the (R)- and (S)-enantiomers were 2.48 h ($r = 0.999$) and 3.14 h ($r = 0.999$), respectively. Both physostigmine and fluoride were effective in blocking ethyl-ester hydrolysis (Figure 6.2). There was negligible hydrolysis of these esters at physiological pH and temperature in either HSA solutions or in protein-free PBS solutions (data not shown).

Rearrangement of ketoprofen glucuronides

Figures 6.3 and 6.4 show the time-dependent degradation of (*i*) the original biosynthetic glucuronides of (R)- and (S)-ketoprofen, (*ii*) the respective appearance of migration isomers of the glucuronides and (*iii*) (R)- and (S)-ketoprofen aglycones arising from conjugate hydrolysis in the incubation media (protein-free PBS and PBS with HSA). In both media there was a rapid disappearance of the biosynthetic glucuronides of (R)- and (S)-ketoprofen, paralleled by the appearance of β-glucuronidase-resistant conjugates. After 8 h incubation in protein-free PBS, approximately 80% of the initial glucuronide concentration for both ketoprofen enantiomers were present as rearrangement isomers. Consistent with the earlier incubation experiments when net hydrolysis alone was monitored, the appearance of hydrolyzed aglycone was low (approximately 10% of the starting metabolite for both (R)- and (S)-enantiomers). The presence of HSA in the incubation media catalyzed the deconjugation of the acyl-linked conjugates of both (R)- and (S)-ketoprofen in a stereoselective fashion. Following 8 h incubation in this media, approximately 10% of (R)-ketoprofen and 40% of (S)-ketoprofen were present as migration isomers relative to the respective starting acyl-glucuronide at time zero.

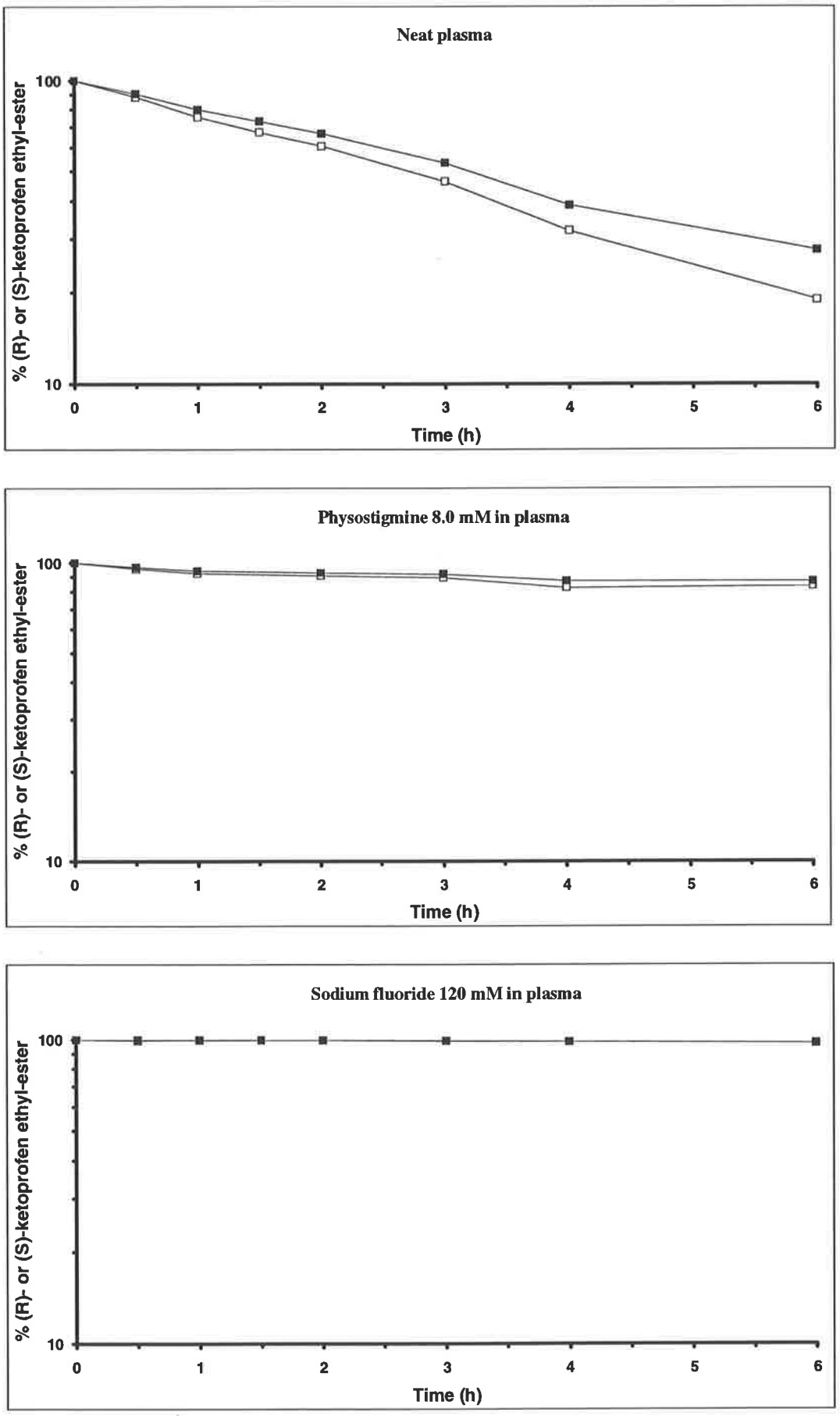


Figure 6.2 Plots of the hydrolysis (pH7.4, 37°C) of (R)-ketoprofen ethyl-ester (❑) and (S)-ketoprofen ethyl-ester (■) in human plasma (expressed as percentage remaining ester as acid equivalents) upon incubation of 15 µg/ml of racemic compound in each of: neat plasma, plasma with 8.0 m*M* physostigmine and plasma with 120 m*M* sodium fluoride.

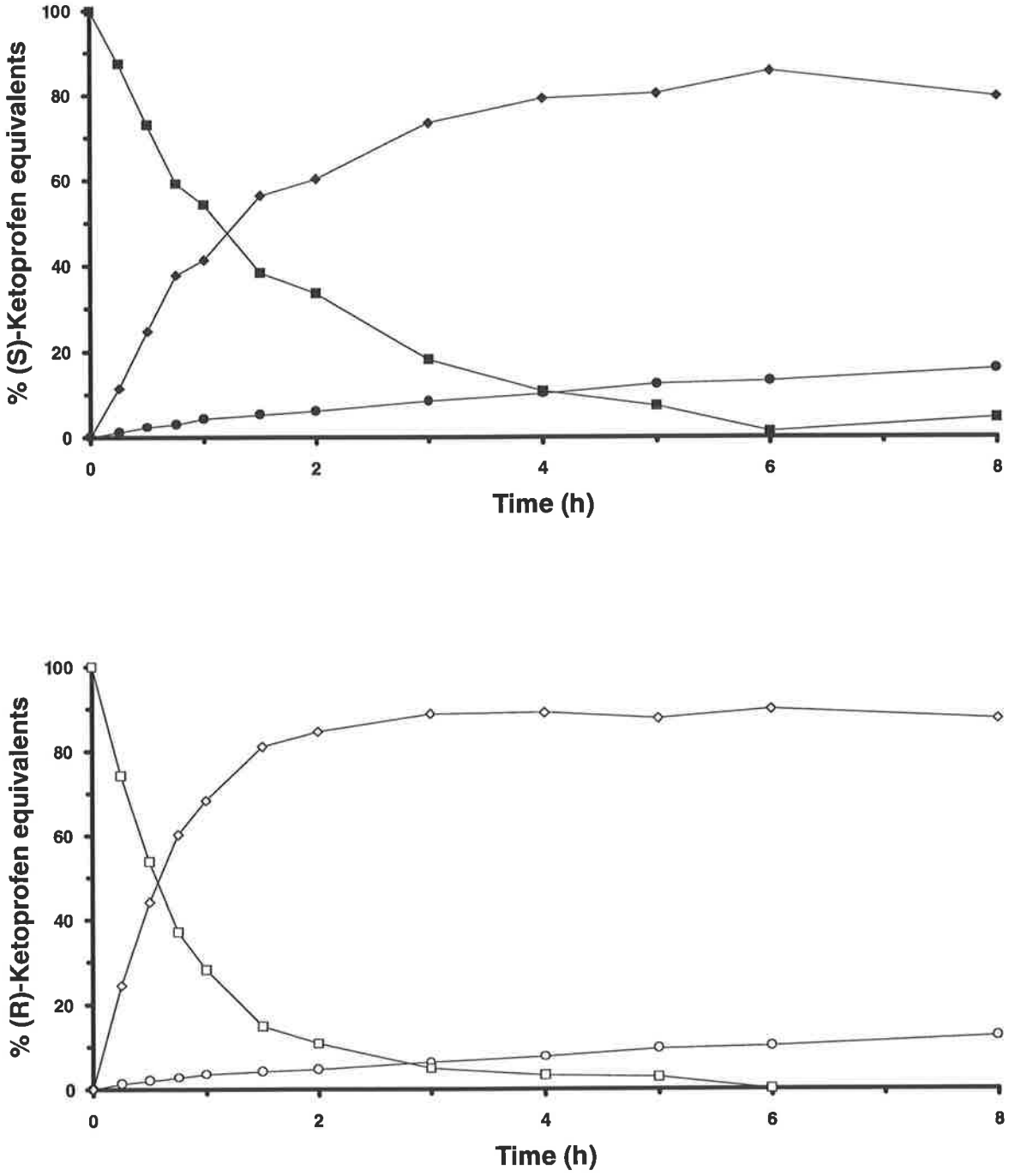


Figure 6.3 Profiles (expressed as aglycone equivalents) of the degradation of biosynthetic glucuronide (■, ❑), the appearance of migration isomers (◆, ◇) and the generated deconjugated drug (●, ❍) in PBS at physiological pH and temperature. The closed symbols represent (S)-ketoprofen (as aglycone or as conjugated drug) and the open symbols (R)-ketoprofen.

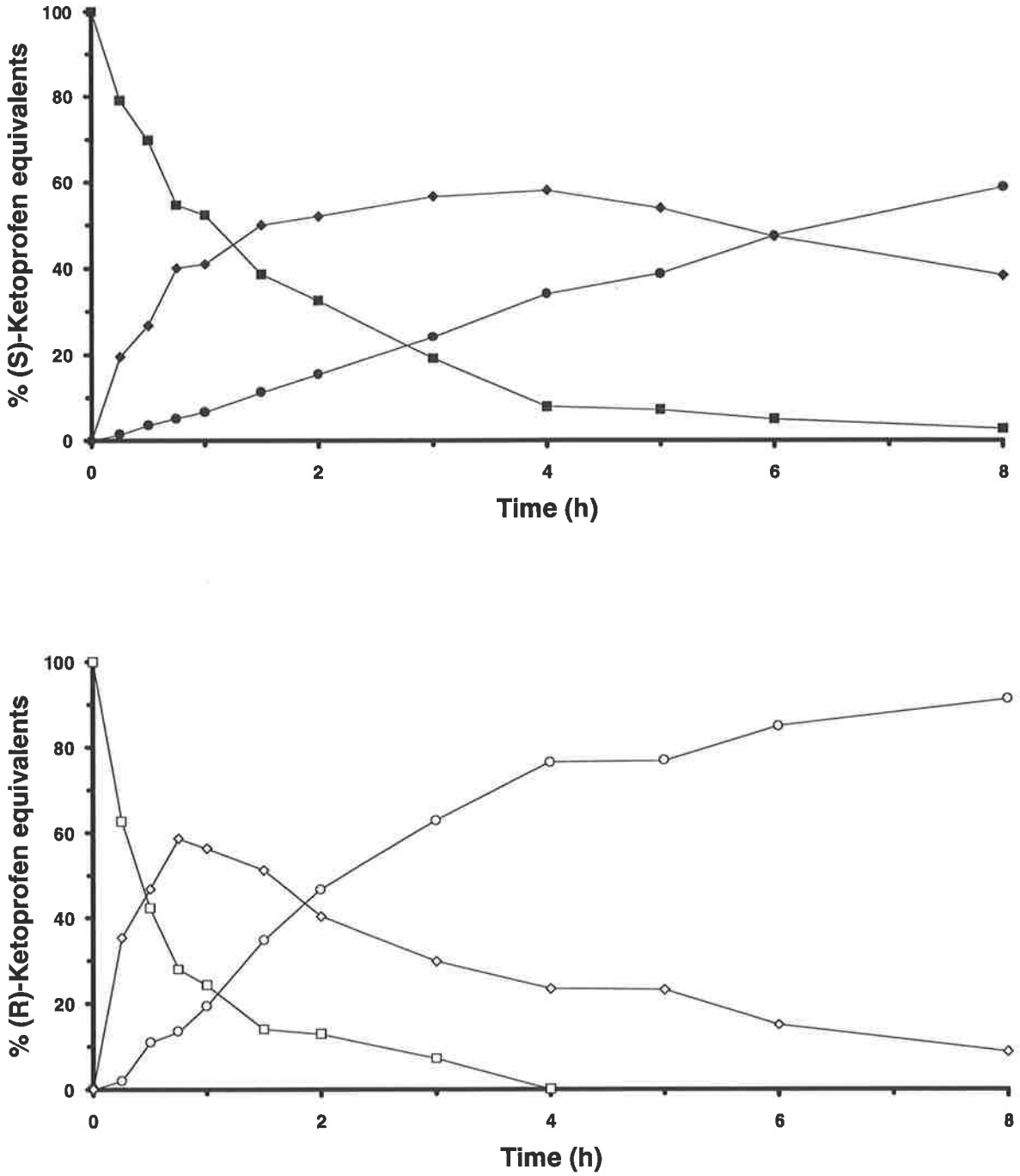


Figure 6.4 Profiles (expressed as aglycone equivalents) of the degradation of biosynthetic glucuronide (■, ❑), the appearance of migration isomers (◆, ◇) and the generated deconjugated drug (●, ❍) in PBS containing 40 g/l HSA (pH7.4, 37°C). The closed symbols represent (S)-ketoprofen (as aglycone or as conjugated drug) and the open symbols (R)-ketoprofen.

6.4 Discussion

These data clearly demonstrate the pivotal role of albumin in the rapid, stereoselective net hydrolysis of the glucuronoconjugates of ketoprofen *in vitro*. The similarity between plasma and HSA solution (40 g/l) suggests that albumin may be the major source of hydrolytic activity in plasma (Tables 6.1 and 6.2). Recent studies examining hydrolysis of conjugates of fenoprofen (Volland *et al.*, 1991), flurbiprofen (Knadler and Hall, 1991) and carprofen (Iwakawa *et al.*, 1988) attributed similar importance to albumin. Of interest, Knadler and Hall (1991) noted preferential hydrolysis of the conjugate of (S)-flurbiprofen in contrast to these studies with ketoprofen and previous studies with fenoprofen (Volland *et al.*, 1991) and carprofen (Iwakawa *et al.*, 1988) where the rate of conjugate hydrolysis was faster for the respective (R)-acyl-glucuronide. In contrast to glucuronide hydrolysis, HSA solutions (40 g/l) did not effect detectable ketoprofen ethyl-ester hydrolysis after 6 h incubations at physiological pH and temperature (data not shown). However, incubations of ethyl-ester with plasma led to significant hydrolysis (Figure 6.2). The marked degree of stereoselectivity elicited by plasma on ketoprofen glucuronide hydrolysis (Figure 6.1, upper panels) was in contrast to the studies with ketoprofen ethyl-esters. In the latter case, this plasma-derived esterase activity was largely indiscriminate of the stereochemical configuration of the acyl moiety. Interestingly, in a study of hydrolysis of various ester prodrugs of (RS)-ibuprofen the plasma-catalyzed hydrolysis of the ethyl-ester of (R)-ibuprofen was reported to be approximately 3-fold faster than that of its optical antipode (Mork and Bundgaard, 1992).

Human serum albumin has been shown to exhibit *in vitro* hydrolytic activity towards the acyl-glucuronides of fenoprofen (Volland *et al.*, 1991), oxaprozin (Ruelius *et al.*, 1986; Wells *et al.*, 1987) and carprofen (Iwakawa *et al.*, 1988) and towards cinnamoylimidazoles (Ohta *et al.*, 1983), phenyl-esters (Kurono *et al.*, 1979) and *p*-nitrophenyl 4-guanidinobenzoate (Kurono *et al.*, 1991). In contrast, an *in vivo* study by Rowe and Meffin (1984) suggested that plasma esterases were responsible for the hydrolysis of clofibric acid glucuronide based on experiments with the irreversible esterase inhibitor, diisopropylfluorophosphate. These workers administered very high doses of the inhibitor to anaesthetized rabbits (approximately 30-fold that of the LD_{50} in conscious animals) and observed an increase in clearance of

clofibric acid, presumed to be due to blockade of esterase-mediated hydrolysis of the glucuronide. Such a large dose of diisopropylfluorophosphate could conceivably lead to alkylphosphorylation of albumin, as observed *in vitro* by Wells *et al.* (1987). This might implicate albumin as the macromolecule responsible for deconjugating clofibric acid in the above study (Rowe and Meffin, 1984). Further, Lin *et al.* (1986) observed a lack of effect of an alternative esterase inhibitor (phenylmethylsulphonyl fluoride) on the clearance of diflunisal (subject to significant acyl-glucuronide formation) in rats with experimentally-induced renal failure. Moreover, when ketoprofen glucuronides were incubated in plasma spiked with classical esterase inhibitors (physostigmine and sodium fluoride) the rates of net hydrolysis of ketoprofen glucuronoconjugates were unaffected (data not shown). These agents were verified to be potent inhibitors of plasma esterases as judged by a marked reduction in the rate of hydrolysis of (R)- and (S)-ketoprofen ethyl-ester (Figure 6.2).

It is interesting to note that the reversible binding of the glucuronide conjugates of ketoprofen to HSA and plasma exhibited significant stereoselectivity, while the protein binding of the aglycones in plasma obtained from similar volunteers was nonenantioselective (see Section 5.3). In the case of carprofen, stereoselectivity was observed, with respect to HSA binding, with both the carprofen glucuronide conjugates and the progenitive aglycones (Iwakawa *et al.*, 1990). It is also likely that incubations of ketoprofen glucuronides in protein solutions leads to the generation of covalent ketoprofen-protein adducts (irreversibly bound drug; see section 3.4) based on previous *in vitro* studies with acyl-glucuronides of other NSAIDs including fenoprofen (Volland *et al.*, 1991), diflunisal (Watt and Dickinson, 1990) and zomepirac (Smith *et al.*, 1986). The degree to which acyl-glucuronides form covalent adducts with proteins is quantitatively minor (1% or less for the three examples cited above) and time dependent. In this study, the degree of reversible protein binding of ketoprofen glucuronides was determined in solutions which had been incubated for less than 15 min. Given this short time interval the estimates of reversible binding would be unlikely to be influenced by covalent drug-protein adduct generation (presumptively) following *in vitro* incubation of ketoprofen glucuronides. It should also be noted that the reversible binding estimates obtained in this study are hybrid estimates, encompassing the protein binding of the biosynthetic glucuronides together with the binding of the rearrangement isomers.

Initially, it was suspected that the site on the albumin molecule at which ketoprofen glucuronide hydrolysis was taking place might be the same site at which the glucuronides bind, as suggested for oxaprozin glucuronide (Ruelius *et al.*, 1986; Wells *et al.*, 1987). However, significant and stereoselective binding of ketoprofen glucuronides to human plasma (Table 6.1 and Figure 6.1) and physiological concentrations of HSA (Table 6.2) was observed; the eudismic ratio for the *%Unbound* values was opposite to that for the $t_{½}$ values for the hydrolysis of the conjugates. This suggested the possible existence of a binding site distinct from the hydrolytic or catalytic site on the albumin molecule with respect to ketoprofen glucuronides. Indeed incubations of ketoprofen glucuronides (15 µg/ml) in very dilute HSA solutions (0.4 g/l or 6 µ*M*) where the ligand to albumin molar ratio was approximately 5:1 for each diastereomer, showed low, non-stereoselective binding yet markedly stereoselective conjugate hydrolysis (Table 6.2). It was also evident from these data (Table 6.2) that as binding of the glucuronides to HSA decreased (in successively more dilute protein solutions), more of these metabolites became available (as unbound glucuronides) for engagement with the catalytic site(s) on HSA molecules (and subsequent hydrolysis to their respective aglycones). A recent study with diflunisal acyl-glucuronide (Watt and Dickinson, 1990) supports the alternative hypothesis of separate catalytic and binding sites on the albumin molecule. It is apparent that diflunisal acyl-glucuronide, a highly protein bound conjugate, is partially protected from hydrolysis in HSA solutions by virtue of this binding site being distinct (and a significant sink for glucuronide) from the catalytic site. Furthermore, the preferential deconjugation of (R)-carprofen glucuronide by HSA (Iwakawa *et al.*, 1988) and the greater percentage unbound value for this diastereomer compared to (S)-carprofen glucuronide (Iwakawa *et al.*, 1990) provides additional support for the hypothesis. It is possible that oxaprozin glucuronide binds to, and is degraded at, a common site on the albumin molecule. Wells *et al.* (1987) reported this to be the benzodiazepine site, or Site II as classified by Sudlow *et al.* (1976). In contrast, carprofen glucuronides have been reported (Iwakawa *et al.*, 1990) to bind preferentially to the warfarin site (Site I).

Minor displacement of (R)- and (S)-ketoprofen glucuronide (*circa* 30 µ*M*) in fraction V and fatty acid-free HSA (4.0 g/l or 60 µ*M*) by warfarin (100 µ*M*) or diazepam (100 µ*M*) (Table

6.3) was observed. In parallel, slight increases were observed in the corresponding conjugate hydrolysis $t_{½}$ values in those solutions where displacement had taken place. While these changes were subtle they are still consistent with the concept of separate hydrolytic and binding sites on the albumin molecule. It is possible that warfarin and diazepam exert an allosteric effect on the hypothesized separate catalytic site(s). Alternatively, these competing ligands might bind to more than one site on the albumin molecule, including the catalytic sites(s).

The rearrangement of acyl-glucuronides from the biosynthetic 1-*O*-β-isomer to other positional isomers has been demonstrated for a number of NSAIDs and other carboxylic acids (see Section 3.3). Ketoprofen glucuronides undergo such reactions (Figures 6.3 and 6.4). Accordingly, the part of this study concerned with glucuronide hydrolysis (analysis by the difference method) describes this process as the net result of hydrolysis (deconjugation) of all possible glucuronide isomers including cleavage of the biosynthetic glucuronide. Caution is required in the interpretation of, for instance, the very slow net hydrolysis of conjugates in protein-free PBS at physiological temperature and pH (Table 6.2). Indeed, additional experiments revealed significant rearrangement of both (R)- and (S)-ketoprofen glucuronides to acyl-linked isomers in PBS both with and without HSA. In the case of protein-free PBS incubations, there was a relatively rapid degradation of biosynthetic glucuronides to isomeric conjugates which was not reflected by measurement of deconjugated drug alone. It was apparent (Figure 6.4) that the addition of HSA to the media catalyzed the deconjugation of the rearrangement isomers as judged by the profile for these conjugates, compared to the profile of the biosynthetic glucuronide disappearance. This is speculative at present in the absence of individual data for purified rearrangement isomers. However, these data (Figures 6.3 and 6.4) are consistent with the generally held concept of the acyl-glucuronide isomerization being a nonenzymatic process. Both in buffer and HSA solutions, the data suggest a faster rate of isomerization of the acyl-glucuronide of (R)-ketoprofen compared to the corresponding reaction for the acyl-glucuronide of (S)-ketoprofen. However, the stereoselectivity of the individual reaction processes for ketoprofen glucuronides remain to be confirmed by studies conducted with individual purified isomers. Moreover, the possibility of an *in vitro* stereoinversion process incorporating conversion of the glucuronide of (R)-ketoprofen to (S)-

ketoprofen glucuronide cannot be ruled out given the reported observation of this process (albeit minor) for the acyl-linked glucuronides of the 2-arylpropanoate fenoprofen (Volland *et al.*, 1991). In any case, fundamentally the purpose of this study has been to examine the net appearance of the enantiomeric aglycones [pharmacologically active in the case of the (S)-enantiomer (see Section 2.2.1 and Chapter 7)].

In summary, acyl-glucuronides are polar compounds generally restricted to the vascular and interstitial fluid compartments, regions containing significant concentrations of albumin. Thus interactions of such metabolites with individual components of these compartments are of particular importance in determining the disposition of both metabolite and pharmacologically active aglycone. These data implicate albumin to be of fundamental importance in the disposition of ketoprofen. Moreover, it is suggested that this macromolecule possesses separate binding and catalytic sites for the acyl-glucuronides of ketoprofen. The significant renal clearance of ketoprofen glucuronides (Upton *et al.*, 1982) implies that in subjects with diminished renal function, the interaction of these accumulated glucuronides with albumin becomes of major pharmacological significance.

Chapter 7

Enantioselective Pharmacodynamics of Ketoprofen: *In Vitro* Inhibition of Human Platelet Cyclo-Oxygenase Activity

7.1 Introduction

While a number of mechanisms have been proposed to explain the pharmacological effects of NSAIDs, it is believed that inhibition of prostanoid biosynthesis is of the greatest importance (see Section 2.2.1). Specifically, NSAIDs act as competitive inhibitors of the cyclo-oxygenase subunit of prostaglandin *H* synthase (Vane, 1971). In every case examined, inhibition of prostaglandin biosynthesis by members of the 2-arylpropanoic acid class of NSAIDs has been highly enantioselective with major or exclusive activity residing with enantiomers of (S)-configuration (Adams *et al.*, 1976; Buttinoni *et al.*, 1983; Cerletti *et al.*, 1990; Evans *et al.*, 1991; Gaut *et al.*, 1975; Ku and Wasvary, 1975; Moreno *et al.*, 1990; Nishizawa *et al.*, 1973; Rubin *et al.*, 1985; see Section 1.5).

To date, no detailed analysis of the potential enantioselective pharmacodynamics of ketoprofen has been performed. Moreno *et al.* (1990) have reported, in abstract form, the effect of ketoprofen enantiomers on arachidonic acid-induced rabbit platelet aggregation. These data were largely qualitative having been carried out at a single drug concentration (10^{-5} *M*); the (S)-enantiomer inhibited platelet aggregation by 83% in contrast to 16% inhibition reported for its optical antipode. The optical purity of the ketoprofen enantiomers was not stated in this report and hence caution is needed in the interpretation of biological activity measured for (R)-ketoprofen (Moreno *et al.*, 1990). As discussed in Section 1.5, experimentally recorded inhibition of eicosanoid-mediated effects by (R)-enantiomeric congeners of 2-arylpropanoates may be due to optical contamination of the test drug by the respective eutomer.

The present study was designed to examine, for the first time in man, the effect of ketoprofen enantiomers on platelet cyclo-oxygenase. The amount of thromboxane A_2 (TXA_2) generated

during the controlled clotting of whole blood was used as an index of cyclo-oxygenase activity (see Figure 2.2 in Section 2.2.1). In this technique, endogenous thrombin is the stimulus for TXA_2 generation. TXA_2 is a labile metabolite of prostaglandin G_2, it is a potent vasoconstrictor and inducer of platelet aggregation and is released in substantial quantities from aggregating platelets (Hamberg *et al.*, 1975; Roberts *et al.*, 1981). Whole blood clotting TXA_2 generation has been previously used as a means of monitoring the anti-platelet activity of NSAIDs (Cerletti *et al.*, 1987; Evans *et al.*, 1991; Longenecker *et al.*, 1985; Nuotto *et al.*, 1983; Patrignani *et al.*, 1982; Patrono *et al.*, 1980). The present investigation was undertaken in healthy volunteers to explore the *in vitro* relationship between the degree of inhibition of TXA_2 formation (drug effect) and the concentration of pharmacologically active unbound (S)-ketoprofen in serum.

7.2 Materials and Methods

Chemicals

(RS)-Ketoprofen was purchased from Sigma Chemical Company (St Louis, MO). The (R)- and (S)-enantiomers of ketoprofen were gifts of Dr Kathy Knights (Flinders University, Bedford Park, South Australia) and Drs Ralph Massy-Westropp and David Hamon (University of Adelaide, Adelaide, South Australia). The optical purity of these ketoprofen enantiomers was determined by an indirect enantioselective HPLC method (see Chapter 4). The optical purity of the HPLC chiral derivatizing reagent ((S)-1-phenylethylamine, Sigma Chem. Co., Lot: 10H3457) was determined by an extension of a ^{1}H-NMR spectroscopy method (Whitesides and Lewis, 1970) [kindly performed by Dr David Hamon, unpublished data]. Briefly, the method involved conversion of (S)-1-phenylethylamine to the less basic acetyl-amide prior to high-field ^{1}H-NMR in the presence of the chiral shift reagent $(hfc)_3Eu$. Comparison of the peak area of the optical impurity ((R)-1-phenylethylacetylamide) α-methyl doublet signal with the ^{13}CH satellite of the (S)-amide [by definition $\frac{1.1}{2}$% of the major signal] enabled an estimation to be made of the area of (R)-amide α-methyl doublet. The percentage optical purity of this chiral derivatizing amine used for assessing the optical purity of the enantiomeric ketoprofen substances was estimated to be in the order of 99.5%. Following replicate analysis (n = 3), the mean (± SD) optical purity of (R)-ketoprofen was 95.7 ± 0.06%

and of (S)-ketoprofen 99.0 ± 0.06%. Suppliers of chemicals used in the experiments when the serum protein binding of (S)-ketoprofen was determined have been cited earlier (see Section 5.2).

Assessment of platelet cyclo-oxygenase inhibition

To monitor the effects of ketoprofen enantiomers on platelet cyclo-oxygenase, whole blood was allowed to clot under controlled conditions. As an index of cyclo-oxygenase activity, the amount of TXA_2 generated was assessed by measuring the concentration of its stable breakdown product, thromboxane B_2 (TXB_2), in the harvested serum as described by Patrono *et al.* (1980). Measurement of serum TXB_2 concentrations was performed by Elizabeth Duncan at the Institute of Medical and Veterinary Science (Adelaide, South Australia). Blood (*circa* 40 ml) was collected by venepuncture from each of four healthy volunteers, none of whom were taking any medication. The mean (± SD) age of the subjects (2 male) was 34.5 (± 13.7) yr. For a given human subject, 1.0 ml of blood was immediately transferred (needle removed) to each of a series of pre-calibrated Pyrex® medium-walled borosilicate culture tubes (10 x 75mm) containing a range of quantities (*vide infra*) of (R)-ketoprofen and/or (S)-ketoprofen. Each tube, which also contained 50 µl of sodium chloride 0.9% injection B.P., had been prewarmed to 37°C in a dry heat bath. The tube contents was gently mixed and returned to the heat bath and maintained at 37°C for 1 h. After the 1 h incubation, the tube was rimmed with a glass rod to detach any unretracted portions of the clot and centrifuged at 2000 *g* (10 min). The harvested serum was stored at -20°C until analysed for TXB_2 content by Ms Elizabeth Duncan at the South Australian Institute of Medical and Veterinary Science (Adelaide) using an established radioimmunoassay method (Fitzpatrick, 1982).

The final blood concentrations of (R)-ketoprofen, (S)-ketoprofen and (RS)-ketoprofen for each subject were as follows: control samples (n = 2), containing no ketoprofen; (R)-ketoprofen at 0.01, 0.1, 0.5, 1, 5, 10 and 20 µg/ml; (S)-ketoprofen at 0.005, 0.01, 0.05, 0.1, 0.5, 1 and 5 µg/ml; and (RS)-ketoprofen at 0.01, 0.02, 0.1, 0.2, 1 and 2 µg/ml. The blood concentration of (S)-ketoprofen in each tube containing racemic drug was 50% (by definition) of the stated (RS)-ketoprofen concentration value. In those tubes containing (R)-ketoprofen alone, the blood concentration of (S)-ketoprofen (present as optical impurity) was derived by

transforming the (R)-ketoprofen value by the fractional impurity value (4.30%, *vide supra*). Thus (S)-ketoprofen was spiked into blood in three fashions: (*i*) as the predominant enantiomer, (*ii*) in equal proportion to its optical antipode and (*iii*) as the minor enantiomer.

Derivation of unbound (S)-ketoprofen concentration in serum

The unbound (S)-ketoprofen serum concentration ($Conc_{(S)\text{-unbound}}$) was derived for each of the spiked blood samples by performing separate *in vitro* drug binding and blood/serum partitioning experiments with each study subject's blood and serum. The $Conc_{(S)\text{-unbound}}$ values could not be measured directly in the serum samples used for TXB_2 analysis as these fell below the assay limits of quantification of the unbound species (see Chapter 5). The fraction unbound of (S)-ketoprofen ($fu_{(S)}$) was determined as the arithmetic average of four values over a spiked serum concentration range of (RS)-ketoprofen of 2.00-12.0 μg/ml for each individual subject. In addition, it was confirmed that the fraction unbound of (S)-ketoprofen was constant over this concentration range (analysis of variance, $P<0.05$). The enantioselective binding methodology is detailed elsewhere where it was also established that the plasma protein binding of both stereoisomers was independent of concentration over the range used in these experiments and nonenantioselective (see Chapter 5). The blood to serum concentration ratio of (S)-ketoprofen ($[B]_{(S)}/[S]_{(S)}$) was calculated as the ratio of the measured concentration of (S)-ketoprofen in serum derived from spiking harvested serum with 2.00 μg/ml of (RS)-ketoprofen to the serum concentration of (S)-ketoprofen subsequent to spiking whole blood with 2.00 μg/ml of (RS)-ketoprofen. Pilot studies revealed that the partitioning of (S)-ketoprofen between blood and serum was independent of drug concentration and that equilibration occurred within 15 min. Consequently, individual subject estimates of $[B]_{(S)}/[S]_{(S)}$ were obtained in duplicate following incubations for 1 h at 37°C. Concentrations of total (bound plus unbound) (S)-ketoprofen were determined by the enantioselective HPLC assay detailed in Chapter 5. The concentration of unbound (S)-ketoprofen in serum following spiking of whole blood with drug ($Conc_{(S)\text{-blood}}$) was calculated according to Equation 7.1.

$$Conc_{(S)\text{-unbound}} = \frac{Conc_{(S)\text{-blood}} \times fu_{(S)}}{[B]_{(S)}/[S]_{(S)}} \qquad (7.1)$$

Pharmacodynamic analysis of TXB_2 data

For each subject, the control serum concentration of TXB_2 was determined by averaging the TXB_2 concentration in the two blood samples containing no ketoprofen. For samples containing ketoprofen, inhibition of TXB_2 production during whole blood clotting was calculated as the percentage decrease in the serum concentration of TXB_2, relative to the control concentration. For each subject, data from each of three approaches for spiking various amounts of (S)-ketoprofen into blood (as predominantly (S)-ketoprofen, as (RS)-ketoprofen, and as predominantly (R)-ketoprofen) were pooled. This was done since it was evident that the logarithmic concentration/effect data for (S)-ketoprofen, for each of the three spiking approaches, were superimposed. Subsequently, the relationship between the unbound serum concentration of (S)-ketoprofen and the percentage inhibition of TXB_2 generation was examined by fitting a standard sigmoidal E_{max} equation to the data with an extended least-squares modelling computer program (Multifit 2.0, Day Computing, Cambridge, U.K.). The sigmoidal E_{max} equation is:

$$E = \frac{E_{max} \times Conc^n}{EC_{50}{}^n + Conc^n} + E_0 \qquad (7.2)$$

where E is the measured effect at drug concentration Conc; E_0 is the basal effect in the absence of drug; E_{max} is the maximal effect; EC_{50} represents the concentration of drug required to cause 50% of E_{max}; and n is the steepness factor for the logarithmic concentration-effect relationship (Holford and Sheiner, 1981). In the present case E was expressed as a percentage of the maximal possible effect of the drug, and hence E_0 was set at zero and E_{max} at 100, thereby simplifying Equation 7.2 to:

$$\%\ \text{Inhibition} = \frac{100 \times (Conc_{(S)\text{-unbound}})^n}{EC_{50}{}^n + (Conc_{(S)\text{-unbound}})^n} \qquad (7.3)$$

Equation 7.3 was applied individually for each subject after pooling data derived from the three approaches to spiking (S)-ketoprofen into blood. Several weighting schemes were explored with the least-squares analysis, the most appropriate being the reciprocal of % TXB_2 inhibition (i.e. $\frac{1}{\%\ \text{Inhibition}}$).

7.3 Results

The effect of (S)-ketoprofen (expressed as pharmacologically active unbound drug) on *in vitro* TXB_2 generation during the controlled clotting of whole blood for each subject is depicted in Figure 7.1. When (RS)-ketoprofen and (R)-ketoprofen were added to blood to assess their effect on platelet TXB_2 production, no effect was attributed to the presence of (R)-ketoprofen. In contrast, (S)-ketoprofen present either alone, as racemic drug, or as a minor enantiomeric impurity elicited a concentration-dependent inhibition of TXB_2 production by platelets. This effect was not modified by the presence of (R)-ketoprofen (Figure 7.1). It was found, for a given subject, that the concentration-response curves (expressed in terms of unbound (S)-ketoprofen in serum) constructed for each of the three ketoprofen spiking approaches were identical. Subsequently, to improve the precision of each subject's model parameter estimates, data derived from adding (S)-ketoprofen to blood as the predominant enantiomer, as racemic drug and as the minor enantiomer, were pooled. When the effect of (S)-ketoprofen on TXB_2 generation was modelled, according to a sigmoidal E_{max} equation, the arithmetic mean unbound serum concentration of (S)-ketoprofen leading to a 50% inhibition of TXB_2 generation was found to be 0.320 ng/ml (Table 7.1). The corresponding slope factor of the concentration-effect curve (n) was 1.52.

Table 7.1 Fraction unbound in serum ($fu_{(S)}$) and the blood to serum concentration ratio ($[B]_{(S)}/[S]_{(S)}$) of (S)-ketoprofen for each subject together with computer generated sigmoidal E_{max} model parameters describing the relationship between the unbound serum concentration of (S)-ketoprofen and the percentage inhibition of TXB_2 generation.

Subject	*Age* (yr) *(Gender)*	$fu_{(S)}$	$[B]_{(S)}/[S]_{(S)}$	EC_{50}(*SE*)* ng/ml	*n* (*SE*)*
#1	28 (f)	0.00745	0.578	0.331 (0.032)	1.93 (0.18)
#2	29 (m)	0.00770	0.578	0.402 (0.059)	1.25 (0.18)
#3	26 (f)	0.00993	0.625	0.286 (0.029)	1.48 (0.16)
#4	55 (m)	0.00782	0.588	0.262 (0.038)	1.41 (0.19)
Mean		0.00823	0.592	0.320	1.52
SD		0.00115	0.022	0.062	0.29

*(*SE*) denotes the standard error of the model parameter estimate

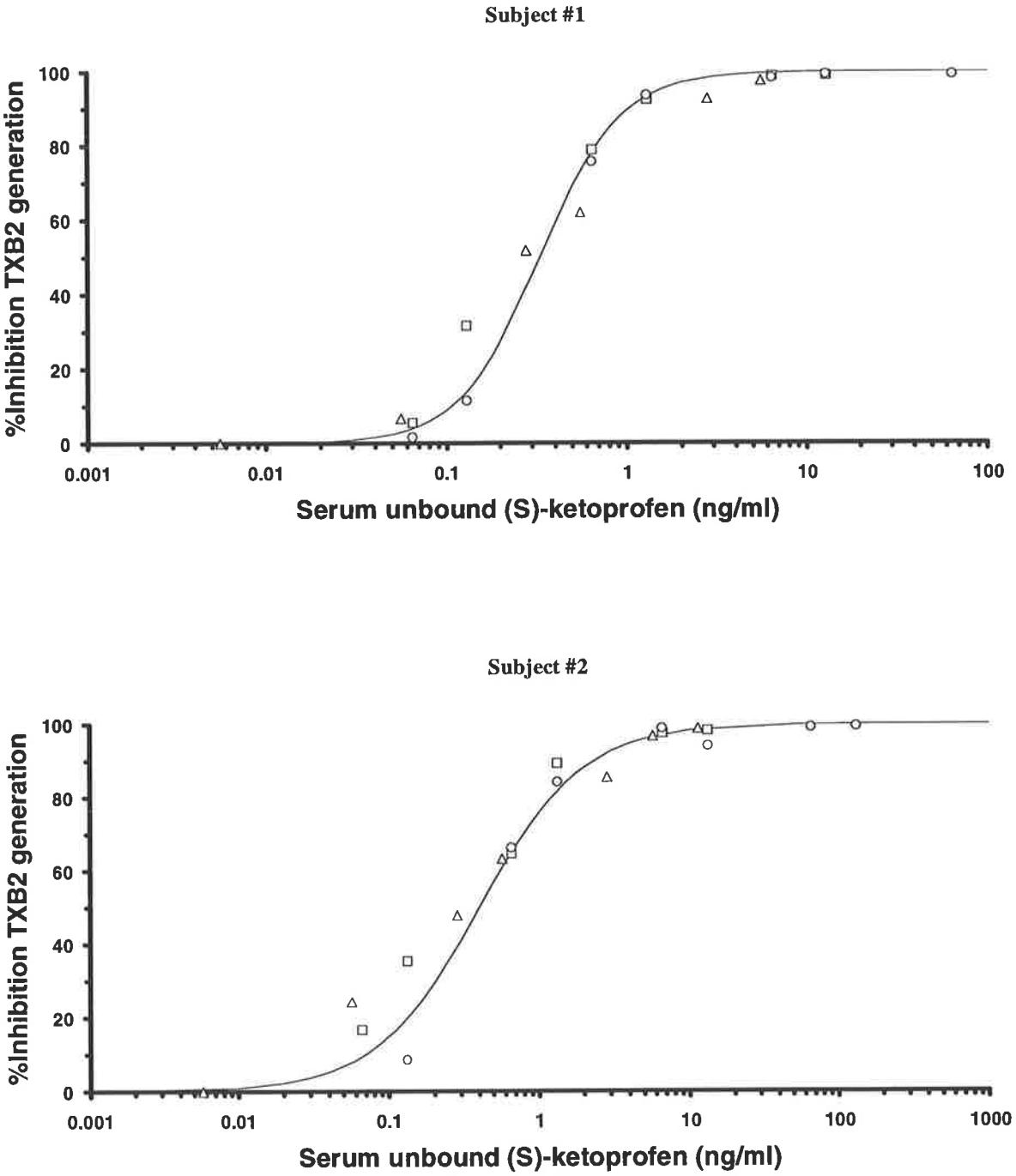


Figure 7.1 *In vitro* relationships between the percentage inhibition of TXB_2 generation and the logarithmic serum concentration of unbound (S)-ketoprofen for study subjects #1 to #4. The symbols are actual data points when (S)-ketoprofen was added to blood as 99.0% optically pure drug (○), as racemic drug (□), and as 4.3% optical impurity together with its antipode (Δ). The line represents the predicted relationship, according to a sigmoidal E_{max} model, from least-squares regression analysis.

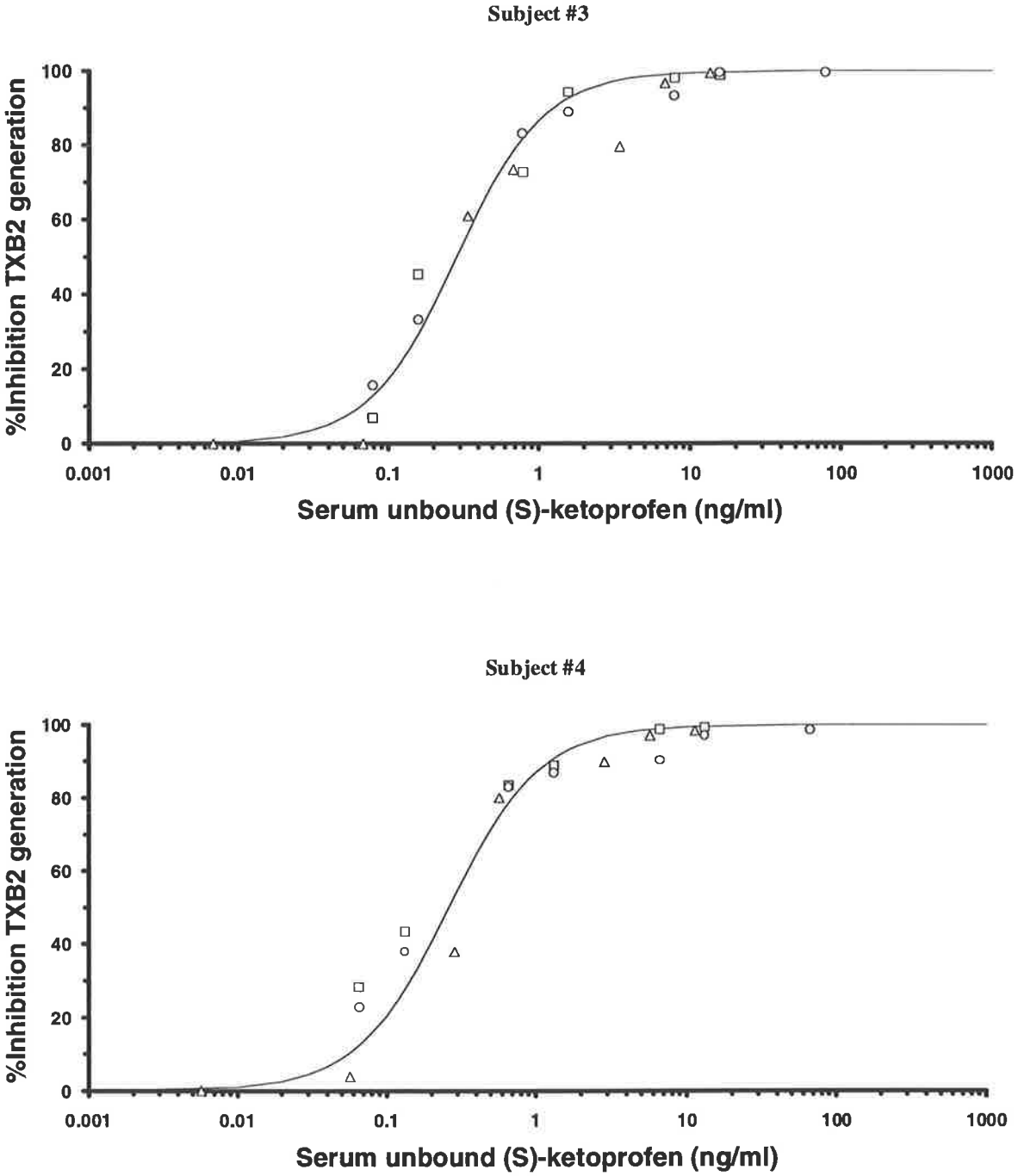


Figure 7.1 (continued) *In vitro* relationships between the percentage inhibition of TXB_2 generation and the logarithmic serum concentration of unbound (S)-ketoprofen for study subjects #1 to #4. The symbols are actual data points when (S)-ketoprofen was added to blood as 99.0% optically pure drug (○), as racemic drug (❑), and as 4.3% optical impurity together with its antipode (Δ). The line represents the predicted relationship, according to a sigmoidal E_{max} model, from least-squares regression analysis.

7.4 Discussion

The optical purity data for the ketoprofen enantiomers were obtained by an indirect HPLC method (see Chapter 4) involving conventional achiral chromatography of the diastereomeric (S)-1-phenylethylamides of (R)- and (S)-ketoprofen. These data were based on the assumption that the chiral derivatization reagent was optically pure. It was estimated that (S)-1-phenylethylamine contained approximately 0.5% of its optical antipode. Thus derived serum concentrations of (S)-ketoprofen, when spiked into blood as the 4.30% impurity in the (R)-ketoprofen test compound, may be subject to a small error (approximately 10%). The net result on the sigmoidal E_{max} model parameter estimates (obtained by combining data from all three spiking approaches) would be expected to be minor.

These data clearly demonstrate the enantiospecificity of inhibition of platelet cyclo-oxygenase-mediated TXB_2 generation, elicited by ketoprofen *in vitro*. Further, (R)-ketoprofen does not modify the activity of the active (S)-enantiomer, even when present as the predominant enantiomeric species. A similar study conducted with ibuprofen (Evans *et al.*, 1991) demonstrated that (R)-ibuprofen did not influence the *in vitro* activity of its pharmacologically active optical antipode when both enantiomers were present as racemic drug. It was previously shown that (R)-ketoprofen does not displace (S)-ketoprofen from plasma protein binding sites at the drug concentrations used in the present study (see Section 5.3). Thus, it is unlikely that indirect dispositional effects can be attributed to the (R)-enantiomer in this *in vitro* pharmacological test system.

A number of studies have reported some minor activity of (R)-enantiomers of 2-arylpropanoate NSAIDs with *in vitro* systems examining inhibition of prostaglandin synthesis and platelet aggregation [see Section 1.5 and reviews by Evans (1992) and Hutt and Caldwell (1983)]. However, it is unclear in the majority of these cases as to whether such effects were due to the presence of some (S)-enantiomer impurity in the respective (R)-enantiomer test compound. Given the brevity of the abstract report of Moreno *et al.* (1990), it is difficult to put their platelet aggregation data for (R)- and (S)-ketoprofen into context with the data generated in this study. Based on the data stated for single ketoprofen enantiomer concentrations (Moreno

et al., 1990), the calculated relative enantiomeric potency ratio [(S)/(R)] for inhibition of aggregation of rabbit platelets (based on enantiomeric EC_{50} values derived from the sigmoidal E_{max} equation by assuming the same slope or *n* value) would be *circa* 26. By comparison, (S)/(R) potency ratios obtained from human platelet aggregation studies performed with carprofen (Gaut *et al.*, 1975) and fenoprofen (Rubin *et al.*, 1985) were >24 and 35, respectively and rat platelet aggregation studies with flurbiprofen (Nishizawa *et al.*, 1973) yielded a (S)/(R) ratio of 200. To suggest that these data are indicative of cyclo-oxygenase-independent mechanisms being involved in the inhibition of platelet aggregation (mechanisms with different eudismic ratios from that suggested by the data herein), or to draw analogies with the inhibition of TXB_2 production would be largely speculative, over and above the fact that data from different animal species would need to be compared.

The results of this study demonstrate the sensitivity of human platelet cyclo-oxygenase to (S)-ketoprofen. By comparison with recent studies with (S)-ibuprofen (Evans *et al.*, 1991) and (S)-naproxen (Williams, 1992), where pharmacologically active unbound drug was examined with an identical *in vitro* test system, ketoprofen (based on EC_{50} of unbound molar concentration of (S)-enantiomer) was approximately 40-fold more potent than (S)-ibuprofen and 100-fold more potent than (S)-naproxen.

It would have been desirable to have monitored the *ex vivo* time-dependency of the anti-platelet effects resulting from ketoprofen dosing, since such information would be clinically useful for making predictions as to when patients receiving the drug should cease taking this medication prior to surgical or dental procedures (where an increased tendency to bleed might compromise the patient). Recent enantioselective concentration/effect studies with ibuprofen (Evans *et al.*, 1991) demonstrated that in subjects administered a range of single racemic doses, the serum TXB_2 concentrations returned to within 10% of the pretreatment concentrations within 24 h of ibuprofen administration. This time-course of TXB_2 inhibition (Evans *et al.*, 1991) was consistent with the fact that binding of ibuprofen to platelet cyclo-oxygenase is a reversible process (Ikeda, 1977).

It was not possible to perform the pharmacodynamic assessment of ketoprofen enantiomers described herein with *ex vivo* blood for two main reasons. Firstly, the potential presence of labile acyl-linked glucuronoconjugates of ketoprofen enantiomers in post-dose plasma (Foster *et al.*, 1988a) necessitated that the controlled blood clotting experiments (performed under physiological conditions, *vide supra*, where acyl-linked conjugates are rapidly hydrolyzed, see Sections 3.3 and 6.3) be performed with metabolite-free blood such that concentration/effect relationships could be calculated. Whilst the pharmacodynamic experiments detailed in this chapter were performed with young healthy subjects in whom metabolite accumulation would be expected to be negligible (see Sections 2.3.3 and 3.5), the method would be applied elsewhere to studies with elderly patients, some of whom had moderately impaired renal function (see Chapter 8). And secondly, the total (bound plus unbound) concentrations of (S)-ketoprofen in serum necessary to produce submaximal inhibition of TXB_2 production (total enantiomeric serum concentrations less than *circa* 0.1 μg/ml) could not be measured since they were at or below the limits of sensitivity of the enantioselective assay (see Chapter 4). Instead, a known spike of (S)-ketoprofen was added to blood and the unbound serum concentration derived as described above (Section 7.2). However, by analogy to ibuprofen (Evans *et al.*, 1991) and in view of the magnitude of (S)-ketoprofen $t_{½}$ (see Section 2.3.3 and Chapter 8), in the absence of *ex vivo* experimental time-dependency data for ketoprofen, it is unlikely that the TXB_2-related effects of this drug would persist to the same extent as those of the irreversible cyclo-oxygenase inhibitor aspirin (Insel, 1991). Unlike reversible arylalkanoate NSAID-mediated cyclo-oxygenase inhibition, the cyclo-oxygenase inactivation of an aspirin-exposed platelet persists for the life-span of that platelet (*circa* 8-11 days; Insel, 1991).

Caution must be taken when extrapolating these data for ketoprofen into the clinical setting. Significant pharmacological activity of the (R)-enantiomer of 2-arylpropanoate NSAIDs can arise indirectly from metabolic chiral inversion (Hutt and Caldwell, 1983), although this is a relatively minor process for ketoprofen in man (see Section 2.3.3). In addition, anti-inflammatory effects may, in part, be mediated by processes independent of cyclo-oxygenase inhibition with potentially different stereochemical determinants (see Section 2.2.1). However, based on estimates of (S)-ketoprofen concentrations achieved with chronic dosing of racemic drug in humans (Jamali and Brocks, 1990) and extrapolating unbound drug concentrations

from *in vitro* binding studies (see Chapter 5), it would appear that the thromboxane-related antiplatelet effects of ketoprofen would persist throughout a standard dosing interval of 100 mg of racemic drug administered twice daily.

Chapter 8

The Influence of Renal Function on the Enantioselective Pharmacokinetics and Pharmacodynamics of Ketoprofen in Patients with Rheumatoid Arthritis

8.1 Introduction

Ketoprofen is a chiral 2-arylpropanoic acid NSAID which, like most structural congeners of this drug class, is marketed for clinical use as the racemate. It is generally accepted that NSAIDs exert their chief pharmacological actions by inhibition of prostaglandin biosynthesis (Brooks and Day, 1991; see Section 2.2.1), yet *in vitro* studies have shown that such activity of chiral NSAIDs resides predominantly or exclusively with enantiomers of the (S)-stereoconfiguration (Evans, 1992; Hutt and Caldwell, 1983; Williams, 1990; see Section 1.5 and Chapter 7). Only drug not bound to plasma protein (unbound drug) is thought capable of interacting with an effector site to elicit an action. Moreover, since ketoprofen (in common with most NSAIDs) has been characterized as a restrictively cleared drug (Lin *et al.*, 1987), only unbound drug will be available for clearance (Wilkinson and Shand, 1975). Consequently, a cardinal element of both the pharmacokinetics and pharmacodynamics of chiral NSAIDs will be the unbound (S)-enantiomer concentration, and hence a perturbation in this moiety may be important clinically (see Section 5.1).

Acyl-glucuronidation is a metabolic pathway common to many 2-arylpropanoic acid NSAIDs including ketoprofen (Upton *et al.*, 1980), fenoprofen (Rubin *et al.*, 1972), carprofen (Rubio *et al.*, 1980) and pirprofen (Luders *et al.*, 1977) [see Chapter 3]. Such drugs have a low percentage of the dose cleared renally in unchanged form yet paradoxically, a number of them, ketoprofen (Advenier *et al.*, 1983; Stafanger *et al.*, 1981), naproxen (Anttila *et al.*, 1980; Upton *et al.*, 1984; Van den Ouweland *et al.*, 1988), ximoprofen (Taylor *et al.*, 1991) and benoxaprofen (Aronoff *et al.*, 1982), have been shown to have diminished clearance in patients with renal dysfunction or in elderly patients in whom renal function is reduced. Moreover, co-

administration of probenecid to subjects receiving ketoprofen (Foster *et al.*, 1989b; Upton *et al.*, 1982), carprofen (Spahn *et al.*, 1989b), benoxaprofen (Spahn *et al.*, 1987) and naproxen (Runkel *et al.*, 1978) induced reductions in the plasma clearances of these NSAIDs. It has been suggested (Meffin, 1985), following mechanistic studies in animals (Meffin *et al.*, 1983a,b), that such observations are consistent with a reduction in the renal elimination of acyl-glucuronoconjugates leading to persistence of these labile metabolites in the body followed by their systemic hydrolysis back to the parent aglycone. With such a reversible cycle of acyl-glucuronidation in operation, net drug clearance of the aglycone is due to conjugation and competition between elimination of glucuronide by renal and hydrolytic clearance pathways (see Section 3.5).

There have been a limited number of studies designed to examine the influence of renal function on the disposition of members of the 2-arylpropanoic acid class of NSAIDs, and still fewer to address this issue specifically with regards to the unbound species (Knadler *et al.*, 1992b; Meffin *et al.*, 1986; Upton *et al.*, 1984). To date, no studies have described the disposition of ketoprofen enantiomers in terms of unbound drug. This study was designed to examine the enantioselective pharmacokinetics and pharmacodynamics of the unbound species in a clinically relevant setting. Longitudinal studies (Lindeman *et al.*, 1985) have shown an overall decline in renal function with age and it is within the ageing population that the greatest consumption of NSAIDs, such as ketoprofen, occurs. Moreover, epidemiological studies have shown that elderly persons are more likely than younger individuals to experience adverse effects from NSAIDs, such as upper gastrointestinal bleeding (Collier and Pain, 1985; Griffin *et al.*, 1991; Strom *et al.*, 1990).

Patients with rheumatoid arthritis were recruited for this single oral dose study and relationships were examined between dispositional parameters and renal function, most notably, in terms of unbound (S)-ketoprofen. While factors such as age, disease severity and concurrent medication may directly influence ketoprofen disposition, in light of recent evidence of diflunisal acyl-glucuronide recycling in patients with renal failure (Dickinson *et al.*, 1991), it was postulated that recycling of labile glucuronides should lead to decreased net clearance of ketoprofen in patients with diminished renal function. In addition, *in vitro* studies were

conducted on pre-dose blood obtained from these patients to investigate the inhibitory effect of unbound (S)-ketoprofen on platelet cyclo-oxygenase and the influence thereon of renal function.

8.2 Methods

Subjects

Fifteen subjects with definite or classical rheumatoid arthritis (Ropes *et al.*, 1959) aged between 51 and 79 years (mean $\pm$ SD = 68.1 $\pm$ 8.5 years) gave written informed consent to participate in the study. The study protocol and patient information leaflet (detailing the study in simple, non-technical language) were approved by the Research and Ethics Committee of the Repatriation General Hospital, Daw Park, South Australia. Subjects were withdrawn from any nonsteroidal anti-inflammatory drug (including aspirin) for a period of seven days prior to the study day (no patients were taking long half-life oxicam NSAIDs). Disease modifying anti-rheumatic agents and other prescribed drugs were continued irrespective of the study. All subjects were admitted to the hospital at least one day prior to study and were fasted from midnight preceding the study and for 4 h after dosing. On study days, fluid intake was restricted to 300 ml till 4 h post-dose when each volunteer received a standardized light lunch. Subject demographics including medications taken as part of continuation of chronic therapy are given in Table 8.1.

Ketoprofen administration and blood and urine sampling

Immediately prior to ketoprofen administration, 60 ml of blood was sampled via an indwelling intravenous cannula sited in a forearm vein. This pre-dose blood was used to conduct the *in vitro* plasma protein binding experiments and the pharmacodynamic assessment of ketoprofen enantiomers (*vide infra*). A single oral 100 mg dose of regular release (RS)-ketoprofen (Orudis®, batch number DD2173, May and Baker, Melbourne, Australia) was given to each subject with 150 ml of water at approximately 08.00am. Venous blood samples (10 ml) were collected into heparinized tubes at 15 min intervals for the first 2 h after dosing then at hourly intervals for the following 4 h and finally at 2-hourly intervals to give a total 10 h post-dose sampling period for each subject. Some deviations in sampling time occurred, as dictated by

clinical circumstances. The intravenous cannula was kept patent over this time with a stylet (Johnson and Johnson, Florida, U.S.A.).

In order to avoid *ex vivo* hydrolysis of labile glucuronoconjugates of ketoprofen (see Section 3.3 and Chapter 6), blood samples were immediately placed on ice then centrifuged (2000 *g*, 10 min, 2°C) within 30 min of collection to allow harvesting of plasma. One millilitre aliquots of plasma were placed in glass culture tubes and pH-stabilized by adding 50 µl of 2.0 *M* sulphuric acid. The tubes were closed with teflon-lined screw-caps and the samples were stored at -20°C prior to stereoselective analysis *(vide infra)*. In preliminary experiments, conducted by adding acyl-glucuronides to plasma and monitoring the appearance of hydrolysed aglycone, it was verified that the sample processing, storage and subsequent analysis within one month of collection did not lead to cleavage of acyl-glucuronide conjugates of (R)- or (S)-ketoprofen.

Urine was collected from each subject over the intervals corresponding to 0-5 h, 5-10 h and 10-24 h after dosing with ketoprofen. For each urine collection the volume was recorded and aliquots stored at -20°C prior to analysis. No attempt was made to prevent spontaneous deconjugation of acyl-linked conjugate metabolites of ketoprofen in urine during either collection or storage.

Creatinine clearance (used as an index of patient renal function) was calculated by dividing the urinary creatinine excretion rate during the combined 0-24 h urine collection by the plasma creatinine concentration in the final blood sample (10 h post-dose). An additional blood sample was taken at this time and allowed to clot prior to harvesting of serum for the purpose of determining the albumin concentration. Both creatinine and albumin concentrations were measured by standard auto-analyzer colourimetric methods (Synchron CX® System, Beckman, Sydney, Australia and BCP System for Hitachi 704®, Boehringer Mannheim, Sydney, respectively).

Enantioselective ketoprofen analyses

Plasma concentrations of total (bound plus unbound) (R)- and (S)-ketoprofen aglycone were measured by a HPLC method which has been detailed in Chapter 4. In order to quantify the urinary concentrations of the combined aglycones plus glucuronoconjugates of ketoprofen enantiomers, urine samples were rendered alkaline by treating aliquots (100 µl volumes of the 0-5 h and 5-10 h collections and 1.00 ml from the 10-24 h collection) with 100 µl of 1.0 *M* sodium hydroxide for 15 min at room temperature. The hydrolyzed urine samples were acidified with 0.50 ml of 2.0 *M* sulphuric acid, extracted together with internal standard and subsequently carried through the enantioselective ketoprofen assay. These conjugate hydrolysis conditions led to complete cleavage without racemization of the aglycone (see Section 6.2). Since the possibility of conjugate hydrolysis taking place in subjects' bladders prior to voiding could not be controlled (Upton *et al.*, 1980 and Section 3.5), no attempt was made to separately quantify aglycone and glucuronide urinary excretion.

Plasma protein binding

The protein binding of ketoprofen enantiomers was investigated in pre-dose plasma from each subject by an *in vitro* method described in detail in Chapter 5. The presence of labile ketoprofen conjugates has been reported in *ex vivo* plasma samples from elderly patients dosed with the drug (Foster *et al.*, 1988a) which necessitated the use of pre-dose (metabolite-free) plasma for determining the protein binding of ketoprofen enantiomers as discussed in Section 5.3. Additionally, given the specific activity of the radiolabelled ketoprofen, the minimum quantity of "tracer" required for radiometric detection of unbound isomers is equivalent to 2.0 µg/ml of (RS)-ketoprofen and hence does not represent a tracer quantity if added to *ex vivo* plasma aliquots. Briefly, the method involved spiking plasma from each subject with 2.00 µg/ml of (RS)-[1-^{14}C]-ketoprofen (purified by HPLC within 48 h of use) and any additional nonradiolabelled (RS)-ketoprofen to yield a series of racemic drug concentrations in plasma of 2.00, 3.25, 4.50, 7.00 and 12.0 µg/ml. These plasma samples were subjected to ultra-filtration at physiological pH and temperature to obtain the unbound species whereupon the enantioselective HPLC method was used to resolve the ketoprofen enantiomers prior to their quantification by radiometric means.

Pharmacodynamic measurements

The pharmacological activity of ketoprofen was determined by an *in vitro* method in pre-dose blood obtained from each subject (see Chapter 7). This involved measurement of the inhibition of platelet thromboxane A_2 (TXA_2) generation during the controlled clotting of whole blood in the presence of a range of (RS)-ketoprofen concentrations (5.00, 10.0, 20.0, 50.0, 100, 200, 1000 and 2000 ng/ml) relative to TXA_2 generation in the absence of drug. As an index of cyclo-oxygenase activity, the amount of TXA_2 generated was assessed by measuring the concentration of its stable breakdown product, thromboxane B_2 (TXB_2), in the harvested serum as described by Patrono and associates (1980). For each subject, the relationship between the percentage inhibition of TXB_2 generation and the unbound concentration of (S)-ketoprofen in serum was subjected to nonlinear least-squares regression (with reciprocal weighting) according to a sigmoidal E_{max} model. Earlier, it had been established that the (S)-enantiomer of ketoprofen was solely active at inhibiting human platelet TXB_2 production and this activity was not modified by the presence of its optical antipode (Section 7.3). The method for deriving the corresponding unbound concentration of (S)-ketoprofen in serum upon spiking racemic drug into blood was based on preliminary studies which confirmed that the unbound fraction of (S)-ketoprofen in plasma ($fu_{(S)}$) *in vitro* was equivalent to the unbound fraction in serum. This has also been demonstrated for the protein binding of naproxen (Upton *et al.*, 1984).

The blood to serum concentration ratio of (S)-ketoprofen ($[B]_{(S)}/[S]_{(S)}$) was calculated for each subject as the ratio of the measured concentration of (S)-ketoprofen in serum derived from spiking serum harvested from pre-dose blood with 2.0 µg/ml of (RS)-ketoprofen to the serum concentration of (S)-ketoprofen subsequent to spiking whole blood with 2.0 µg/ml of racemic drug. This parameter was used in conjunction with $fu_{(S)}$ to derive the unbound (S)-ketoprofen concentration in serum from the corresponding whole blood spike concentration of drug as detailed previously (see Section 7.2).

Pharmacokinetic analyses

For each subject, the area under the plasma concentration-time curve from time zero to infinity for total (bound plus unbound) (R)-ketoprofen ($AUC_{(R)\text{-total}}$) and (S)-ketoprofen ($AUC_{(S)\text{-total}}$) were calculated using the linear trapezoidal rule for the area from time zero to the last sampling time point, to which was added the extrapolated area to infinite time. The extrapolated area was determined by dividing the final plasma concentration (interpolated from log-linear regression analysis) by the terminal rate constant, and in all cases this area accounted for no more than 15% of the total area. The terminal slope, determined by log-linear regression of at least the final five data points, was used to calculate the terminal rate constant and half-life ($t_{½}$) for each enantiomer.

The areas under the curves for unbound (R)- and (S)-ketoprofen ($AUC_{(R)\text{-unbound}}$ and $AUC_{(S)\text{-unbound}}$, respectively) were calculated by multiplying the unbound fraction of drug enantiomer in plasma ($fu_{(R)}$, $fu_{(S)}$) by the respective AUC for total enantiomer. Values of $fu_{(R)}$ and $fu_{(S)}$ were calculated for each subject as the arithmetic mean of five individual unbound fraction estimates from pre-dose plasma spiked with (RS)-ketoprofen over the enantiomer concentration range from 1.00 to 6.00 µg/ml *(vide supra)*.

The cumulative (0-24 h) recovery of (R)- and (S)-ketoprofen in alkaline hydrolysed urine (as combined glucuronide conjugate and aglycone) was expressed as the amount of each enantiomer excreted in urine ($Ae_{(R)}$ and $Ae_{(S)}$, respectively).

Statistical methods

Correlations between subject characteristics (creatinine clearance and serum albumin) and various pharmacokinetic and pharmacodynamic parameters were investigated using the Spearman rank-order correlation coefficient, or where appropriate, linear regression. Other data sets were compared statistically by analysis of variance or the Student's paired *t*-test, as appropriate, with differences considered significant at $P<0.05$ for 2-tailed distributions.

Table 8.1 Study patient demographics

Patient	*Gender*	*Age* (years)	*Weight* (kg)	*Serum albumin** (g/l)	*Creatinine clearance* (ml/min)	*Medications during the study*
1	F	67	53	31	26	Theophylline, cyclosporin, salbutamol.
2	M	71	81	19	45	Verapamil, paracetamol.
3	M	74	77	31	50	Isosorbide dinitrate, hydroxychloroquine, chlorothiazide, nifedipine, glyceryl trinitrate.
4	F	74	69	36	55	Sulphasalazine, paracetamol, temazepam.
5	F	60	62	39	65	Methotrexate.
6	M	72	82	34	70	Amiodarone, chlorothiazide, diltiazem, dothiepin, prednisolone, warfarin.
7	M	79	83	28	81	Indapamide, glicazide, temazepam, methyldopa, salbutamol.
8	M	67	82	36	87	Methotrexate, paracetamol, nitrazepam.
9	F	51	46	32	69	Ranitidine, frusemide, paracetamol, potassium chloride, prednisolone, dextropropoxyphene.
10	F	75	57	32	75	Methotrexate.
11	M	74	61	34	87	Metformin, glicazide, digoxin, temazepam, paracetamol, aurothioglucose.
12	F	77	63	34	83	Atenolol, calcitriol, thyroxine, glyceryl trinitrate.
13	M	68	62	26	91	Paracetamol, methotrexate.
14	M	57	91	33	120	Aurothiomalate.
15	M	56	83	29	159	Paracetamol, methylprednisolone.

* Laboratory reference range: 32 - 42 g/l.

8.3 Results

Plasma protein binding of (R)- and (S)-ketoprofen

The fraction unbound of ketoprofen enantiomers was determined *in vitro* in pre-dose plasma spiked with a range of concentrations (1.00-6.00 μg/ml) of each enantiomer present together. Figure 8.1 depicts the percentage unbound of (R)- and (S)-ketoprofen in plasma with each data point representing the mean of individual determinations carried out in the 15 subjects. Analysis of variance was used to assess: (*i*) whether the protein binding of ketoprofen was enantioselective and (*ii*) whether the binding of either enantiomer exhibited concentration-dependence over the clinically relevant range of total (bound plus unbound) drug concentrations. Firstly, the percentage unbound of (S)-ketoprofen exceeded the corresponding value for its optical antipode at all but the lowest (1.00 μg/ml) spike concentration ($P<0.05$, Fisher's least significant difference (LSD) test). Secondly, there was no significant change in the percentage unbound of (R)-ketoprofen across the concentration range examined but the percentage unbound of (S)-ketoprofen was significantly greater ($P<0.05$, Fisher's LSD test) at an enantiomer concentration of 6.00 μg/ml compared with corresponding values at 3.50 μg/ml and below.

The percentage unbound values obtained for each enantiomer as the average of determinations across the spike concentration range are given for individual subjects in Table 8.2. There was a significant difference ($P<0.0002$, paired *t*-test) between values for each enantiomer; the mean ± SD percentage unbound values of (R)- and (S)-ketoprofen were 0.724 ± 0.149% and 0.801 ± 0.194%, respectively. There were no correlations ($P>0.05$) between the percentage unbound of either enantiomer of ketoprofen and either creatinine clearance (CL_{CR}) or serum albumin concentration.

Disposition of ketoprofen enantiomers

Plasma concentration-time profiles of total (bound plus unbound) (R)- and (S)-ketoprofen for the subjects are provided in Figure 8.2 and individual tabulated data in the Appendix. Pharmacokinetic parameters are listed for individual subjects in Table 8.2. Post-dose blood

collections were incomplete for one subject (patient #9) due to failure of the indwelling intravenous cannula.

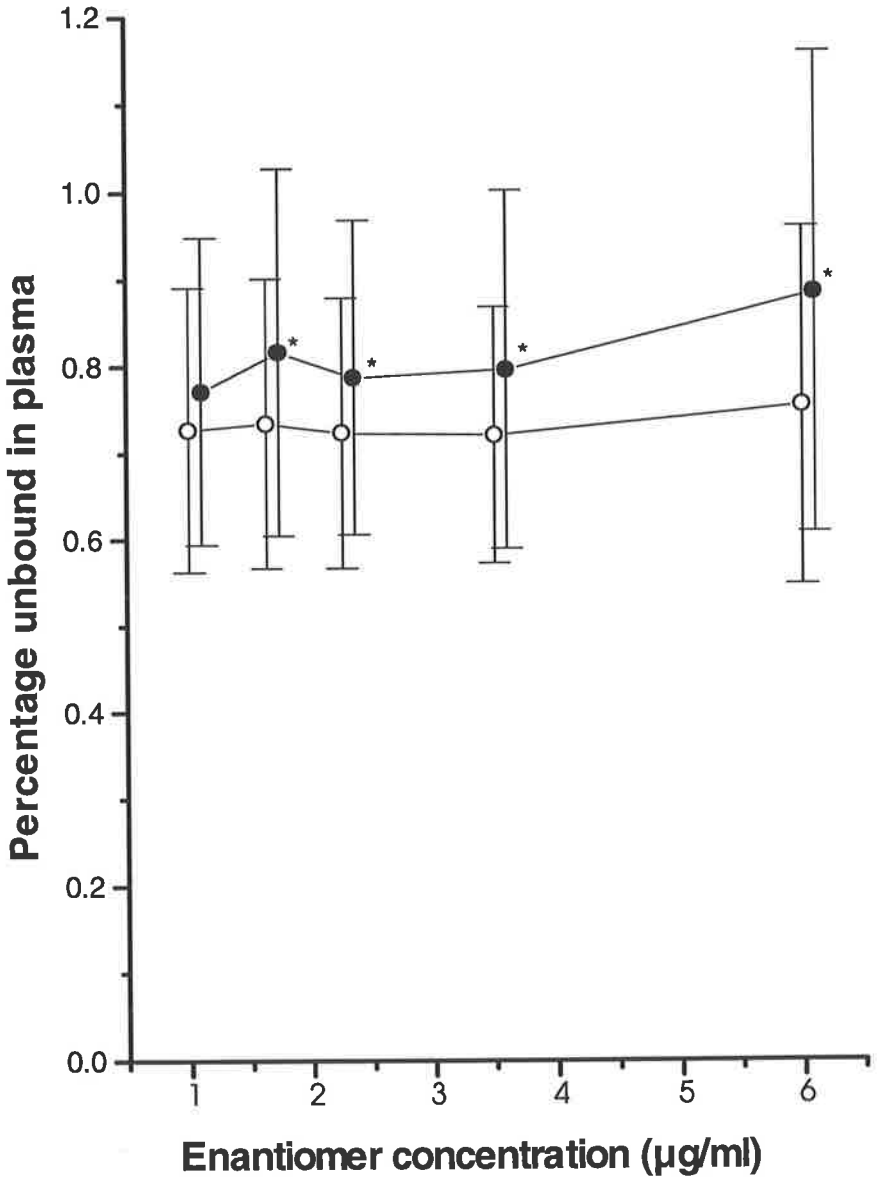


Figure 8.1 Mean (SD bars) percentage unbound of (R)-ketoprofen (○) and (S)-ketoprofen (●) in plasma from the 15 study patients after addition of (RS)-ketoprofen *in vitro*. To enhance graphic clarity the data have been displaced horizontally.
*Indicates a difference (ANOVA, $P<0.05$) between $fu_{(R)}(\%)$ and $fu_{(S)}(\%)$ at a given plasma concentration of enantiomeric ketoprofen.

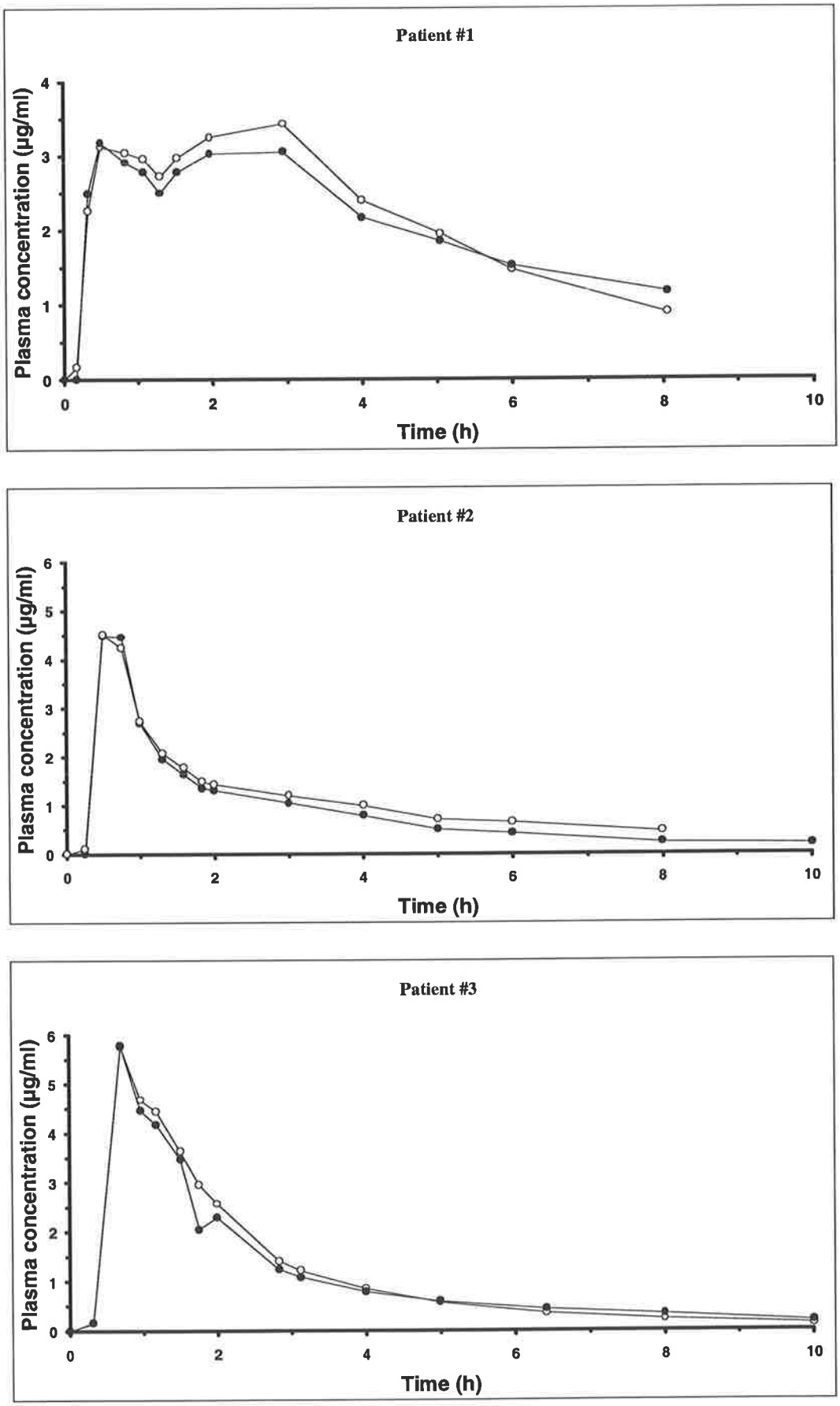


Figure 8.2 Plasma concentration-time profiles for total (bound plus unbound) (R)-ketoprofen (❍) and (S)-ketoprofen (●) in patients #1 to #15 following oral ingestion of single 100 mg doses of (RS)-ketoprofen.

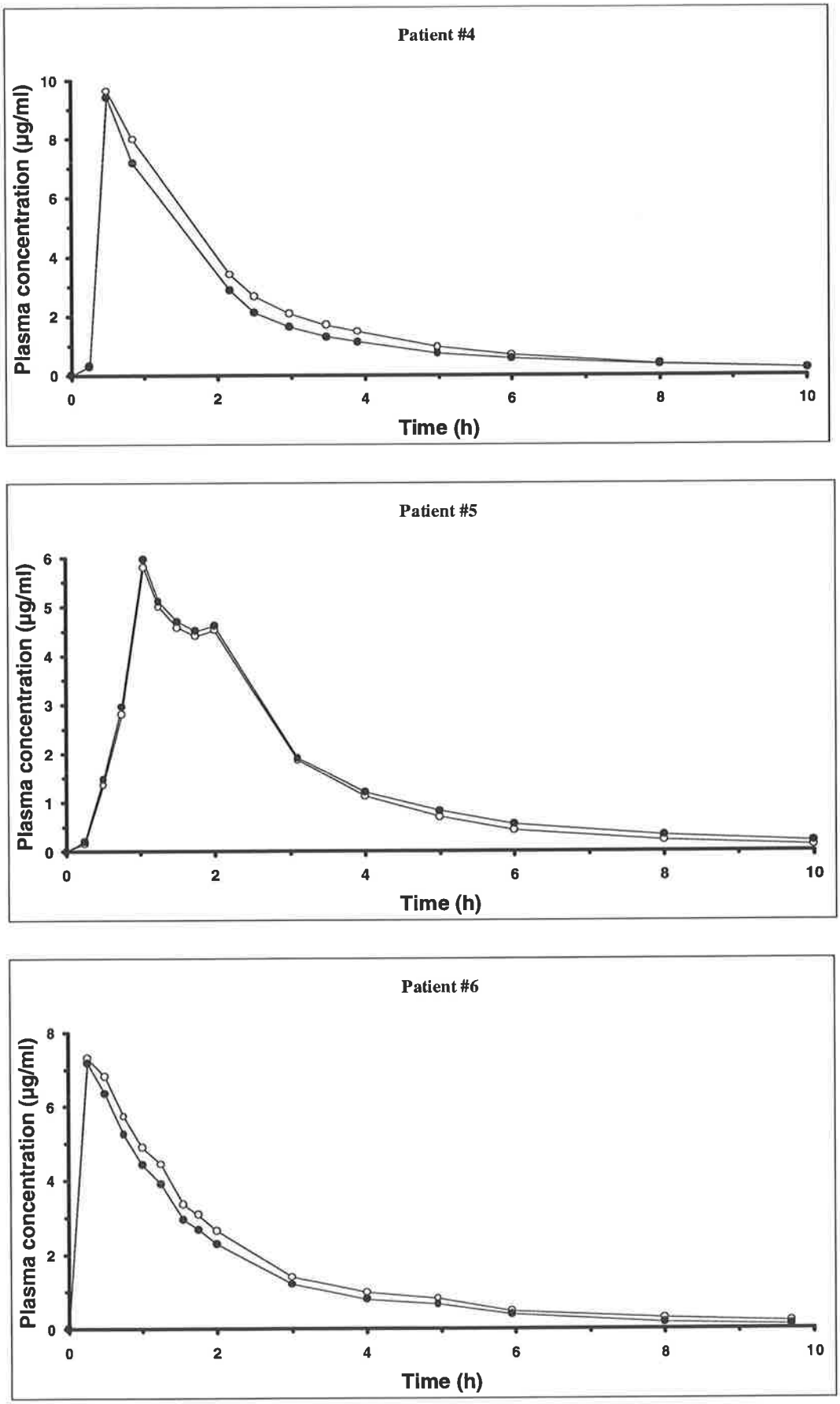


Figure 8.2 (continued) Plasma concentration-time profiles for total (bound plus unbound) (R)-ketoprofen (❍) and (S)-ketoprofen (●) in patients #1 to #15 following oral ingestion of single 100 mg doses of (RS)-ketoprofen.

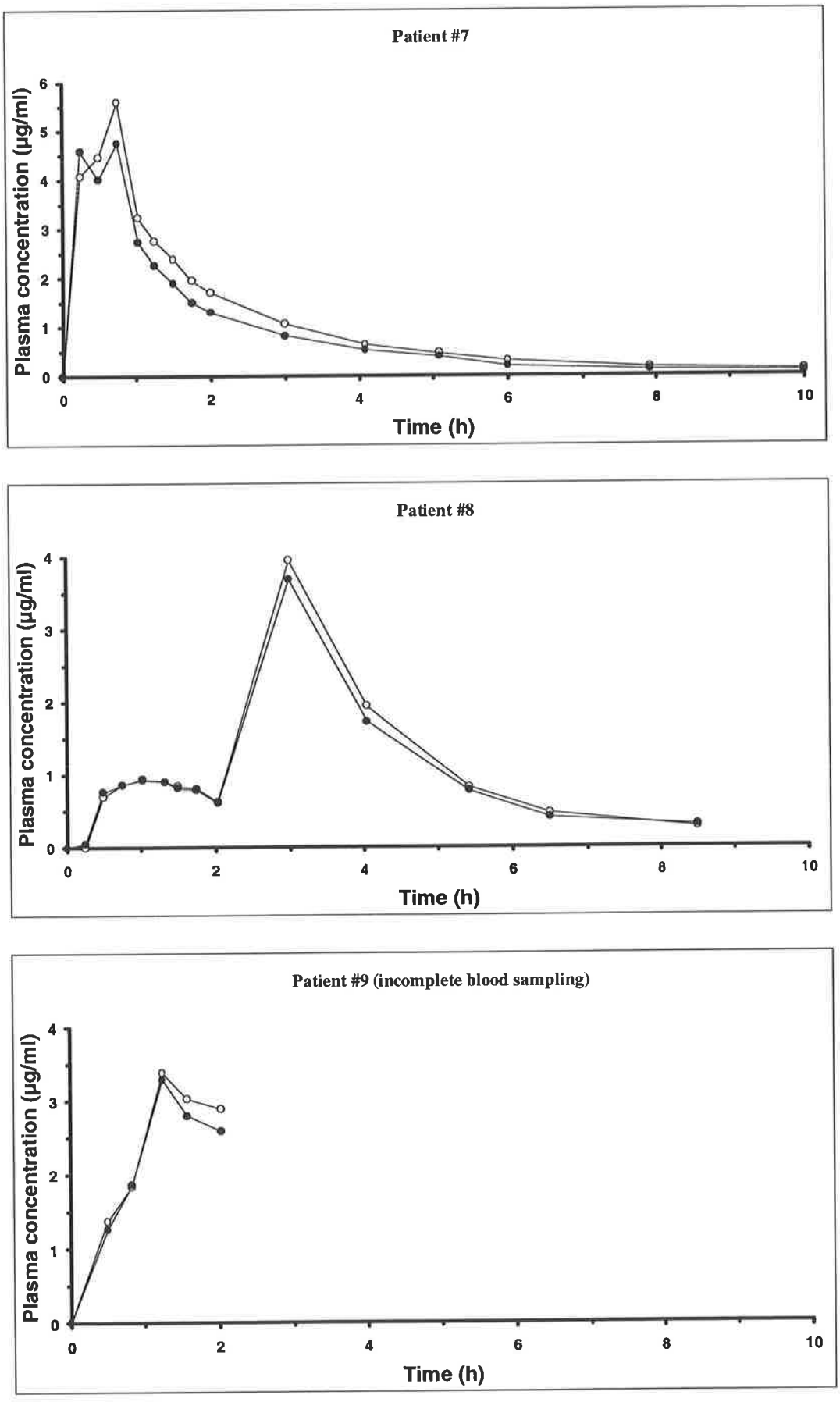


Figure 8.2 (continued) Plasma concentration-time profiles for total (bound plus unbound) (R)-ketoprofen (❍) and (S)-ketoprofen (●) in patients #1 to #15 following oral ingestion of single 100 mg doses of (RS)-ketoprofen.

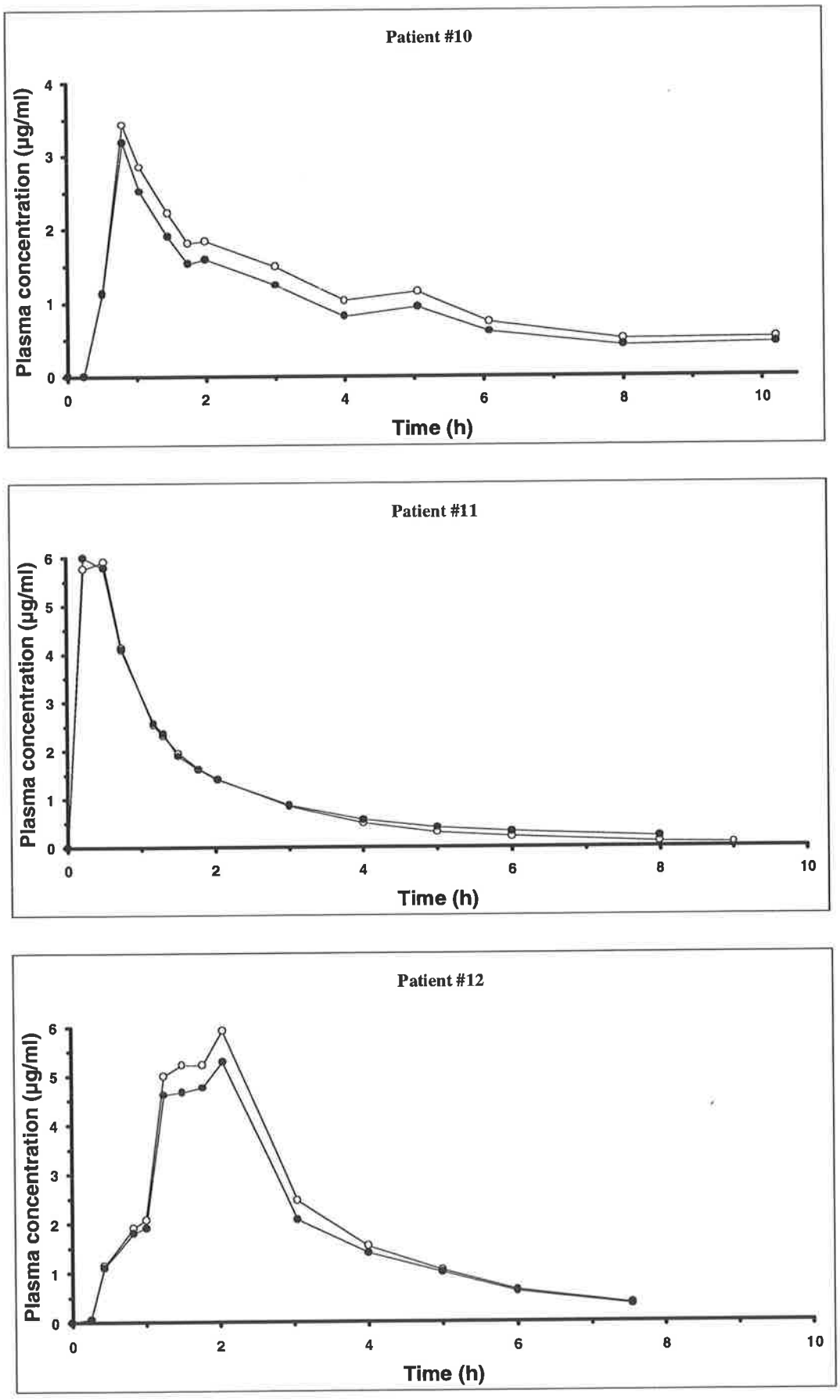


Figure 8.2 (continued) Plasma concentration-time profiles for total (bound plus unbound) (R)-ketoprofen (○) and (S)-ketoprofen (●) in patients #1 to #15 following oral ingestion of single 100 mg doses of (RS)-ketoprofen.

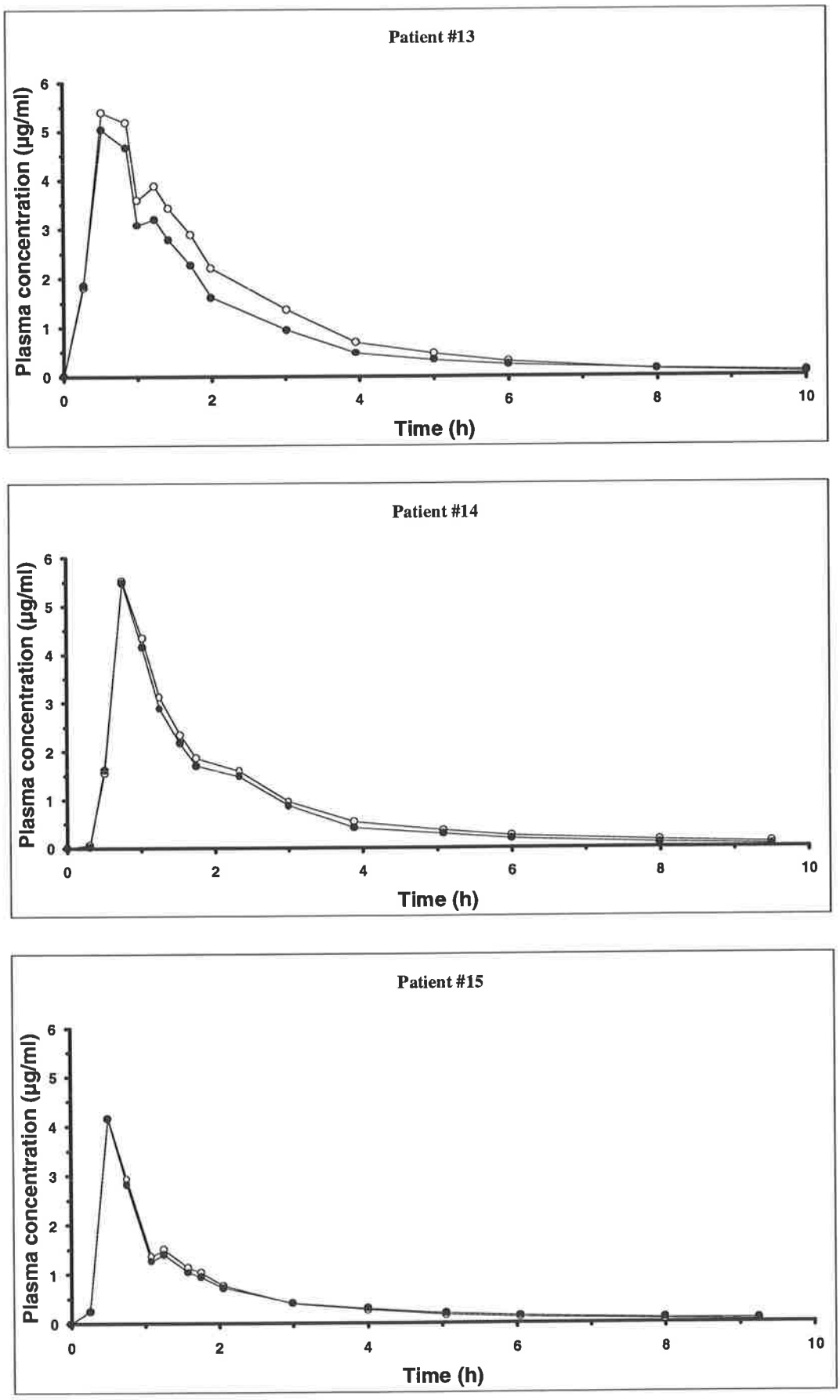


Figure 8.2 (continued) Plasma concentration-time profiles for total (bound plus unbound) (R)-ketoprofen (❍) and (S)-ketoprofen (●) in patients #1 to #15 following oral ingestion of single 100 mg doses of (RS)-ketoprofen.

Table 8.2 Individual pharmacokinetic parameters for (R)- and (S)-ketoprofen

Patient	$fu_{(R)}$ (%)	$fu_{(S)}$ (%)	$AUC_{(R)\text{-total}}$ (mg.h/l)	$AUC_{(S)\text{-total}}$ (mg.h/l)	$AUC_{(R)\text{-unbound}}$ (μg.h/l)	$AUC_{(S)\text{-unbound}}$ (μg.h/l)	$t_{½(R)}$ (h)	$t_{½(S)}$ (h)	$Ae_{(R)}$ (mg)	$Ae_{(S)}$ (mg)
1	0.890	1.01	19.2	19.9	171	201	2.66	3.82	13.5	23.9
2	0.990	1.17	8.98	10.4	88.9	122	2.55	3.20	5.80	13.3
3	0.830	0.870	11.6	11.4	93.1	99.2	2.23	2.95	9.23	17.5
4	0.752	0.747	19.8	17.3	149	129	2.34	2.74	23.2	31.7
5	0.697	0.755	14.0	15.1	97.6	114	1.87	2.33	27.5	35.1
6	0.526	0.556	15.3	13.3	80.5	73.9	1.88	1.63	23.1	30.6
7	0.528	0.656	10.4	8.74	54.9	57.3	1.83	1.76	17.1	23.2
8	0.693	0.728	10.2	9.64	70.7	70.2	1.41	1.48	29.6	36.3
9	0.738	0.766	*	*	*	*	*	*	14.9	24.8
10	0.625	0.683	12.4	10.6	77.5	72.4	4.14	4.23	23.0	32.5
11	0.760	0.885	9.35	9.99	71.1	88.4	1.77	2.48	24.9	33.7
12	0.618	0.660	15.5	14.0	95.8	92.4	1.42	1.47	25.4	33.4
13	1.01	1.21	11.2	9.31	113	113	1.69	2.58	24.1	30.0
14	0.598	0.630	8.45	7.61	50.5	47.9	1.64	1.41	25.0	34.0
15	0.639	0.686	4.82	4.88	30.8	33.5	1.57	1.89	20.7	29.5

*Patient withdrew from blood sampling part of study 3 h after receiving ketoprofen dose due to cannula failure and insufficient data points were obtained to adequately define the terminal portion of semi-logarithmic plasma concentration-time profile. Urine collections (up to 24 h post-dose) continued for this patient.

8.3 Results (continued)

Disposition of ketoprofen enantiomers (continued)

Significant negative correlations existed between $AUC_{(S)\text{-unbound}}$ and CL_{CR} (r_s = -0.761, P<0.005) and between $AUC_{(R)\text{-unbound}}$ and CL_{CR} (r_s = -0.647, P<0.02) [Figure 8.3(a)]. In addition, there were negative correlations between $AUC_{(S)\text{-total}}$ and CL_{CR} (r_s = -0.783, P<0.002) and between $AUC_{(R)\text{-total}}$ and CL_{CR} (r_s = -0.594, P<0.05) [Figure 8.4(a)]. Transformed enantiomeric AUC data (1/AUC) for unbound and total ketoprofen versus CL_{CR} are shown in Figures 8.3(b) and 8.4(b), respectively. The linear correlation coefficients (r) for the reciprocal of $AUC_{(S)\text{-unbound}}$ versus CL_{CR} and the reciprocal of $AUC_{(R)\text{-unbound}}$ versus CL_{CR} were 0.900 (P<0.0001) and 0.878 (P<0.0001), respectively. The corresponding linear correlation coefficients for the reciprocal of AUC for total (S)- and total (R)-ketoprofen versus CL_{CR} were 0.885 (P<0.0001) and 0.808 (P<0.0005), respectively.

Negative associations of statistical significance were observed between the half-lives of (R)- and (S)-ketoprofen and CL_{CR} (r_s = -0.805, P<0.001 and r_s = -0.625, P<0.02; respectively) [Figure 8.5]. There were no correlations (P>0.05) between the $AUC_{(R)}$:$AUC_{(S)}$ ratios for either unbound or total drug and CL_{CR} [Figures 8.6 and 8.7, respectively]. A significant positive correlation was observed between $Ae_{(R)}$ and CL_{CR} (r_s = 0.554, P<0.05) but not between $Ae_{(S)}$ and CL_{CR} (r_s = 0.481, 0.05<P<0.10) [Figure 8.8].

Paired comparisons (t-test) were made between (R)- and (S)-ketoprofen for the dispositional parameters listed for individual subjects in Table 8.2. Significant differences were found between enantiomers for $t_{½}$ (P<0.01) and Ae (P<0.0001). However, there were no differences in AUC for either total or unbound drug.

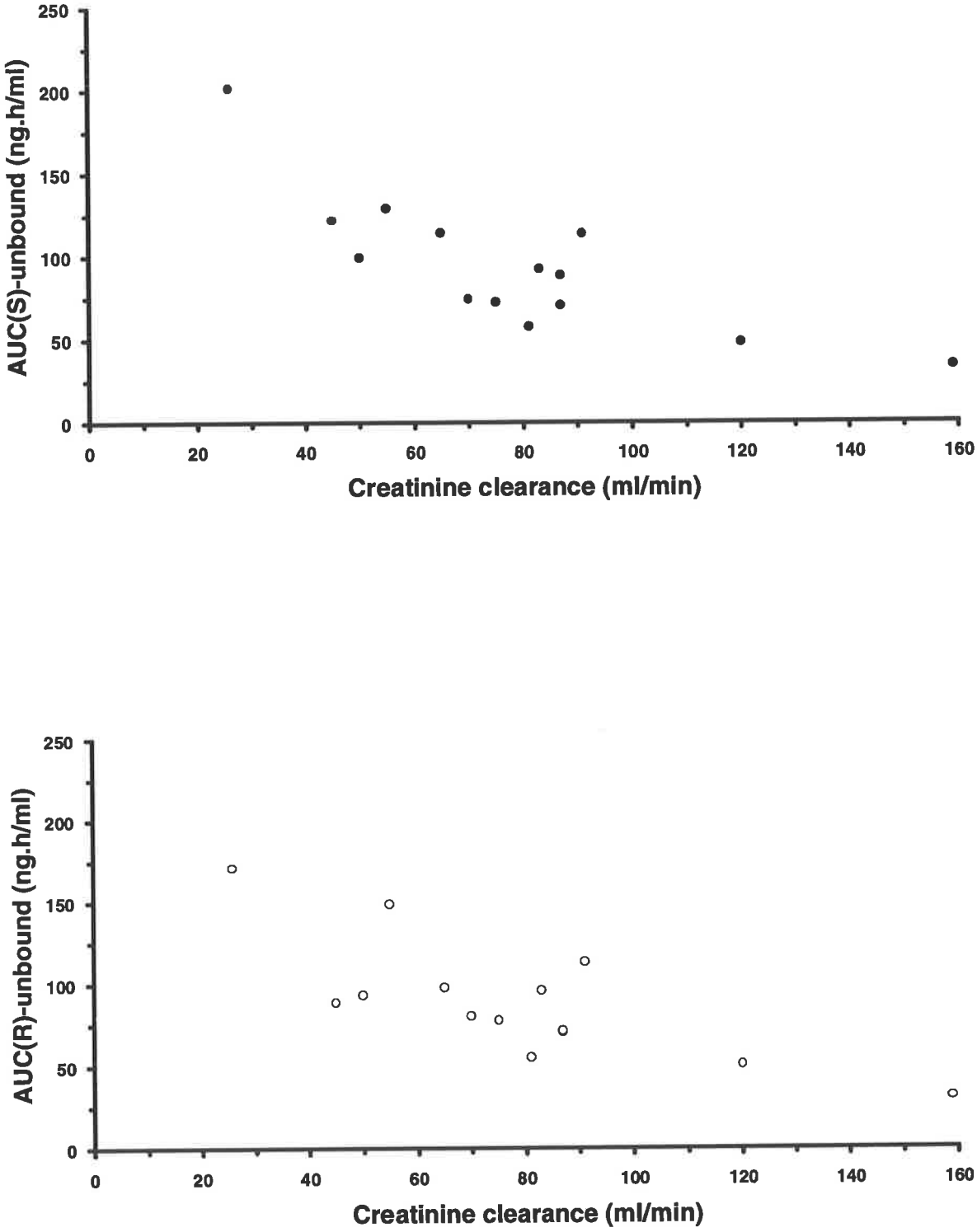
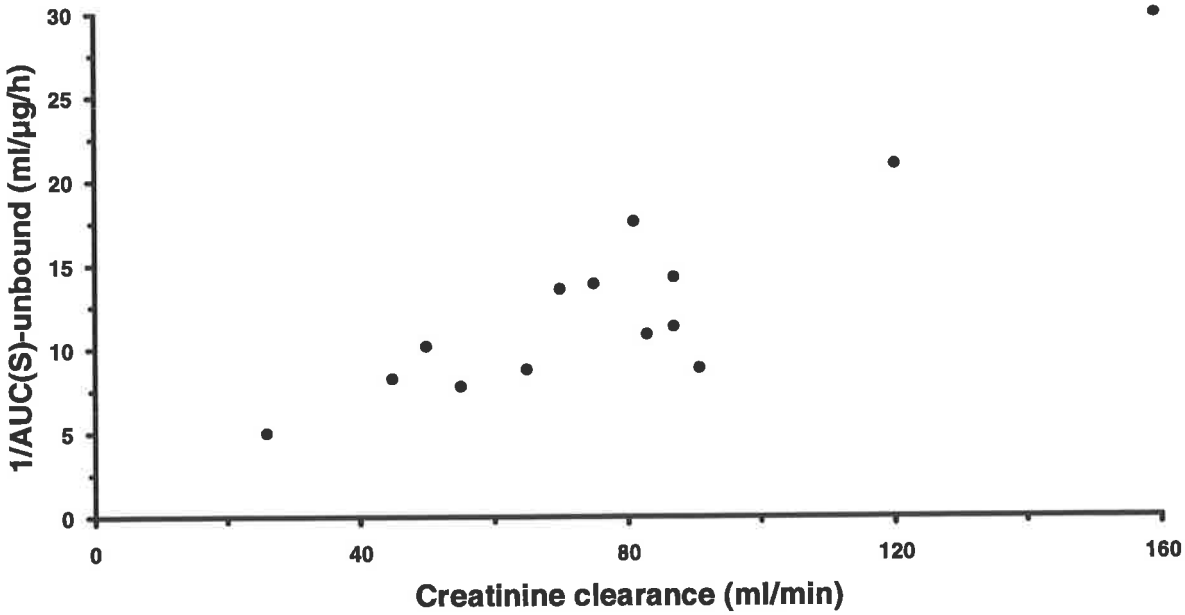


Figure 8.3(a) Plots of $AUC_{(S)\text{-unbound}}$ (●) (r_s = -0.761, P<0.005) and $AUC_{(R)\text{-unbound}}$ (○) (r_s = -0.647, P<0.02) versus CL_{CR} for 14 study patients.



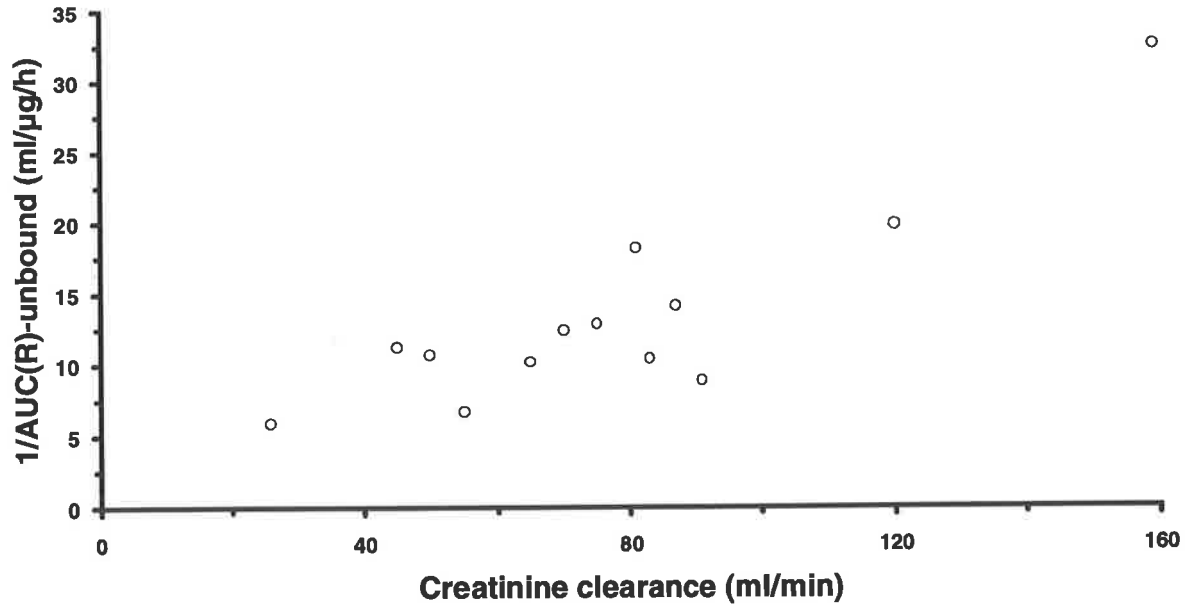


Figure 8.3(b) Relationships (linear regression coefficient and probability value) between the reciprocal of $AUC_{(S)\text{-unbound}}$ (r = 0.900, P<0.0001) and CL_{CR} and the reciprocal of $AUC_{(R)\text{-unbound}}$ (r = 0.878, P<0.0001) and CL_{CR}.

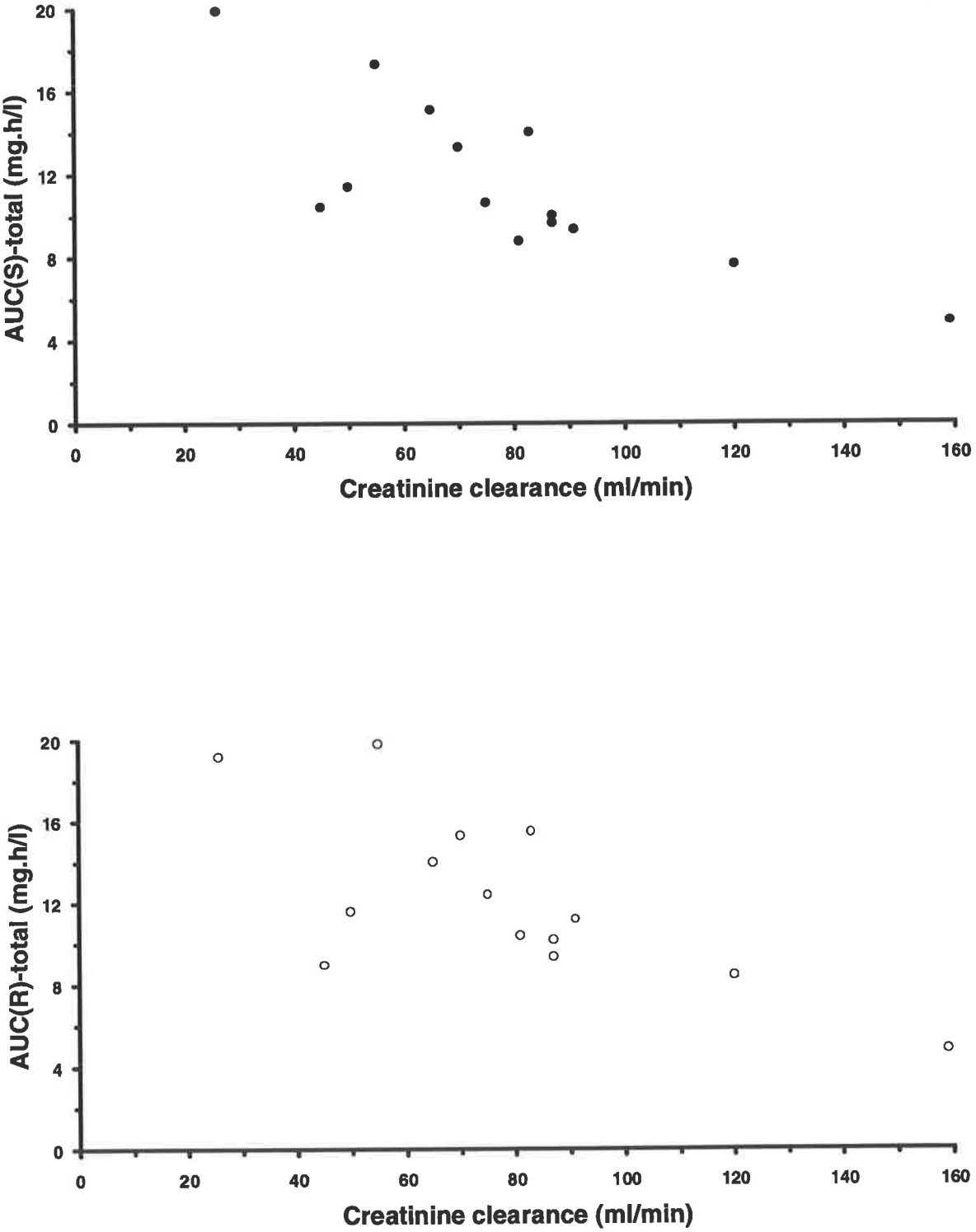
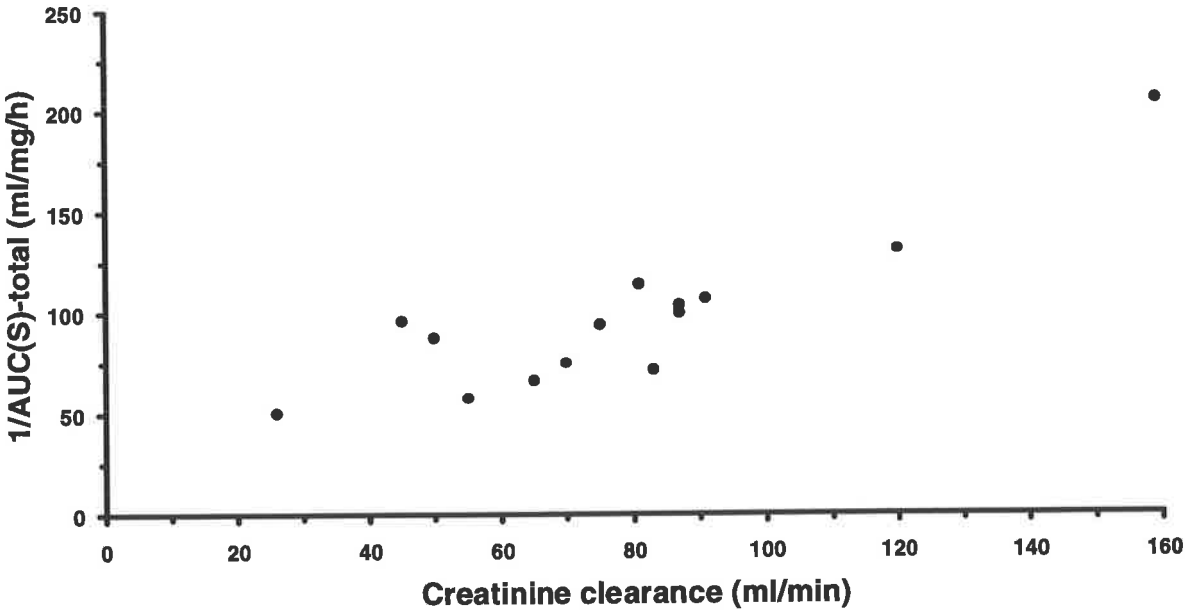


Figure 8.4(a) Plots of $AUC_{(S)\text{-total}}$ (●) (r_s = -0.783, P<0.002) and $AUC_{(R)\text{-total}}$ (○) (r_s = -0.594, P<0.05) versus CL_{CR} for 14 study patients.



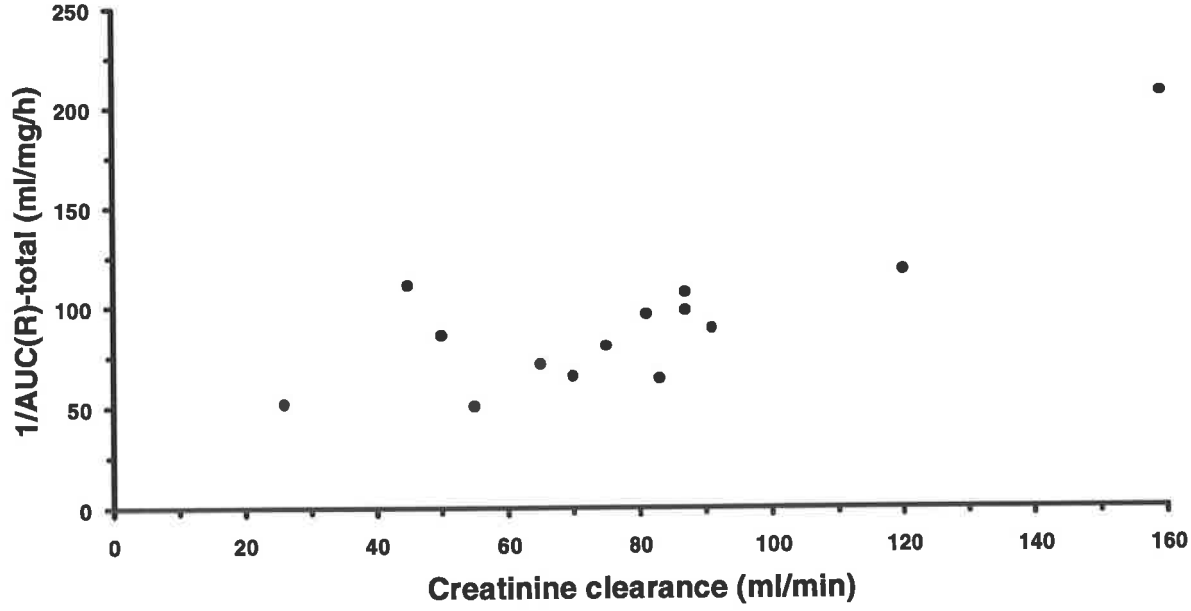


Figure 8.4(b) Relationships (linear regression coefficient and probability value) between the reciprocal of $AUC_{(S)\text{-total}}$ ($r = 0.885$, $P<0.0001$) and CL_{CR} and the reciprocal of $AUC_{(R)\text{-total}}$ ($r = 0.808$, $P<0.0005$) and CL_{CR}.

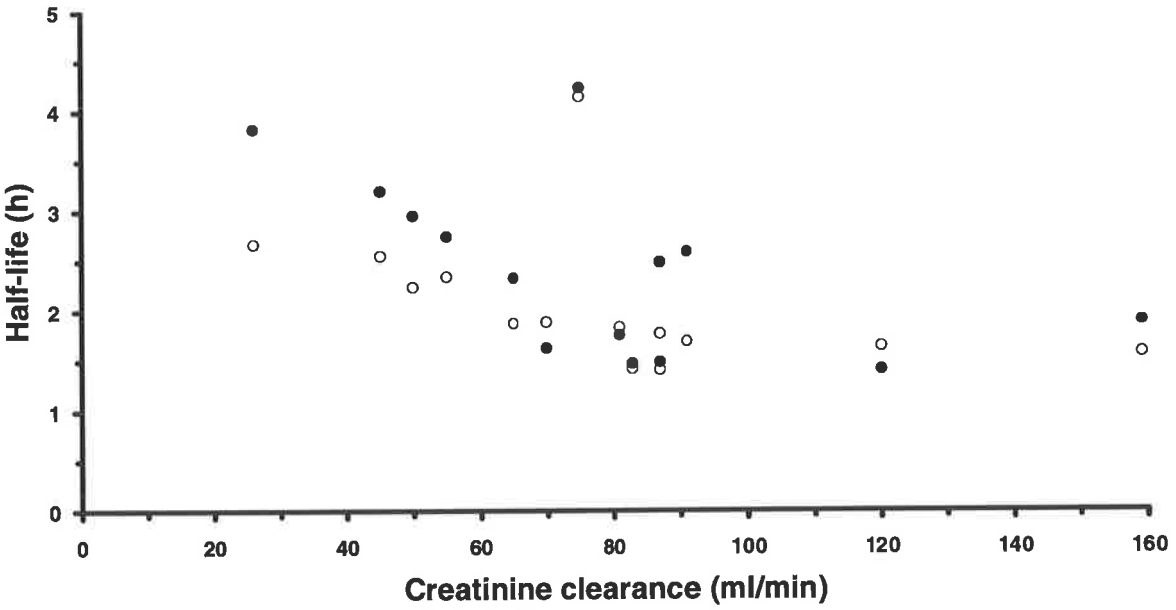


Figure 8.5 Plots of $t_{½(S)}$ (●) (r_s = -0.625, P<0.02) and $t_{½(R)}$ (○) (r_s = -0.805, P<0.001) versus CL_{CR} for 14 study patients.

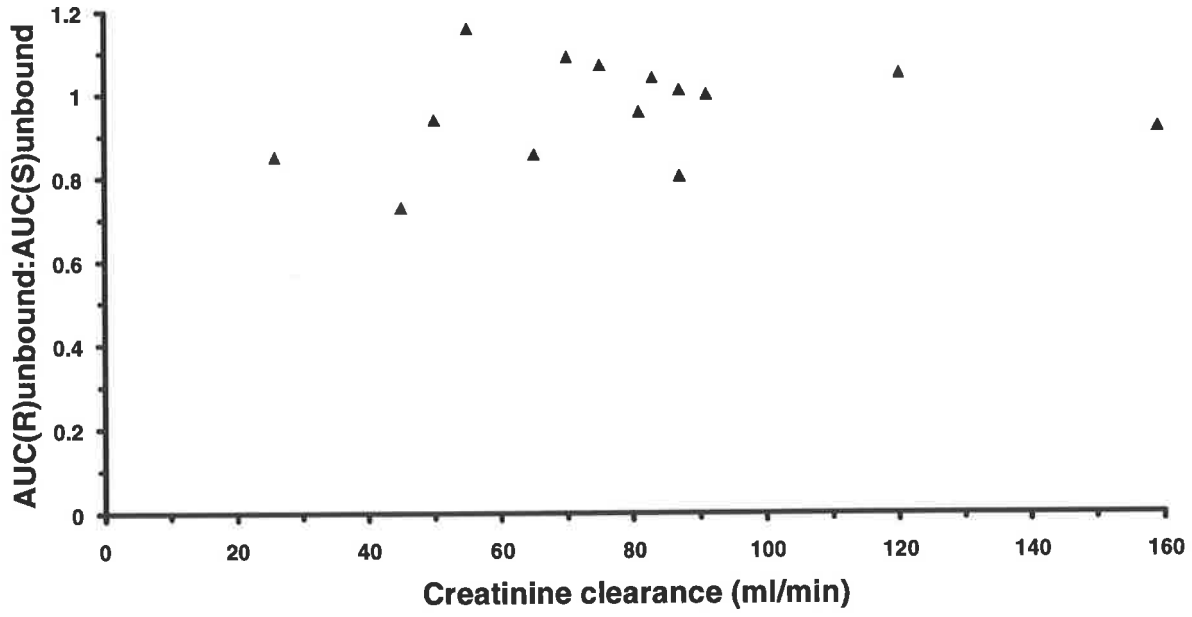


Figure 8.6 Plot of the ratio of $AUC_{(R)}$:$AUC_{(S)}$ for unbound enantiomeric ketoprofen versus CL_{CR} for 14 study patients (r_s = -0.192, P>0.05).

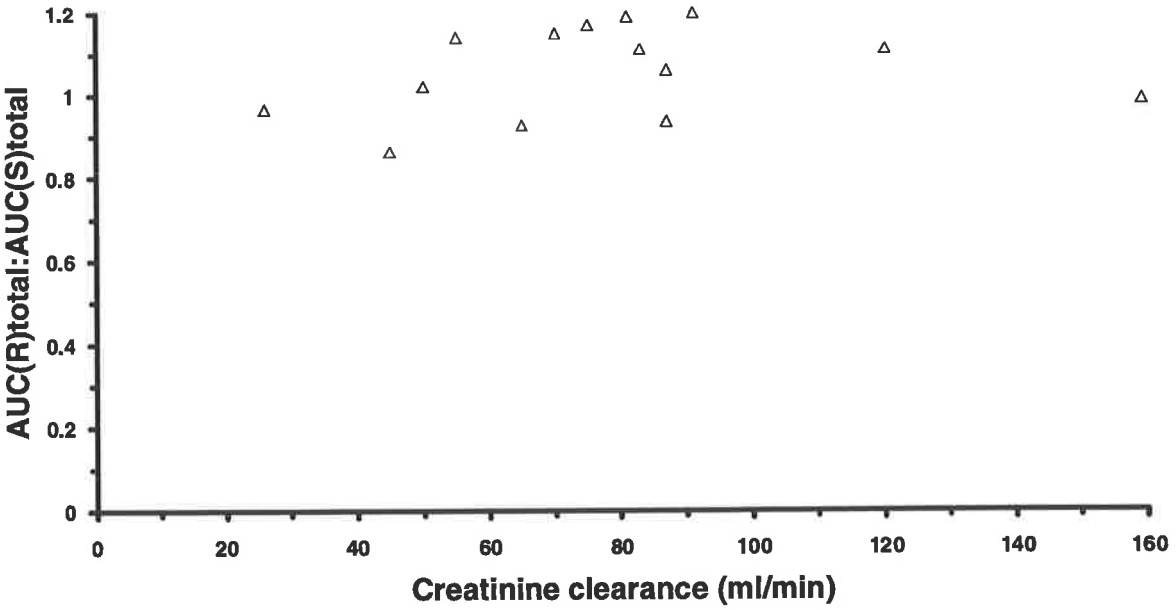


Figure 8.7 Plot of the ratio of $AUC_{(R)}$:$AUC_{(S)}$ for total (bound plus unbound) enantiomeric ketoprofen versus CL_{CR} for 14 study patients (r_s = -0.319, P>0.05).

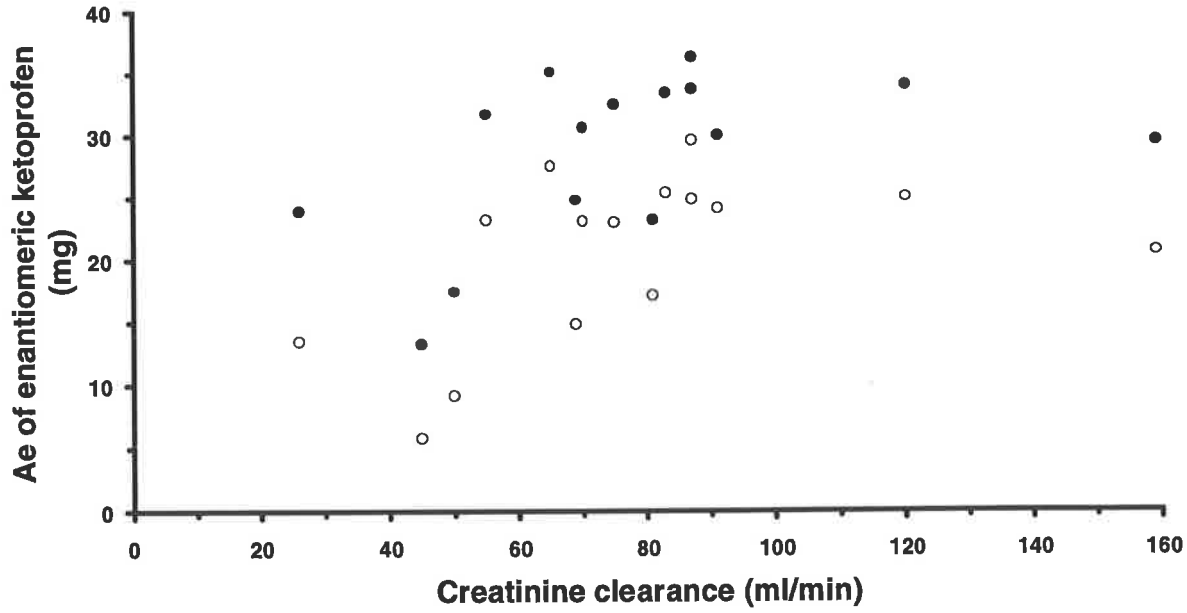


Figure 8.8 Plot of $Ae_{(S)}$ (●) (r_s = 0.481, P>0.05) and $Ae_{(R)}$ (○) (r_s = 0.554, P<0.05) as a function of CL_{CR} for all 15 study patients.

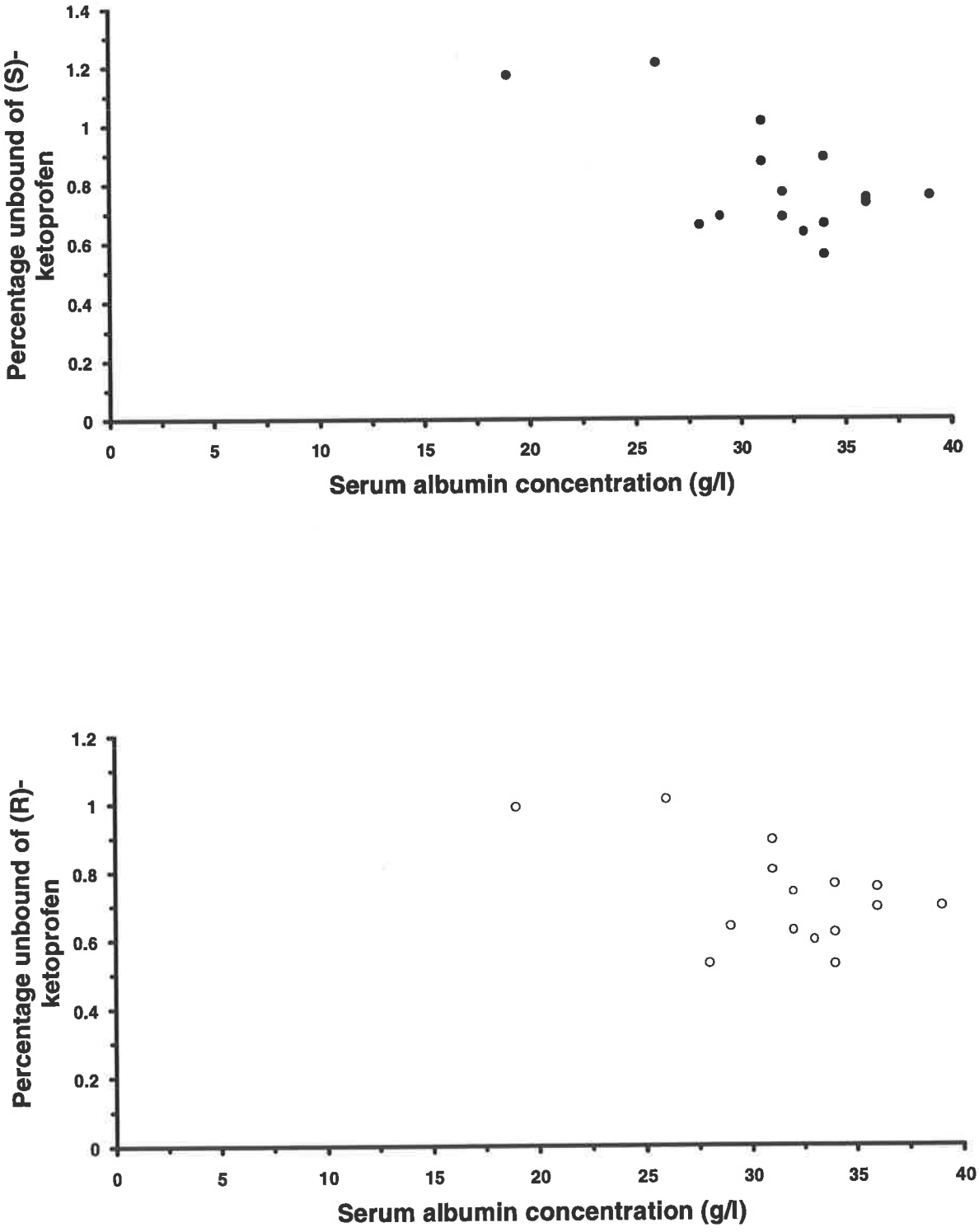


Figure 8.9 Plots of the mean percentage unbound of (S)-ketoprofen in plasma (●) (r_s = -0.345, P>0.05) and the mean percentage of (R)-ketoprofen (○) (r_s = -0.327, P>0.05) as functions of the serum albumin concentration for all 15 patients.

8.3 Results (continued)

Pharmacodynamics of (S)-ketoprofen

The relationship between the percentage inhibition of TXB_2 generation and serum unbound (S)-ketoprofen concentration is shown for the subjects in Figure 8.10 and individual data tabulated in the Appendix. The effect of pharmacologically active unbound (S)-ketoprofen, added as (RS)-ketoprofen *in vitro*, on platelet TXB_2 generation during the controlled clotting of whole blood is presented for individual subjects in Table 8.3. These data are presented as sigmoidal E_{max} model parameter estimates. The data from one subject (patient #13) were not included since these data were not amenable to modelling.

No correlation existed between the concentration of unbound (S)-ketoprofen required to cause a 50% inhibition of TXB_2 generation (EC_{50}) and CL_{CR} (r_s = -0.129, P>0.05) [Figure 8.11]. The mean $\pm$ SD EC_{50} for the 14 subjects examined was 0.216 $\pm$ 0.143 ng/ml and the corresponding steepness factor (n) for the concentration-effect relationship was 2.26 $\pm$ 1.97.

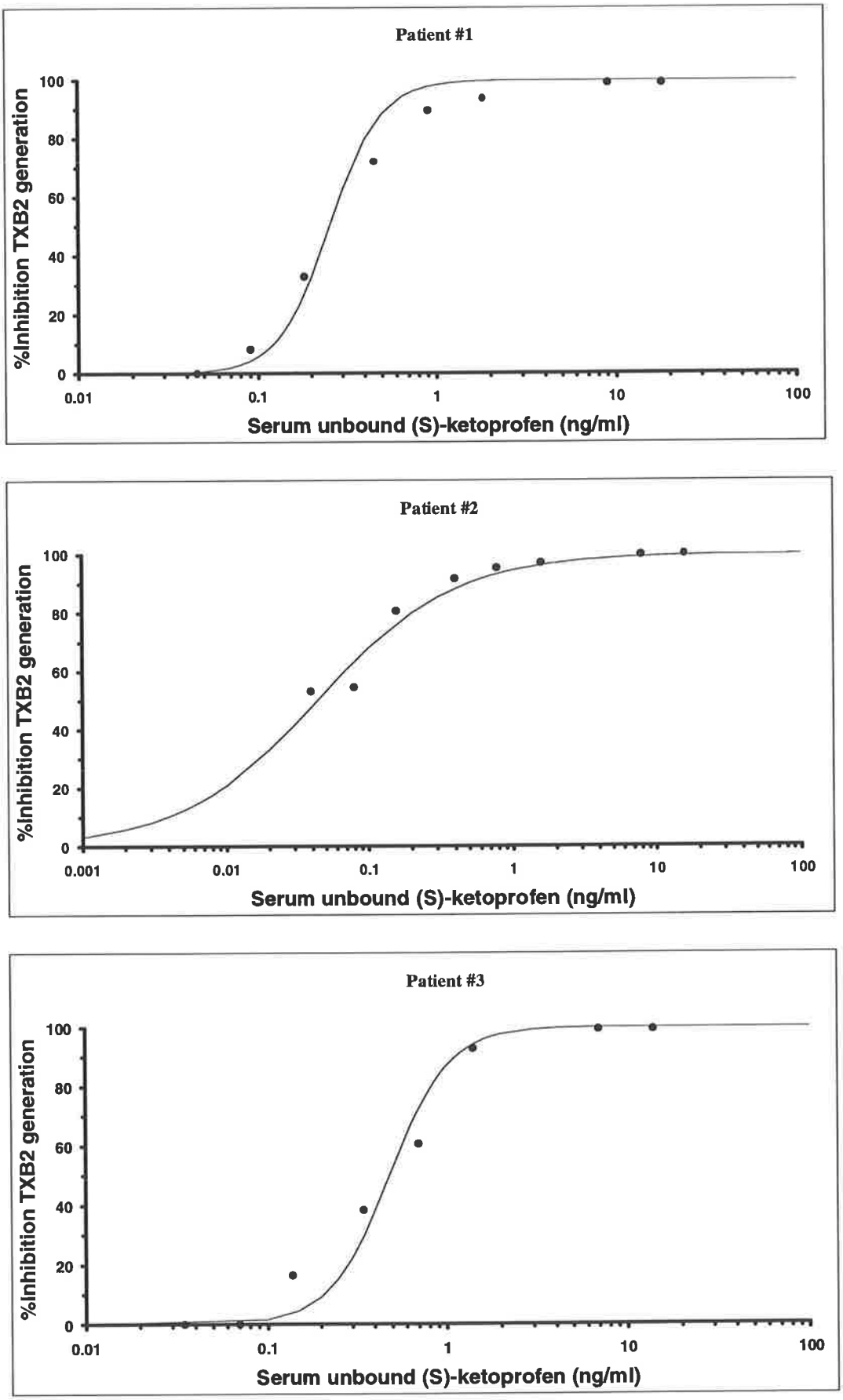


Figure 8.10 Relationships between the percentage inhibition of TXB_2 generation and the concentration of unbound (S)-ketoprofen in serum for patients #1 to #15 (the data from patient #13 were not amenable to modelling). The symbols are actual data points and the line is the sigmoidal E_{max} model-predicted relationship.

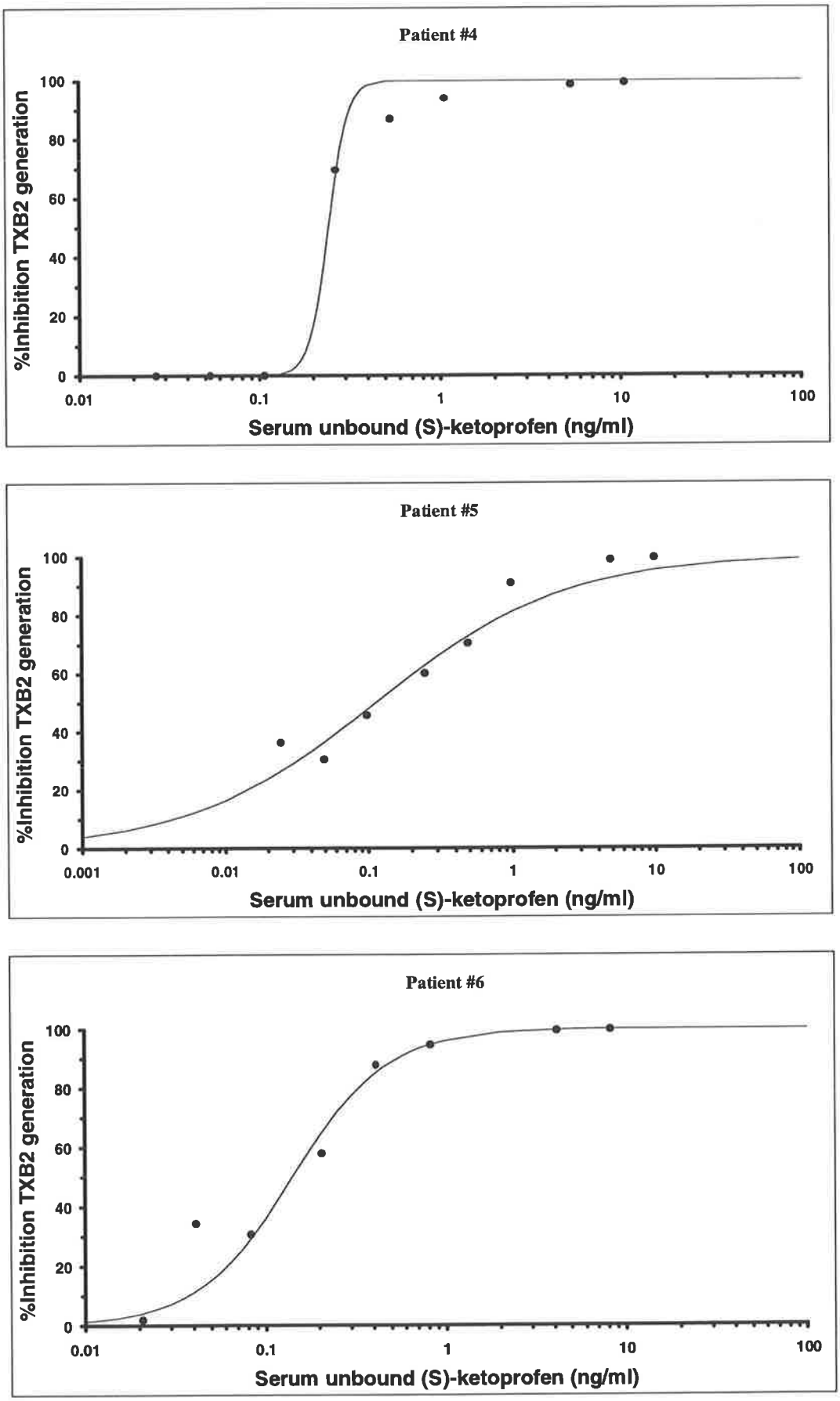


Figure 8.10 (continued) Relationships between the percentage inhibition of TXB_2 generation and the concentration of unbound (S)-ketoprofen in serum for patients #1 to #15 (the data from patient #13 were not amenable to modelling). The symbols are actual data points and the line is the sigmoidal E_{max} model-predicted relationship.

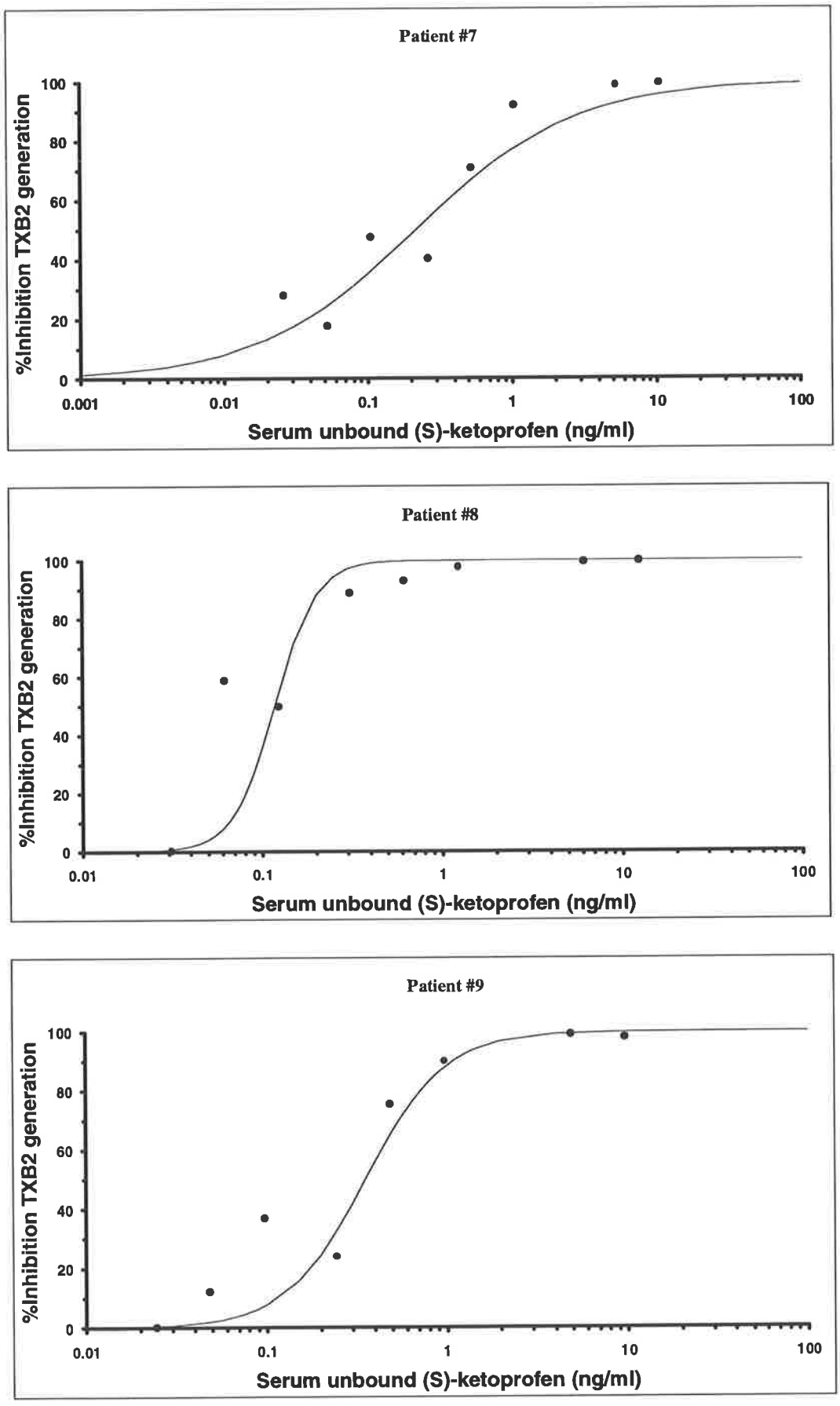


Figure 8.10 (continued) Relationships between the percentage inhibition of TXB_2 generation and the concentration of unbound (S)-ketoprofen in serum for patients #1 to #15 (the data from patient #13 were not amenable to modelling). The symbols are actual data points and the line is the sigmoidal E_{max} model-predicted relationship.

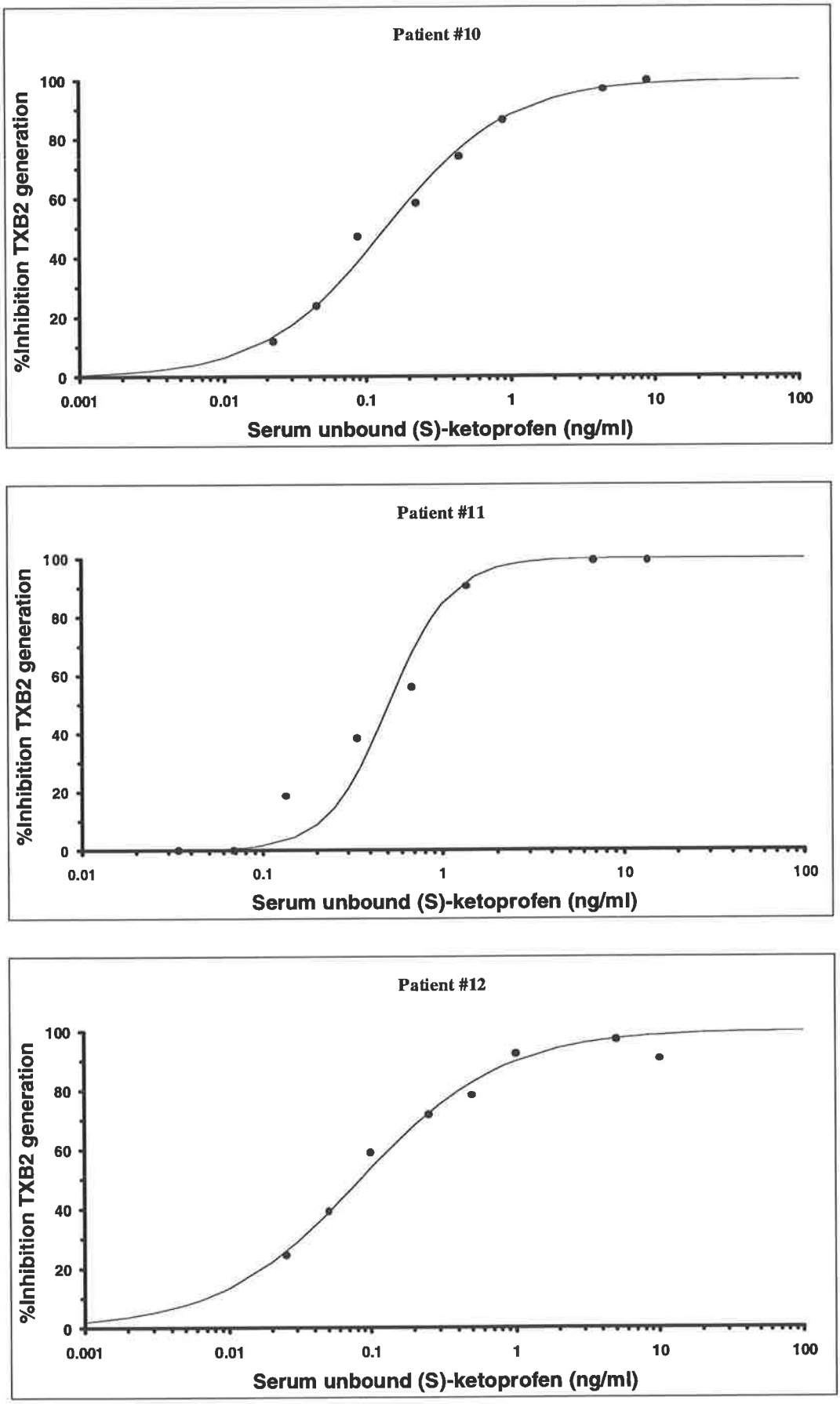
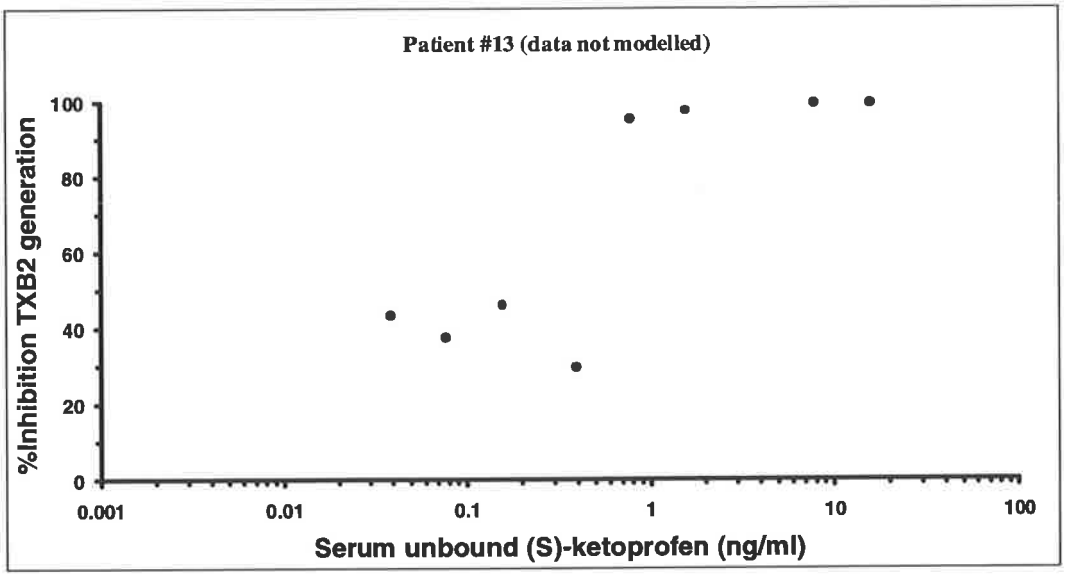
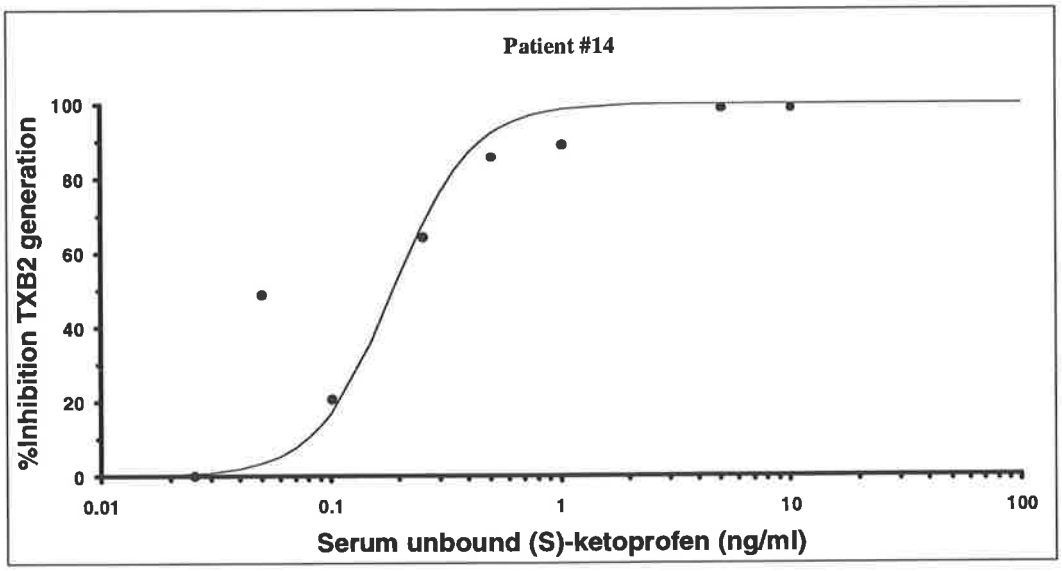


Figure 8.10 (continued) Relationships between the percentage inhibition of TXB_2 generation and the concentration of unbound (S)-ketoprofen in serum for patients #1 to #15 (the data from patient #13 were not amenable to modelling). The symbols are actual data points and the line is the sigmoidal E_{max} model-predicted relationship.





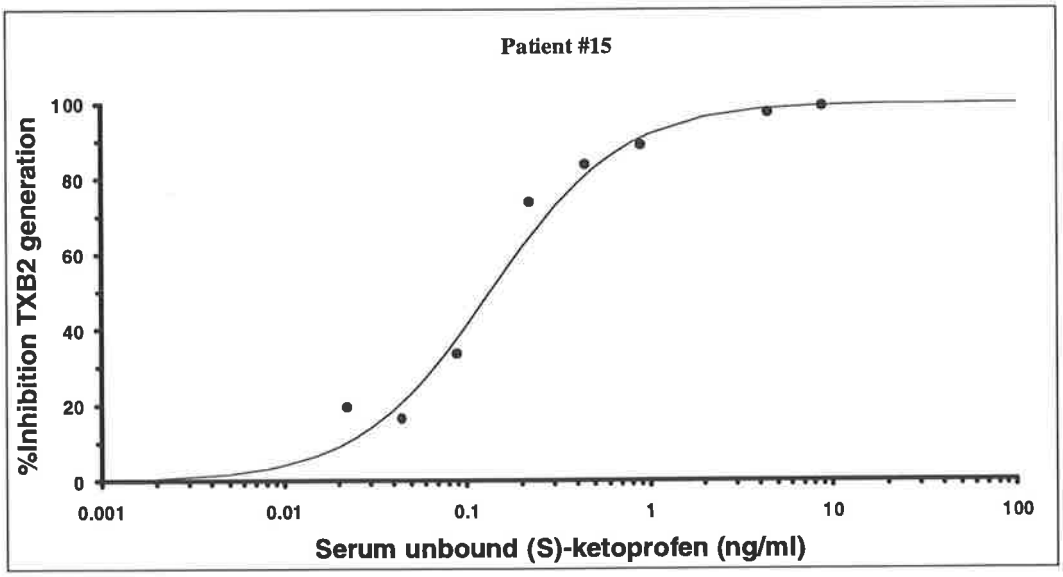


Figure 8.10 (continued) Relationships between the percentage inhibition of TXB_2 generation and the concentration of unbound (S)-ketoprofen in serum for patients #1 to #15 (the data from patient #13 were not amenable to modelling). The symbols are actual data points and the line is the sigmoidal E_{max} model-predicted relationship.

Table 8.3 Pharmacodynamic parameters (± standard error of the estimate) derived from the sigmoidal E_{max} modelling of individual subject data together with the experimentally derived blood to serum concentration ratio of (S)-ketoprofen used (together with $fu_{(S)}$) for calculating corresponding unbound serum concentrations after spiking whole blood with drug.

Patient #	$[B]_{(S)}/[S]_{(S)}$	EC_{50} (ng/ml)	*n*
1	0.554	0.254 ± 0.030	2.99 ± 0.41
2	0.742	0.044 ± 0.007	0.91 ± 0.17
3	0.622	0.484 ± 0.072	2.61 ± 0.43
4	0.695	0.243 ± 0.009	8.25 ± 1.99
5	0.766	0.113 ± 0.022	0.67 ± 0.11
6	0.670	0.142 ± 0.030	1.64 ± 0.32
7	0.626	0.218 ± 0.066	0.79 ± 0.20
8	0.588	0.117 ± 0.024	3.72 ± 1.12
9	0.783	0.355 ± 0.090	1.96 ± 0.53
10	0.769	0.138 ± 0.012	1.02 ± 0.08
11	0.644	0.508 ± 0.087	2.50 ± 0.45
12	0.654	0.084 ± 0.007	0.87 ± 0.08
13	0.773	*	*
14	0.618	0.189 ± 0.053	2.52 ± 0.72
15	0.769	0.135 ± 0.021	1.21 ± 0.19
Mean	0.685	0.216	2.26
SD	0.077	0.143	1.97

* Data for this subject were not amenable to modelling.

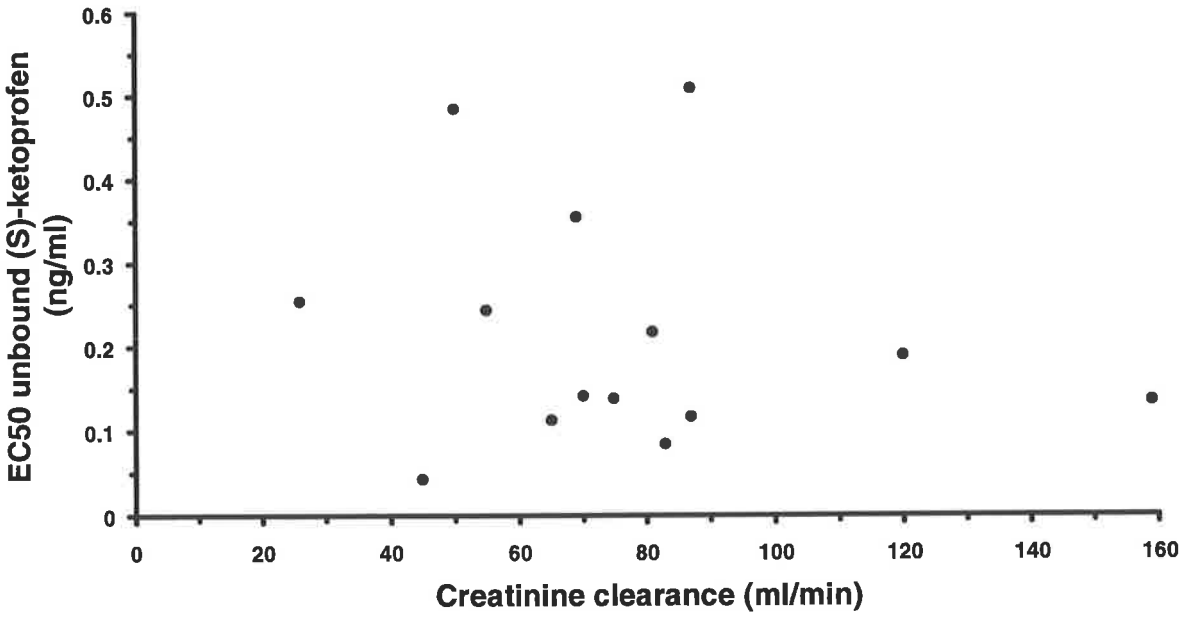


Figure 8.11 Plot of concentration of unbound (S)-ketoprofen in serum required to cause a 50% inhibition of TXB_2 generation as a function of CL_{CR} for 14 study patients (r_s = -0.129, P>0.05).

8.4 Discussion

The mean percentage unbound values of (R)- and (S)-ketoprofen for each patient (Table 8.2) were calculated from the respective measured values obtained from the *in vitro* addition of (RS)-ketoprofen to pre-dose plasma over the enantiomer concentration range of 1.00 to 6.00 μg/ml. Given the concentration-dependent protein binding of (S)-ketoprofen in these patients at some total drug concentrations achieved within the first hour of dosing (binding of (S)-ketoprofen was linear at concentrations of 3.50 μg/ml and below), it would have been desirable to estimate $fu_{(S)}$ (and subsequently, $AUC_{(S)\text{-unbound}}$) from serially-determined *ex vivo* samples. However, this was not possible given the presence of ketoprofen glucuronoconjugates in post-dose plasma. While these metabolites do not directly influence the protein binding of the parent enantiomers (see Section 5.3), they are susceptible to deconjugation under the handling conditions (physiological pH and temperature) necessary to obtain the unbound species (see Chapters 3 and 6). Furthermore, manifestly stereoselective hydrolysis of ketoprofen conjugates was observed *in vitro* in plasma under physiological conditions (Section 6.3), thereby spuriously altering the (R):(S) aglycone ratio (in addition to increasing the parent drug concentration) in non-quenched *ex vivo* samples. The calculated $AUC_{(S)\text{-unbound}}$ values may differ slightly from true values; however, the magnitude of this discrepancy would be expected to be small based on a mean 11% increase in $fu_{(S)}$ at 6.00 μg/ml of (S)-ketoprofen versus the corresponding value at 3.50 μg/ml (Figure 8.1) and the fact that $fu_{(S)}$ was linear for most *ex vivo* plasma enantiomer concentrations.

It is of interest to note that the percentage unbound of (S)-ketoprofen in plasma is greater than that of its optical antipode in these elderly patients while a lack of stereoselectivity in the binding of ketoprofen to plasma protein from healthy young volunteers was noted (Chapter 5). Moreover, in those young subjects (none of whom were taking any medication), concentration-dependent binding of (S)-ketoprofen was evident only at a supra-therapeutic enantiomer concentration of 19.0 μg/ml. The magnitude of percentage unbound estimates of ketoprofen enantiomers is similar in both groups of subjects. Recent studies with flurbiprofen (Knadler *et al.*, 1992a,b) have shown a reduction in plasma protein binding of both enantiomers in uraemic patients, compared to normal subjects, which was attributed to a

significantly lower plasma albumin concentration in the former patient group. Some of the study patients herein (none of whom were classified clinically as uraemic) had serum albumin concentration values below the normal range (Table 8.1). However, there was no correlation between the percentage unbound of each enantiomer and either serum albumin (Figure 8.9) or CL_{CR}. An unavoidable complicating factor in this and most other studies examining protein binding in elderly patients with rheumatoid arthritis is the potential effects of concurrent medication. Perhaps this, together with reported alterations to binding of acidic drugs in patients with reduced renal function and accumulated endogenous anions (Lindup *et al.*, 1991) and the low serum albumin concentrations in this elderly patient group, might explain the appearance of non-linear protein binding of (S)-ketoprofen at lower total concentrations than that observed earlier in healthy young medication-free subjects (Chapter 5).

For ketoprofen, which is cleared almost exclusively by metabolism (Upton *et al.*, 1980), AUC_{total} after a single oral dose (D) is determined by the fraction of the administered dose absorbed from the gastrointestinal tract (f_a), the fraction of drug unbound in plasma, and the intrinsic clearance of unbound drug from plasma (CLu_{int}) (Wilkinson and Shand, 1975). For each enantiomer, given the minimal chiral inversion of this 2-arylpropanoate in man (*vide infra*),

$$AUC_{total} = \frac{f_a.D}{fu.CLu_{int}} \qquad (8.1)$$

The corresponding relationship for the unbound species of each enantiomer simplifies to

$$AUC_{unbound} = \frac{f_a.D}{CLu_{int}} \qquad (8.2)$$

Equation 8.2 predicts that disease state-induced changes to $AUC_{unbound}$ take place as a result of alterations to either oral bioavailability or intrinsic drug clearance or both and are independent of changes to the fraction unbound in plasma.

The data shown in Figures 8.3(a) and 8.3(b) demonstrate that although a negligible amount of ketoprofen is cleared renally as unchanged drug (Upton *et al.*, 1980; see Section 2.3.3) the AUC of both unbound (R)- and unbound (S)-ketoprofen were inversely related to patient renal

function. While the potential confounding influence of co-medication is recognised, these findings support the mechanism of accumulation of renally eliminated, labile acyl-glucuronides and their subsequent systemic hydrolysis back to the parent aglycone, demonstrated by Meffin and coworkers for clofibric acid and termed a "futile cycle" (1983a,b), and originally proposed for the reduced clearance of diflunisal observed in patients with impaired renal function (Faed, 1980). As discussed in Section 2.3.4.1, the disposition of ketoprofen (in terms of total enantiomers) appears to be unaffected by the arthritis disease process. Additionally, the unbound fraction of enantiomers in plasma is quantitatively similar in both healthy and arthritic volunteers and consequently, the $AUC_{unbound}$ versus CL_{CR} relationships observed in this study are less likely to be functionally related to the degree of severity of the arthritic condition. In the present case, declining renal function induces an apparent reduction in net CLu_{int} (*i.e.* the result of conjugative and other clearances of ketoprofen enantiomers and their regeneration by hydrolysis of the unstable glucuronoconjugates) and hence an increase in $AUC_{unbound}$ (Equation 8.2). Sallustio *et al.* (1988b) were unable to establish a relationship between AUC, in terms of total (bound plus unbound) ketoprofen enantiomers at steady state, and CL_{CR} in an elderly cohort of patients with rheumatoid arthritis upon multiple dosing with a sustained-release formulation of the drug. In the absence of protein binding data for those patients, the likelihood exists that undetected changes to the fraction unbound negated alterations to net CLu_{int}, thereby masking potential renal dysfunction-induced perturbations in AUC_{total}, as suggested by inspection of Equation 8.1. However, of interest in the present study, both total and unbound AUC (for each enantiomer) were inversely correlated with CL_{CR} (Figures 8.4 and 8.3, respectively). Furthermore, one would anticipate with a cycle of reversible glucuronidation in operation, that renal dysfunction would cause greater elevations in $AUC_{unbound}$ for those drugs (such as ketoprofen) for which clearance is predominantly via acyl-glucuronidation relative to drugs subject to significant phase I (non-conjugative) metabolism. Consequently, it is not surprising that Knadler and associates (1992b) were unable to demonstrate "futile cycling" of unbound flurbiprofen enantiomers in uraemic patients, presumably since elimination of parent drug proceeds by substantial oxidative metabolic pathways in addition to glucuronide conjugation.

An alternative postulate to explain the relationships in Figure 8.3 might be changes to oral bioavailability as a function of degree of renal impairment. Lack of availability of an intravenous formulation of ketoprofen prevented describing the disposition of unbound enantiomers independent of potential changes to oral drug absorption. However, the oral bioavailability of ketoprofen in healthy subjects has been reported to be close to unity (Jamali and Brocks, 1990; see Section 2.3.1) and hence it would be highly unlikely for absorption of ketoprofen enantiomers to be enhanced in patients with reduced renal function which could otherwise explain these relationships. Indeed the urinary recovery of (R)-ketoprofen, as combined aglycone and conjugate, declined with diminishing renal function (Figure 8.8 and *vide infra*) and if this is indicative of reduced (rather than increased) oral bioavailability, then the absolute relationships between $AUC_{unbound}$ and CL_{CR} would have been dampened by changes to f_a and hence these data (Figure 8.3) could be more compelling in support of a decrease in net CL_{int} with declining renal function. Previously, it has been suggested that decreased systemic clearance of ketoprofen in elderly subjects is due to a diminished capacity to conjugate aglycone with glucuronic acid (Advenier *et al.*, 1983). While declining renal function might be associated with a synchronous reduction in liver function, studies of a number of drugs (forming stable ether glucuronides) administered to patients with renal failure have shown normal glucuronoconjugation capacity (Verbeeck, 1982). As stated above, a practically unavoidable complication with this study are the potential effects of co-medication on ketoprofen disposition. However, in light of current knowledge of pharmacokinetic drug interactions with this drug (Verbeeck, 1990; see Section 2.3.4.2), there appeared to be no obvious drug interaction trend which might otherwise explain the variation in AUC data in the patients (co-medications listed in Table 8.1).

Consistent with the acyl-glucuronide recycling hypothesis, a reduction in the urinary recovery of combined (conjugated and nonconjugated) (R)-ketoprofen was observed as renal function decreased (the correlation between the urinary recovery of (S)-ketoprofen and CL_{CR} just failed to reach statistical significance) [Figure 8.8]. Urine collection periods were not extended beyond 24 h after dosing since most of the aglycone and metabolites recovered were found in the initial 0-5 h aliquot with only 14% of the total 0-24 h excretion of ketoprofen taking place in the final (10-24 h) collection period (expressed as the mean result from the 5 patients with

the lowest CL_{CR}). In addition, a previous study examining the disposition of tritiated ketoprofen in elderly human subjects showed negligible urinary excretion of radioactivity after 24 h (Delbarre *et al.*, 1976) and a study of ketoprofen pharmacokinetics in patients with chronic renal failure also demonstrated a lack of excreted drug aglycone or conjugate reclaimed in urine collections beyond 24 h (Stafanger *et al.*, 1981). This latter study together with enantioselective examinations of ketoprofen disposition in elderly arthritic patients in the present investigation and other studies (Foster *et al.*, 1988a; Sallustio *et al.*, 1988b) showed considerable reductions in the urinary recovery of nonconjugated and conjugated ketoprofen compared to studies with young healthy subjects (Foster *et al.*, 1988b; Upton *et al.*, 1980). The biliary excretion of ketoprofen or its acyl-glucuronide has been ruled out as a significant route of elimination in man (Foster *et al.*, 1989b). It is possible that hydroxylation, as originally reported in early studies with ketoprofen (Populaire *et al.*, 1973), may be of greater importance in patients with diminished renal function.

While metabolic chiral inversion of the (R)- to the (S)-enantiomer has been demonstrated for ketoprofen in man (Jamali *et al.*, 1990), the extent of this inversion process was reported to be small (*circa* 9-12% in healthy subjects). Obviously, the (R):(S) AUC ratio is not a measure of the absolute extent of chiral inversion. However, based on the "futile cycle" model proposed for a chiral structural homologue of ketoprofen, 2-phenylpropanoic acid (Meffin *et al.*, 1986), a renally induced reduction in net clearance of the (R)-enantiomer would disproportionately increase the plasma concentrations of its optical antipode (see Section 3.5). In this case, the extent to which impaired renal function predictively increases the exposure to (S)- relative to (R)-aglycone will be dependent on the degree of chiral inversion of the particular 2-arylpropanoate. In the absence of direct influences of renal function on the intrinsic inversion process and given the minor role of inversion for ketoprofen in man, it is not surprising that no relationship was detected between the $AUC_{(R)}$:$AUC_{(S)}$ ratio for either total or unbound ketoprofen and CL_{CR} (Figures 8.7 and 8.6, respectively).

Attempts were made to quantify the AUC for the acyl-glucuronides of (R)- and (S)-ketoprofen from indirect conjugate analysis (see Chapter 6) of *ex vivo* plasma samples, since it was suspected that these parameters might be elevated in those patients with impaired renal

function. Foster *et al.* (1988a,b) observed elevated plasma glucuronide concentrations of enantiomeric ketoprofen in elderly arthritic subjects and were unable to detect these metabolites in the plasma of young healthy and arthritic subjects. Unfortunately, it was not possible to reliably quantify the acyl-linked conjugates in the plasma from the patients in the present study.

No attempt was made to derive clearance or volume of distribution estimates of ketoprofen enantiomers for the patients in this study given both the apparent "futile cycling" of conjugate and the observed metabolic chiral inversion of the drug in humans (Jamali *et al.*, 1990). In general, the concepts of clearance and distribution volume, for both total and unbound drug, pertain to circumstances where elimination occurs from the pharmacokinetic compartment into which the dose was loaded. As noted by Upton and coworkers (1984), this principle would be violated by aglycone-conjugate recycling; ketoprofen conjugates in plasma constitute a peripheral compartment in equilibrium with aglycone in the dosed (central) compartment, which in turn constitutes the site from which urinary excretion of conjugates occurs. Furthermore, the conversion of (R)-ketoprofen to (S)-ketoprofen *in vivo* (albeit minor in humans, *vide supra* and Section 2.3.3) precludes knowledge of the actual amount of (S)-ketoprofen in the body in the absence of fractional inversion data for individual subjects. In this case, following racemic drug dosing, calculation of both clearance and volume of distribution cannot be performed for the (S)-enantiomer.

The unbound and total AUC estimates for (R)- and (S)-ketoprofen (Table 8.2) show a lack of enantioselectivity in terms of the plasma concentration-time course of this drug in humans. In terms of total drug, dose-normalised AUC values for ketoprofen enantiomers in this study were similar to those obtained in elderly patients with arthritis as were the half-life estimates for (R)- and (S)-ketoprofen (Foster *et al.*, 1988a). It is interesting to note the universally higher $Ae_{(S)}$ values in comparison with the $Ae_{(R)}$ values (Table 8.2), an observation also made by others (Foster *et al.*, 1988a,b; Sallustio *et al.*, 1988b). Additionally, rapid and stereoselective hydrolysis of ketoprofen glucuronides was observed in *in vitro* experiments conducted with human plasma under physiological conditions (see Section 6.3). Extrapolated to the *in vivo* situation, the faster deconjugation of (R)-ketoprofen glucuronide, prior to its

urinary excretion, is possibly reflected (at least in part) in the higher urinary recovery of (S)-ketoprofen observed in this study. Spahn and coworkers (1989b) have previously noted that stereoselective degradation of acyl-glucuronide conjugates could be misinterpreted as a difference in glucuronide formation rates (see Section 3.2).

With respect to the *in vitro* inhibition of platelet cyclo-oxygenase by unbound (S)-ketoprofen, it appeared that renal function was not a determinant of this drug effect, based on the estimated concentration of pharmacologically active moiety required to inhibit the production of platelet TXA_2 by 50% (Figure 8.11). Moreover, the mean value of EC_{50} was similar in magnitude (although discrete estimates were subject to greater inter-individual variability) to values obtained by identical means in young healthy subjects (Section 7.3). It was also apparent, based on estimates of (S)-ketoprofen concentrations achieved with chronic dosing of racemic drug in humans (Foster *et al.*, 1988a,b) and applying these *in vitro* data and the data obtained in healthy volunteers (Section 7.3), that the thromboxane-related antiplatelet effects of ketoprofen would persist throughout the dosing interval. It was not possible to monitor the *ex vivo* time-dependency of the TXB_2-mediated anti-platelet effects in these patients for the reasons stated in Section 7.4.

In conclusion, the exposure to pharmacologically active unbound (S)-ketoprofen is increased and the corresponding elimination half-life is protracted in those patients with diminished renal function. Based on these data, a dosage adjustment may be warranted in this patient group, the magnitude being dependent upon the degree of impairment of renal function.

Chapter 9

General Conclusion

Many chiral drugs, including the vast majority of chiral NSAIDs, are currently used clinically as racemates. In the case of congeners of the 2-arylpropanoic acid NSAIDs, it would appear unlikely that there would be a uniform shift towards the marketing of enantiomerically pure drugs given the widespread popularity, low cost and apparent safety of the racemic therapeutic entities, even though enantioselectivity is more often the rule rather than the exception, with respect to the pharmacology of these drugs. Thus in clinically relevant settings, it is essential to individually characterize the pharmacokinetic and pharmacodynamic properties of the enantiomers and moreover, in nearly all cases, measure these properties when both enantiomers are present together.

Clearly, potentially misleading drug disposition and concentration-effect data may be generated for a chiral drug if nonstereoselective analytical methods are used. In order to examine various aspects of the clinical pharmacology of ketoprofen, assays were developed for determining both the total and unbound concentrations of (R)- and (S)-ketoprofen, most notably, when the enantiomers were present together in the biological sample of concern.

From the results of *in vitro* protein binding studies, developed and applied in a cohort of healthy, young volunteers, it was shown that at clinically relevant drug concentrations, the unbound fraction of ketoprofen did not exhibit enantioselectivity. Furthermore, the unbound fraction of each enantiomer in plasma was invariant (over this concentration range) and was not modified either by the presence of the respective optical antipode or by the acyl-glucuronide metabolites.

Ketoprofen is subject to significant metabolic transformation to renally excreted labile acyl-glucuronides. Biosynthetic acyl-glucuronides of ketoprofen were purified and characterized prior to the performance of *in vitro* studies aimed to address the hydrolysis, reversible protein

binding and the rearrangement reactions of these metabolites. It was apparent that albumin, rather than plasma esterases, catalysed the hydrolysis of collectively, the glucuronides and their rearrangement isomers *in vitro*, and further, facilitated this deconjugation reaction in a highly stereoselective fashion. Somewhat surprisingly, the degree of plasma protein binding of the ketoprofen glucuronides was relatively high (*circa* 90% bound) and unlike the protein binding of the aglycones (in plasma from comparable volunteers) exhibited marked stereoselectivity. It was postulated that the sites on the albumin molecule at which deconjugation and reversible binding of the conjugates takes place are dissimilar or functionally distinct. When the rearrangement process for ketoprofen glucuronides was monitored *in vitro*, it was apparent that this reaction predominated quantitatively (over the hydrolysis reaction) in protein-free buffer at physiological pH and temperature. The addition of albumin to the incubation medium enhanced the hydrolysis of collectively, the biosynthetic acyl-glucuronides and the rearranged isomers. It is speculative to extrapolate from the data obtained from *in vitro* incubations of the labile glucuronides to the situation *in vivo;* however, it appeared likely that in clinical situations where these metabolites might accumulate systemically, their interaction with albumin should be of pharmacological significance.

Ketoprofen reduces platelet aggregation via a prostaglandin-dependent mechanism involving reversible (presumptively) inhibition of cyclo-oxygenase. *In vitro* studies were performed to assess the relative inhibitory activity of (S)- and (R)-ketoprofen on platelet cyclo-oxygenase. This activity resided exclusively with the (S)-enantiomer; the recorded activity of enantiomeric (R)-ketoprofen was attributed to optical contamination of the test compound. In addition to being devoid of activity, (R)-ketoprofen did not modify the potency of its optical antipode. Based on the ability of (S)-ketoprofen to inhibit platelet thromboxane A_2 production during the controlled clotting of whole blood, the sigmoidal E_{max} modelled data (in terms of the pharmacologically active unbound (S)-enantiomeric species) indicated that ketoprofen was a potent inhibitor. Upon extrapolation to chronic dosing with ketoprofen, this effect on platelet cyclo-oxygenase would be predicted to be maximal across an entire standard dose interval.

A clinical study was performed with a group of fifteen patients with rheumatoid arthritis (receiving co-medication) to examine the influence of renal function on the enantioselective

pharmacokinetics and pharmacodynamics of ketoprofen upon administration of a single oral dose of racemic ketoprofen. It was hypothesized that the exposure to pharmacologically active unbound (S)-ketoprofen in plasma should be inversely related to the degree of renal function and moreover, the apparent sensitivity of platelet cyclo-oxygenase might be influenced by the degree of renal impairment. These studies revealed significant associations between the reciprocal of AUC for both unbound and total (S)-ketoprofen and creatinine clearance and between the reciprocal of AUC for both unbound and total (R)-ketoprofen and creatinine clearance. However, there was no correlation between the EC_{50} of unbound concentration of (S)-ketoprofen in serum required to inhibit platelet cyclo-oxygenase and creatinine clearance. Indeed, estimates of the EC_{50} in these patients were similar in magnitude to values obtained earlier in healthy volunteers. Unlike the protein binding results for healthy volunteers, in plasma from these arthritic patients the *in vitro* unbound fraction of (S)-ketoprofen exceeded the corresponding value of its optical antipode and there was evidence of nonlinearity in the unbound fraction of (S)-ketoprofen towards higher clinically relevant plasma concentrations. It was concluded from this study that diminished renal function was associated with an increased exposure to unbound (S)-ketoprofen in plasma, presumably due to regeneration of parent aglycone arising from the hydrolysis of systemically accumulated acyl-glucuronides. The apparent sensitivity of platelet cyclo-oxygenase to the inhibitory effect of (S)-ketoprofen was not modified by renal function. Thus, based on pharmacokinetic grounds, dosage adjustment for ketoprofen may be warranted in patients with decreased renal function.

Remaining to be answered are a number of questions pertaining to the clinical pharmacology of ketoprofen. Further studies are needed to investigate the generation of irreversibly bound drug-protein adducts for ketoprofen, particularly in clinical situations where the acyl-glucuronides accumulate systemically. While the immunotoxicological sequelae upon exposure to these covalently bound adducts is speculative at present, an enhanced body load, as might be expected in elderly patients or in patients with renal dysfunction, might be responsible for decreased drug tolerability. Of interest would be further investigations of the reversible binding of ketoprofen enantiomers to plasma protein and serum albumin, and the influence thereon of factors such as fatty acid content, albumin concentration and protein binding site specific probes. It would be interesting to extend the pharmacodynamic studies of inhibition of

platelet cyclo-oxygenase by ketoprofen to examine the *in vivo* time course of this anti-platelet effect. Such data would be clinically useful in making predictions as to when chronic recipients of this drug should cease taking ketoprofen prior to surgical procedures. Moreover, on the issue of ketoprofen pharmacodynamics, it would be extremely useful to be able to demonstrate relationships between drug concentration (in plasma and/or synovial fluid) and anti-inflammatory and analgesic effects. And finally, a question somewhat distantly related to the current clinical application of the drug, is whether the apparent sensitivity of platelet cyclo-oxygenase to ketoprofen affords recipients of this drug with cardio-protection. Perhaps those patients currently receiving ketoprofen need not co-medicate with a cardio-protective agent, such as aspirin, since such defence might already be provided by ketoprofen.

Appendix

Tabulated Study Patient Data

Tabulated raw data for each rheumatoid arthritic patient who participated in the study of renal function influences on the enantioselective pharmacokinetics and pharmacodynamics of ketoprofen (Chapter 8). Cited below are: (*i*) the *ex vivo* plasma concentration-time data for total (bound plus unbound) enantiomeric ketoprofen and (*ii*) the *in vitro* percentage inhibition data for platelet TXB_2 production as a function of the concentration of (S)-ketoprofen not bound to serum protein.

Patient identification number: #1

(*i*) Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.167	.162	ND (not detected)
.333	2.26	2.49
.500	3.12	3.18
.833	3.04	2.91
1.08	2.96	2.78
1.30	2.72	2.50
1.53	2.97	2.78
1.97	3.25	3.03
2.95	3.43	3.05
4.00	2.40	2.17
5.05	1.95	1.85
6.00	1.47	1.52
8.05	.886	1.17

(*ii*) Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
0.00	.0455
7.80	.0910
32.8	.182
71.7	.455
89.1	.910
93.3	1.82
98.9	9.10
98.8	18.2

Patient identification number: #2

(*i*) Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.250	.015	.106
.500	4.49	4.52
.750	4.46	4.25
1.00	2.70	2.73
1.30	1.96	2.08
1.58	1.64	1.78
1.83	1.36	1.49
2.00	1.31	1.43
3.00	1.05	1.20
4.00	.786	.992
5.00	.510	.707
6.00	.419	.647
8.00	.228	.457
10.0	.196	ND

(*ii*) Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
52.9	.0395
54.2	.0790
80.3	.158
91.4	.395
95.2	.790
96.9	1.58
99.7	7.90
100	15.8

Patient identification number: #3

(*i*) Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.317	.154	.164
.700	5.76	5.78
.967	4.68	4.46
1.18	4.44	4.17
1.50	3.63	3.47
1.75	2.95	2.05
2.00	2.57	2.29
2.83	1.41	1.24
3.12	1.21	1.08
4.00	.845	.783
5.00	.568	.589
6.42	.350	.429
8.00	.221	.325
10.0	.129	.182

(*ii*) Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
0.00	.0350
0.00	.0700
16.4	.140
38.2	.350
60.6	.700
92.6	1.40
99.2	7.00
99.1	14.0

Patient identification number: #4

(*i*) Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.250	.298	.328
.500	9.64	9.41
.850	7.99	7.18
2.17	3.41	2.87
2.50	2.66	2.11
2.97	2.08	1.63
3.47	1.69	1.30
3.90	1.48	1.13
4.98	.955	.741
6.00	.677	.565
8.00	.381	.351
10.0	.237	.232

(*ii*) Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
0.00	.0268
0.00	.0535
0.00	.107
69.4	.268
86.8	.535
93.8	1.07
98.4	5.35
99.1	10.7

Patient identification number: #5

(*i*) Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.250	.155	.201
.500	1.36	1.47
.750	2.81	2.96
1.05	5.81	5.96
1.25	5.01	5.11
1.50	4.58	4.70
1.75	4.40	4.50
2.00	4.52	4.61
3.10	1.86	1.90
4.00	1.12	1.21
5.00	.698	.823
6.00	.425	.553
8.00	.215	.318
10.0	.118	.200

(*ii*) Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
36.2	.0247
30.5	.0493
45.5	.0986
60.0	.247
70.2	.493
90.8	.986
98.8	4.93
99.4	9.86

Patient identification number: #6

(*i*) Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.270	7.31	7.17
.500	6.82	6.35
.750	5.73	5.24
1.00	4.90	4.43
1.25	4.44	3.91
1.55	3.36	2.94
1.75	3.07	2.68
2.00	2.64	2.29
3.00	1.39	1.20
4.00	.979	.792
4.95	.812	.659
5.95	.478	.387
8.00	.291	.164
9.70	.191	.105

(*ii*) Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
1.85	.0208
34.4	.0415
30.4	.0830
57.9	.208
87.6	.415
94.3	.830
99.2	4.15
99.5	8.30

Patient identification number: #7

***(i)* Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers**

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.250	4.08	4.59
.500	4.46	4.01
.750	5.60	4.75
1.03	3.23	2.73
1.25	2.76	2.26
1.50	2.39	1.89
1.75	1.94	1.50
2.00	1.70	1.30
3.00	1.06	.822
4.08	.637	.523
5.08	.460	.393
6.00	.308	.194
7.92	.167	.117
10.0	.111	.081

***(ii)* Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration**

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
27.9	.0263
17.6	.0523
47.2	.105
40.2	.263
70.5	.525
91.7	1.05
98.7	5.25
99.4	10.5

Patient identification number: #8

(*i*) Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.250	ND	.050
.500	.697	.759
.750	.858	.863
1.03	.930	.939
1.33	.910	.903
1.50	.843	.815
1.75	.809	.795
2.03	.630	.615
3.00	3.95	3.69
4.05	1.94	1.73
5.42	.827	.773
6.50	.470	.409
8.50	.272	.296

(*ii*) Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
.120	.0310
58.7	.0620
49.8	.124
88.7	.310
92.9	.620
97.5	1.24
99.4	6.20
99.7	12.4

Patient identification number: #9 (Incomplete post-dose sampling)

(*i*) Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.500	1.37	1.26
.833	1.84	1.86
1.25	3.38	3.29
1.58	3.03	2.80
2.03	2.89	2.59

(*ii*) Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
0.00	.0245
11.9	.0489
36.9	.0978
23.9	.245
75.3	.489
89.8	.978
98.9	4.89
97.9	9.78

Patient identification number: #10

(*i*) Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.233	ND	ND
.500	1.13	1.12
.800	3.43	3.19
1.05	2.85	2.52
1.45	2.23	1.91
1.75	1.81	1.53
2.00	1.84	1.59
3.00	1.49	1.24
4.00	1.03	.814
5.05	1.15	.945
6.08	.737	.606
8.00	.499	.414
10.2	.517	.449

(*ii*) Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
11.7	.0222
23.7	.0444
46.9	.0888
58.3	.222
73.9	.444
86.4	.888
96.7	4.44
99.6	8.88

Patient identification number: #11

***(i)* Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers**

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.233	5.76	6.00
.500	5.91	5.78
.733	4.14	4.09
1.17	2.53	2.57
1.30	2.31	2.35
1.50	1.95	1.89
1.78	1.62	1.61
2.03	1.41	1.40
3.00	.842	.869
4.00	.499	.562
5.00	.296	.402
6.00	.211	.315
8.00	.091	.208
9.00	.071	ND

***(ii)* Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration**

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
0.00	.0343
0.00	.0685
18.6	.137
38.3	.343
55.9	.685
90.4	1.37
99.1	6.85
99.2	13.7

Patient identification number: #12

(*i*) Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.250	.061	.057
.430	1.14	1.11
.830	1.91	1.80
1.00	2.07	1.91
1.25	5.00	4.62
1.50	5.23	4.67
1.78	5.22	4.76
2.05	5.93	5.29
3.05	2.47	2.07
4.00	1.53	1.39
5.00	1.04	1.00
6.00	.639	.616
7.55	.366	.339

(*ii*) Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
24.4	.0253
39.1	.0505
58.9	.101
71.6	.253
78.2	.505
92.2	1.01
96.8	5.05
90.5	10.1

Patient identification number: #13

(*i*) Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.267	1.80	1.85
.517	5.38	5.03
.850	5.18	4.65
1.00	3.59	3.08
1.23	3.87	3.20
1.42	3.42	2.78
1.72	2.89	2.26
2.00	2.20	1.61
3.02	1.36	.943
3.95	.687	.474
5.00	.467	.334
6.00	.295	.234
8.00	.132	.143
10.0	.059	.092

(*ii*) Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
43.1	.0391
37.5	.0783
45.8	.157
29.4	.391
95.1	.783
97.4	1.57
99.4	7.83
99.2	15.7

Patient identification number: #14

(*i*) Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.300	.067	.025
.500	1.55	1.62
.750	5.52	5.48
1.02	4.34	4.15
1.25	3.12	2.88
1.53	2.34	2.16
1.75	1.86	1.70
2.33	1.59	1.48
3.00	.958	.873
3.88	.530	.414
5.08	.361	.284
6.00	.247	.179
8.00	.138	.088
9.50	.094	.048

(*ii*) Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
0.00	.0255
48.8	.0510
20.6	.102
64.0	.255
85.5	.510
88.8	1.02
98.6	5.10
98.6	10.2

Patient identification number: #15

(*i*) Plasma concentration-time data for total (bound plus unbound) ketoprofen enantiomers

Time (h)	Plasma concentration (μg/ml)	
	(R)-ketoprofen	(S)-ketoprofen
0		
.250	.246	.239
.500	4.16	4.15
.750	2.92	2.82
1.08	1.35	1.26
1.25	1.50	1.39
1.58	1.13	1.03
1.75	1.03	.944
2.05	.760	.719
3.00	.394	.401
4.00	.269	.291
5.05	.163	.199
6.05	.113	.144
8.00	.061	.088
9.25	.044	.072

(*ii*) Percentage inhibition of TXB_2 generation versus serum unbound (S)-ketoprofen concentration

% Inhibition TXB_2 generation	Serum unbound (S)-ketoprofen (ng/ml)
19.4	.0223
16.3	.0446
33.4	.0892
73.6	.223
83.7	.446
88.8	.892
97.3	4.46
99.0	8.92

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Corrigendum

Page number	*Line number**	*Incorrect word/phrase*	*Correction*
2	3	amoung	among
13	-2	dispostion	disposition
14	9	-hyroxy-	-hydroxy-
16	-3	suggest	suggests
21	-6	being (sentence lacks verb)	replace with "is"
25	10	focused	focussed (and on p 30: line -2, and p 36: line 6)
25	-10	inhibitior	inhibitor
30	-7		delete "of"
30	-3	is	are
31	3	route	routes
36	10		delete "significant"
43	10	positonal	positional
60	-2	is	are

*negative line numbers are counted up from foot of page

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Short Communication

Enantiospecific analysis of ketoprofen in plasma by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic (HPLC) assay for the determination of the *R*- and *S*-enantiomers of ketoprofen is described. Facile ketoprofen extraction from plasma and derivatization to the diastereomeric *S*-1-phenylethylamides was followed by normal-phase HPLC. The ketoprofen diastereomeric amides eluted within 8 min. The limit of quantification of the assay was 0.15 mg/l of each enantiomer (signal-to-noise ratio = 5).

INTRODUCTION

Ketoprofen, 2-(3′-benzoylphenyl)propanoic acid, is a member of the 2-arylpropanoic acid series of non-steroidal anti-inflammatory drugs. Ketoprofen is marketed as the racemate; however, in keeping with other studied members of this structural drug class, it exhibits enantioselective pharmacodynamic and pharmacokinetic properties (recently reviewed by Jamali and Brocks [1]). The importance of discriminating between the separate enantiomers of this drug and of other 2-arylpropanoates, when studying the pharmacology in human subjects, has become self-evident [2].

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Earlier reports of analytical methods for ketoprofen in biological matrices utilized achiral techniques for the determination of unresolved drug [3–5]. Previous enantiospecific high-performance liquid chromatographic (HPLC) methods [6–8] entailed either extensive sample preparation or lengthy chromatography times. Sallustio *et al.* [6] utilized a protracted sample clean-up, employing reversed-phase HPLC isolation and quantification of unresolved ketoprofen followed by derivatization and subsequent normal-phase HPLC analysis of the diastereomeric *S*-1-phenylethylamides of ketoprofen.

Bjorkman [7] and Foster and Jamali [8] have developed reversed-phase HPLC methods for the L-leucinamide diastereomers of ketoprofen with single chromatographic run times of 16 and 20 min, respectively.

We report here major modifications to the method of Sallustio *et al.* [6] including simple extraction of unresolved drug from acidified plasma and subsequent normal-phase HPLC separation of the diastereomeric *S*-1-phenylethylamides within 8 min.

EXPERIMENTAL

Reagents and chemicals

Racemic ketoprofen was a gift from May and Baker (Sydney, Australia) and *S*-naproxen (internal standard) of F. H. Faulding (Adelaide, Australia). Pure *R*- and *S*-ketoprofen were kindly supplied by Dr. Kathy Knights of The Flinders University of South Australia (Bedford Park, Australia). Racemic [1-^{14}C]ketoprofen was synthesized and authenticated [9]. HPLC-grade heptane, isopropyl alcohol and methanol were purchased from BDH (Poole, UK) as were analytical-grade sulphuric acid, *n*-hexane, ethyl acetate and dichloromethane for sample extraction. Thionyl chloride (redistilled over linseed oil within four weeks of use) was purchased from May and Baker (Dagenham, UK), sodium hydroxide and linseed oil were from Ajax (Sydney, Australia) and *S*-1-phenylethylamine was obtained from Sigma (St. Louis, MO, USA). Racemic fenoprofen calcium was obtained from Eli Lilly (Indianapolis, IN, USA), racemic ibuprofen was a kind gift of Boots (Nottingham, UK), mefenamic acid was obtained from Parke Davis (Sydney, Australia) and salicylic acid from F. H. Faulding.

Instrumentation and chromatographic conditions

The liquid chromatograph consisted of a Model 510 pump, Wisp autoinjector, Model 490 variable-wavelength UV absorbance detector and Model 840 data station, all from Waters Assoc. (Milford, MA, USA).

An SGE (Sydney, Australia) glass-lined HPLC column (250 mm × 4 mm I.D.) containing 5 μm silica was operated at ambient temperature (22°C) through which the mobile phase (isopropyl alcohol–*n*-heptane, 8:92, v/v) was pumped at 1 ml/min. This solvent was filtered (0.22 μm) and degassed immediately prior to use and an in-line 2 μm filter (Waters Assoc.) was positioned ahead of the column.

The column was monitored for UV absorbance at a detection wavelength of 254 nm.

Sample preparation and derivatization

In a culture tube (100 mm × 16 mm) equipped with a PTFE-lined screw cap were added 1.0 ml of plasma, 0.05 ml of internal standard solution (*S*-naproxen, 200 mg/l in methanol), 0.5 ml of 2 *M* sulphuric acid and 8 ml of extracting solvent (10% ethyl acetate in *n*-hexane). Each sample was gently mixed for 10 min on a rotary mixer (30 rpm) and then centrifuged for 10 min (1500 *g*). The organic layer was transferred to a fresh culture tube and evaporated to dryness at 45°C under a stream of purified nitrogen (Zymark, Hopkinton, MA, USA). The dried residue was reconstituted with 0.1 ml of 1.5% thionyl chloride in *n*-hexane (freshly prepared) and the tube firmly capped and heated for 1 h at 75°C in a dry heat bath. The sample was subsequently allowed to cool to room temperature before adding 0.5 ml of 2% *S*-1-phenylethylamine in dichloromethane (freshly prepared) and re-capping the tube for a further 15 min. Final extraction of the lipophilic amides was accomplished by the addition of 0.5 ml of 2 *M* sulphuric acid and 5 ml of *n*-hexane. Mixing, centrifugation and drying of the organic layer were as described above for the initial extraction step. The dried residue was reconstituted with 0.25 ml of mobile phase and 0.2 ml was injected onto the HPLC column.

Calibration, precision and accuracy

Racemic ketoprofen plasma standards were prepared by adding 1.0 ml of drug-free plasma to culture tubes containing dried methanolic extracts of ketoprofen such that the final concentration range of 0.156–10.00 mg/l for each enantiomer was achieved. These standards were taken through the sample preparation and derivatization methods described above. Separate calibration curves for each ketoprofen enantiomer were constructed as the peak-area ratios of the diastereomers to the internal standard diastereomer, and least-squares linear regression analysis was performed to determine slopes, intercepts and regression coefficients.

The concentration/normalised peak-area ratios for each enantiomeric ketoprofen standard over the concentration range were also calculated for each set of standards.

The accuracy and precision of the method were assessed by preparing methanolic solutions of racemic ketoprofen from weighings independent of those used for preparing the calibration standards. Addition of drug-free plasma to dried methanolic extracts yielded ketoprofen enantiomeric concentrations in plasma of 0.200 and 9.00 mg/l. Aliquots (1 ml) of these plasma samples were analysed to determine intra-day accuracy and precision, and inter-day (eight-week period) accuracy and precision of the assay.

Extraction efficiency

In order to optimize and quantitate the initial extraction of unresolved ketoprofen enantiomers and internal standard from plasma a reversed-phase non-stereoselective HPLC method was used [9]. Briefly, this achiral method involved simple extraction of ketoprofen and internal standard from acidified plasma and chromatography on a radially compressed (RCM-100) phenyl cartridge (4 μm, 100 mm × 8 mm I.D., Waters Assoc.) through which the mobile phase (acetonitrile–10 m*M* acetate buffer, pH 3, 50:50, v/v) was pumped at 2 ml/min (UV wavelength detection at 260 nm).

Instrumentation was identical to that described above for the enantiospecific ketoprofen assay.

The peak areas after injection of unresolved ketoprofen and internal standard extracted from plasma were compared to those generated from direct injections of aqueous solutions of these compounds. Extraction efficiency was assessed at the upper and lower ends of the calibration range (18 and 0.4 mg/l unresolved racemic ketoprofen) and of the internal standard.

Derivatization efficiency

Racemic [1-^{14}C]ketoprofen was synthesized and subsequently purified by the non-stereoselective reversed-phase HPLC method detailed under *Extraction efficiency* (described in detail elsewhere [9]). A methanolic aliquot of this radiochemically pure ketoprofen (specific activity 8.70 mCi/mmol) was added to drug-free plasma to achieve an enantiomeric concentration of 1.25 mg/l and taken through the enantiospecific assay extraction and derivatization steps. The HPLC eluates corresponding to the [1-^{14}C]-*R*- and [1-^{14}C]-*S*-ketoprofen diastereomeric amide peaks were collected in glass tubes, evaporated to dryness under nitrogen (45°C), reconstituted and counted in a xylene-based liquid scintillant (PCS II, Amersham, U.K.).

Counting was carried out in a Nuclear-Chicago (Des Plaines, IL, USA) liquid scintillation system (Unilux III) and quench correction was performed with the internal standard method ([^{14}C]toluene, Amersham). These counts were compared with counts from pure radiolabelled racemic ketoprofen. The combined extraction and derivatization efficiency for ketoprofen could thus be determined together with a measure of potential chiral discrimination during the derivatization reaction between enantiomeric ketoprofen acyl chlorides and *S*-1-phenylethylamine.

Assigning absolute configuration

Each authentic pure enantiomer of ketoprofen was taken through the complete sample preparation method to establish its retention time. Potential racemization was also checked by this method.

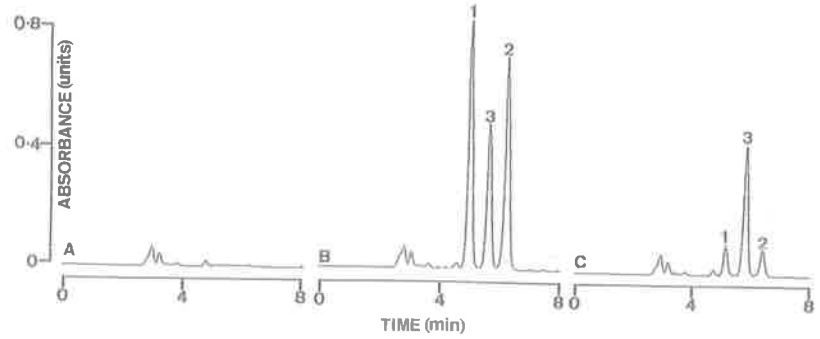

Fig. 1. Chromatograms of derivatized samples. (A) Extract of drug-free plasma (1 ml) without internal standard. (B) Plasma sample obtained 2 h after a 100-mg dose of racemic ketoprofen (Orudis, May and Baker) containing 6.21 mg/l *R*-ketoprofen and 4.82 mg/l *S*-ketoprofen. (C) Plasma sample obtained 6.5 h after this ketoprofen dose containing 0.521 mg/l *R*-ketoprofen and 0.484 mg/l *S*-ketoprofen. Peaks 1 and 2 represent the diastereomeric *S*-1-phenylethylamides of *R*-ketoprofen and *S*-ketoprofen, respectively, and peak 3 the *S*-1-phenylethylamide of *S*-naproxen (internal standard).

RESULTS AND DISCUSSION

Chromatograms resulting from the analysis of drug-free plasma and plasma obtained 2 and 6.5 h after a single 100-mg dose of racemic ketoprofen (Orudis, May and Baker, Sydney, Australia) are depicted in Fig. 1. No interfering endogenous plasma peaks were observed. Under the chromatographic conditions described for the enantiospecific assay, the retention times for the *R*- and *S*-ketoprofen diastereomeric amides were 5.2 and 6.6 min, respectively. *S*-Naproxen-*S*-1-phenylethylamide conveniently eluted between the ketoprofen peaks at 5.9 min.

Calibration curves generated over the enantiomeric concentration range from 0.156 to 10.00 mg/l (supplied as racemic drug) were linear for both enantiomers of ketoprofen. Linear least-squares regression analysis for twelve calibration

TABLE I

LINEAR LEAST-SQUARES REGRESSION ANALYSIS FOR TWELVE CALIBRATION CURVES FOR EACH KETOPROFEN ENANTIOMER CONSTRUCTED INDEPENDENTLY OVER AN EIGHT-WEEK PERIOD

Parameter	Value (mean ± S.D.)	
	R-Ketoprofen	*S*-Ketoprofen
Slope	2.87 ± 0.19	2.94 ± 0.21
y-Intercept	0.0120 ± 0.00054	0.0169 ± 0.00062
Regression coefficient	0.999 ± 0.0008	0.999 ± 0.0007
Concentration range (mg/l)	0.156 – 10.00	0.156 – 10.00

curves is depicted in Table I. In every case the correlation coefficient was >0.999.

The intra-day accuracy and precision of the assay was assessed by analysing six separate aliquots of two independently prepared plasma samples covering the upper and lower limits of the ketoprofen enantiomeric concentration range. For the first quality control sample containing 9.00 mg/l of each enantiomer, the mean concentrations of *R*- and *S*-ketoprofen were determined to be 9.25 mg/l (coefficient of variation, C.V. = 3.81%) and 9.08 mg/l (C.V. = 2.96%), respectively. The second sample contained 0.200 mg/l *R*-ketoprofen and *S*-ketoprofen. The concentrations determined were 0.217 mg/l (C.V. = 7.92%) and 0.193 mg/l (C.V. = 8.90%), respectively.

The inter-day accuracy and precision were determined by analysing the quality control plasma samples containing 9.00 and 0.200 mg/l of each enantiomer (supplied as the racemate) over an eight-week period. The mean (n = 12) concentrations of *R*- and *S*-ketoprofen for the lower plasma concentration samples were 0.191 mg/l (C.V. = 4.70%) and 0.196 mg/l (C.V. = 4.21%), respectively. The accuracy (and precision) values (n = 12) for the 9.00 mg/l plasma samples were 9.24 mg/l (C.V. = 3.90%) and 9.36 mg/l (C.V. = 5.00%) for the *R*- and *S*-enantiomers of ketoprofen, respectively.

The mean (±S.D.) extraction efficiency of 10% ethyl acetate in hexane–sulphuric acid (*vide supra*) was assessed (n = 6) for 18.0 mg/l racemic ketoprofen in plasma and found to be 83.9 ± 1.4% relative to an unextracted aqueous aliquot of ketoprofen utilizing the non-stereospecific assay described above. The efficiency of extraction (n = 6) for 0.400 mg/l racemic ketoprofen was 86.5 ± 3.3%. The respective value (n = 6) for the internal standard (*S*-naproxen) was 95.5 ± 2.9%.

Assessment of the combined extraction and derivatization methodologies of the enantiospecific assay entailed analysis of plasma sample containing 1.25 mg/l of each enantiomer of ketoprofen (supplied as purified racemic [1-^{14}C]ketoprofen) over a two-week period (n = 8). The mean (±S.D.) yield for unresolved ketoprofen was 79.6 ± 6.1% and the ratio of *R*- to *S*-ketoprofen diastereomeric amide counts was 1.002 ± 0.021. Thus, derivatization to the diastereomeric amides with *S*-1-phenylethylamine was non-stereoselective and largely quantitative.

Validation of the method in terms of potential racemization of ketoprofen enantiomers during sample processing was examined by analysis of plasma samples containing 5.00 mg/l pure *R*-ketoprofen and pure *S*-ketoprofen over a two-week period (n = 8 for each). The concentration of the contaminant antipode remained less than 2.5% in each case providing evidence that no racemization was taking place during sample extraction and derivatization.

It is important to establish the specificity of any analytical method which is designed for measurement of drug concentrations in biological matrices, since the possibility exists for subjects to be coadministered other drugs. Given the acidic environment in which extraction of xenobiotics takes place with this assay, a

range of acidic drugs were assessed in terms of their retention times relative to the elution profiles of ketoprofen and internal standard diastereomers. The retention times for the diasteromeric amides of the *R*- and *S*-enantiomers of ibuprofen were 3.3 and 4.1 min and for fenoprofen, 3.7 and 4.6 min, respectively. Mefenamic acid eluted as its respective amide at 3.0 min while salicylic acid had a retention time of 3.8 min.

In addition, plasma samples were obtained from a number of human subjects who had been prescribed a variety of xenobiotics not including non-steroidal anti-inflammatory agents. These included ranitidine, nifedipine, penicillamine, midazolam, diazepam, theophylline, methylprednisolone and digoxin. None of these parent compounds nor any observed metabolite peak coeluted with the *S*-1-phenylethylamides of ketoprofen and *S*-naproxen (internal standard).

In summary, we have described a rapid, sensitive and specific HPLC assay for the determination of ketoprofen enantiomers in plasma. The method performs with adequate accuracy and precision.

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STEREOSELECTIVE INTERACTIONS OF KETOPROFEN GLUCURONIDES WITH HUMAN PLASMA PROTEIN AND SERUM ALBUMIN

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Abstract—A clearance pathway common to many aryl alkanoic acids is the generation of renally eliminated ester glucuronides. These metabolites are susceptible to systemic hydrolysis which generates the parent aglycone. We have conducted *in vitro* studies with biosynthetic *R*- and *S*-ketoprofen glucuronides to elucidate the mechanism of this phenomenon. These conjugates were incubated in human plasma, various concentrations of human serum albumin (HSA) and protein-free buffer. It was apparent that albumin, rather than plasma esterases, catalysed the hydrolysis of the glucuronides. The albumin-catalysed hydrolysis of ketoprofen glucuronides was highly stereoselective. The mean (±SD) hydrolysis half-life of *R*-ketoprofen glucuronide in plasma (N = 4) at physiological pH and temperature was 1.37 (±0.30) hr. The corresponding value for *S*-ketoprofen glucuronide, 3.46 (±0.84) hr, was significantly different ($P < 0.005$). In contrast, synthetic ethyl esters of *R*- and *S*-ketoprofen were hydrolysed by plasma esterases, but not by HSA, and with little stereoselectivity. The reversible protein binding of ketoprofen glucuronides was determined at physiological pH and temperature by a rapid ultra-filtration method. The binding of *R*- and *S*-ketoprofen glucuronide to human plasma protein was independent of concentration ($P > 0.05$) over the range of 1–20 μg/mL. The mean (±SD) percentage unbound in plasma (N = 4) of *R*-ketoprofen glucuronide was 12.6 (±1.4) %. The corresponding value for *S*-ketoprofen glucuronide, 9.12 (±0.54) %, was significantly different ($P < 0.005$). *S*-Ketoprofen glucuronide was also more avidly protein bound in physiological concentrations of HSA. However, this stereoselectivity decreased in more dilute HSA solutions. Based on the hydrolysis and protein binding data for ketoprofen glucuronides, we propose the existence of separate binding and catalytic sites on the albumin molecule for these metabolites.

Ketoprofen is a non-steroidal anti-inflammatory drug (NSAID‖) and is eliminated predominantly (approximately 70% of administered dose in humans) as acyl (ester) glucuronides in urine [1] (see Fig. 1 for structure). This is a clearance process common to many NSAIDs including fenoprofen [2], indoprofen [3], carprofen [4] and pirprofen [5]. Glucuronoconjugation of xenobiotics has been assumed to lead to abolition of the pharmacological activity of the aglycone due to reduced lipophilicity and subsequent renal or biliary excretion of the conjugate [6].

A series of elegant experiments [7, 8] examined the disposition of clofibric acid in an animal model of diminished renal function and in animals coadministered probenecid. The existence of a cycle of reversible glucuronide conjugation for acyl glucuronides and their respective aglycones was deduced. This provided a mechanistic explanation for the reduced clearances of the predominantly non-renally cleared NSAIDs ketoprofen [9, 10], naproxen [11], ximoprofen [12] and benoxaprofen [13] in patients with renal dysfunction or in elderly patients in whom renal function was diminished. In such a cycle, net clearance is due to glucuronoconjugation and competition between elimination of glucuronide by renal and hydrolytic clearances. This cycle predicts that inhibition of the renal elimination of the acyl glucuronide will lead to a reduction in net drug clearance due to accumulation and deconjugation of the physiologically labile glucuronide metabolite [14].

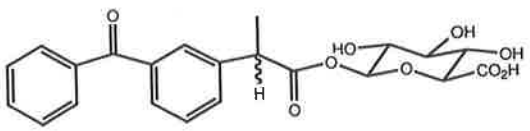

Fig. 1. Structure of 1-*O*-ketoprofen-β-D-glucuronide.

In addition to direct hydrolysis of the biosynthetic 1-*O*-acyl-β-glucuronide to the corresponding aglycone, intra-molecular rearrangement via acyl migration of the drug moiety to other positions on the glucuronic acid ring and subsequent hydrolysis of these isomers are potential sources of deconjugated drug [14, 15]. Moreover, a quantitatively minor competing pathway is covalent binding via the acyl glucuronide forming a drug–protein adduct [15]. Each of these processes involves potential regeneration of pharmacologically

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‖ Abbreviations: NSAID, non-steroidal anti-inflammatory drug; HSA, human serum albumin; PBS, isotonic phosphate-buffered (0.067 M, pH 7.4) saline.

BP 44:2-G

active aglycone and has been reported for a number of NSAIDs [15] including fenoprofen (Ref. 16 and references to other compounds therein). Whereas the hydrolysis of covalent drug–protein adducts has been a quantitatively minor contributor to regenerated aglycone, the hydrolysis of positional isomers of glucuronides has been shown to be a significant pathway for fenoprofen [16], oxaprozin [17] and diflunisal [18].

The aim of the present study was to examine the mechanisms involved in *in vitro* deconjugation of acyl glucuronoconjugates using ketoprofen glucuronides as model metabolites. Significant concentrations of ketoprofen glucuronides have been detected in the plasma of elderly patients with reduced renal function [19] and in young healthy subjects coadministered probenecid [20]. Specifically, we have studied the relationship between the reversible binding of these conjugates to human plasma or human serum albumin and net hydrolysis (i.e. hydrolysis of biosynthetic glucuronide and the positional isomers together with, possibly, hydrolysis of drug–protein adduct). A further aim was to investigate factors which might influence these processes. Moreover, since ketoprofen (in common with most NSAIDs possessing a chiral carbon) is marketed for clinical use as a racemate, we have examined the stereoselectivity of glucuronide net hydrolysis and reversible binding.

MATERIALS AND METHODS

Purification and characterisation of ketoprofen glucuronides. The purification procedure for biosynthetic ketoprofen glucuronides has been described in detail elsewhere [21]. Characterization of the purified *R*- and *S*-ketoprofen glucuronides was carried out by both chemical and physical means. The extent of hydrolysis of the glucuronides by purified β-glucuronidase (type VII-A, Sigma Chemical Co., St Louis, MO, U.S.A.; 100 U/mL, 37°, 60 min) was identical to that of complete alkaline hydrolysis (1.0 M sodium hydroxide, 22°, 15 min). This suggested exclusive C-1 attachment of ketoprofen to the glucuronic acid moiety with a β-linkage; and consequently, a lack of regioisomeric compounds in the purified material. In addition, enantioselective analysis of the aglycone (*vide infra* and [22]) confirmed the proportion of *R*:*S* ketoprofen to be 44.6:55.4 following hydrolysis by either chemical or enzymatic means of the purified biosynthetic metabolites.

Confirmation of structure as 1-*O*-(*R*,*S*)-ketoprofen-β-D-glucopyranosiduronate (glucuronide) was obtained by high-field (300 MHz) ^{1}H-NMR spectroscopy. Two overlapping doublets at 5.4δ (relative to tetramethylsilane) of unequal heights (corresponding to the enantiomeric ratio of aglycone) with coupling constants (5.7 and 6.3 Hz) the same for each and consistent with trans diaxial hydrogen atoms (anomeric protons) confirmed acyl attachment at C-1 of D-glucuronic acid in the β-configuration. Hydroxyl protons were not seen, presumably replaced by deuterium from the solvent (CD_3OD). Two overlapping doublets, again of unequal height, at 1.45 δ with coupling constants of 6.3 and 6.6 Hz corresponded to the α-methyl protons of the two diastereomers split by the corresponding α protons.

Fast-atom bombardment mass spectroscopy of glucuronides dispersed in thioglycerol showed fragments consistent with a glucuronide conjugate of ketoprofen. Major ions at *m*/*z* 453 and 276 corresponded to sodium adducts of both the molecular ion and fragment aglycone ion. In addition, fragment ions were recorded at *m*/*z* 253 (aglycone ion), 209 and 105.

Synthesis of ketoprofen ethyl ester. Racemic ketoprofen (200 mg, 0.79 mmol) in 5 mL of dichloromethane and 0.5 mL of thionyl chloride (previously distilled over quinoline and boiled linseed oil, successively) was refluxed for 2 hr, and the solvent and excess thionyl chloride removed under a stream of purified nitrogen. The residue was dissolved in 2 mL of dry toluene and evaporated to dryness; this azeotroping process was repeated.

The resulting crude racemic ketoprofen acid chloride was dissolved in 5 mL of dichloromethane and added dropwise with stirring (4 hr) over an ice-bath to 1 mL of anhydrous ethanol containing 20 μL of dry pyridine. The solution was filtered, evaporated to dryness and redissolved in 5 mL of dichloromethane. This solution was washed thrice with 10 mL of phosphate buffer (0.1 M, pH 6). The organic layer was dried (magnesium sulphate) and the solvent removed under a stream of nitrogen to yield ketoprofen ethyl ester (167 mg, 75%). A portion of this material was subjected to complete alkaline hydrolysis (1 M sodium hydroxide, 50°, 2 hr) and yielded an acid with physical properties identical to those of authentic racemic ketoprofen.

Hydrolysis experiments. The rates of net hydrolysis of the glucuronide or ethyl esters of *R*- and *S*-ketoprofen were determined in a number of different matrices. The concentrations of the diastereomeric glucuronides or enantiomeric ethyl esters were determined by a difference method. The concentration of each hydrolysed product (*R*- and *S*-ketoprofen) after a given incubation time, was subtracted from the initial (time zero) concentration of ester (expressed as ketoprofen equivalents) and the difference was subsequently normalized as a percentage of the initial ester concentration.

For the net hydrolysis of the glucuronoconjugates of ketoprofen, the HPLC-purified diastereomeric glucuronides were added to various media to achieve a concentration of approximately 15 μg/mL (*ca.* 8.3 and 6.7 μg/mL of *S*- and *R*-ketoprofen glucuronide, respectively) expressed as ketoprofen equivalents. The media comprised; (i) fraction V human serum albumin (HSA) (Calbiochem, San Diego, CA, U.S.A.; lot 902736, 99.5% pure by electrophoresis) at concentrations of 40, 4.0 and 0.40 g/L in isotonic phosphate-buffered (0.067 M, pH 7.4) saline (PBS); (ii) essentially fatty acid-free fraction V HSA (Sigma; lot 118F9311) 4.0 g/L in PBS; and (iii) plasma obtained via arm vein venepuncture from four healthy volunteers. The media were adjusted to a final pH of 7.4 with orthophosphoric acid (2.5%) and temperature of 37° (oscillating waterbath, 20 cpm). In addition, the hydrolysis of ketoprofen glucuronides was determined in protein-free PBS at pH 7.4 and 9.0. The influence of warfarin sodium

(100 μM; Sigma) and diazepam (100 μM; Roche, Sydney, Australia) on the rates of hydrolysis of the diastereomeric glucuronides was examined in HSA solutions by pre-incubating with warfarin or diazepam for 15 min prior to addition to ketoprofen glucuronides. The experiments were performed throughout by adding fresh temperature- and pH-adjusted medium (10.0 mL) to a glass culture tube containing the glucuronides (from a methanolic solution evaporated to dryness under nitrogen immediately prior to addition of medium). The solution was mixed and rapidly aliquoted (1.00 mL) into individual glass tubes which were closed with PTFE-lined screw caps and returned to the waterbath. One of the aliquots of glucuronides was hydrolysed to completion (1.0 M sodium hydroxide, 100 μL, 15 min, 22°) to determine the initial (time zero) *R*- and *S*-ketoprofen glucuronide concentration. Thereafter, timed samples were removed from the waterbath and the hydrolysis reaction quenched (pH decreased to 3.0; [14]) with 0.025 mL of 2 M sulphuric acid. Incubations of glucuronides were carried out for up to 6 hr in the case of plasma and 40 g/L HSA, up to 8 hr for 4.0 g/L HSA and up to 24 hr for 0.4 g/L HSA and protein-free PBS solutions. The quench conditions were verified experimentally for ketoprofen glucuronides. The liberated aglycones were immediately extracted into hexane/ethyl acetate prior to enantioselective analysis as described below.

Hydrolysis experiments with the ethyl esters of ketoprofen were essentially the same as described for the glucuronides, with the following exceptions. The synthesized material spiked into media (15 μg/mL) was racemic and complete hydrolysis (time zero aliquot) was effected by 100 μL of 1 M sodium hydroxide at 50° for 2 hr. Fraction V HSA (40 g/L), human plasma from a single volunteer and protein-free PBS (all at 37° and pH 7.4) were the media tested. The influence of physostigmine (8 mM; Sigma) and sodium fluoride (120 mM; Ajax Chemical, Sydney, Australia) on the rates of hydrolysis of the enantiomeric ethyl esters was examined. Incubations of these esters were carried out for up to 6 hr.

Protein binding of ketoprofen glucuronides. The *in vitro* reversible protein binding of *R*- and *S*-ketoprofen glucuronide was determined in human plasma and in various concentrations of HSA (both fraction V and fatty acid free) with and without warfarin (100 μM) or diazepam (100 μM). Binding was determined at physiological pH and temperature. The unbound species were obtained by ultra-filtration (Centrifree^R, Amicon Division, Danvers, MA, U.S.A.) of 1-mL aliquots in a pre-warmed (37°) centrifuge (2000 *g*, 10 min; Heraeus Suprafuge 22 with HFA 20.16 fixed-angle rotor, Osterode, Germany). Less than 15 min elapsed between the addition of glucuronides to plasma or HSA and the acid-quenching of the corresponding ultra-filtrate. The concentrations of unbound diastereomeric glucuronides were determined by the difference method (*vide supra*). Typically, the aglycone concentration in the non-hydrolysed (acid-quenched) ultra-filtrate was less than 10% of that in the alkaline hydrolysed ultra-filtrate. There was no detectable sorption of glucuronides onto the ultra-filtration membrane.

In the experiments conducted with human plasma, the protein binding of *R*- and *S*-ketoprofen glucuronide (present together as 44.6% *R*- and 55.4% *S*-) was examined over the plasma concentration range of approximately 2–40 μg/mL (expressed as unresolved ketoprofen equivalents). Plasma (harvested within 1 hr of use) was obtained from each of four healthy volunteers, none of whom was taking any medication.

The binding of ketoprofen glucuronides, at a constant concentration of 15 μg/mL (8.3 and 6.7 μg/mL of *S*- and *R*-ketoprofen glucuronide, respectively) was investigated in the HSA solutions. When the influence of warfarin or diazepam on the binding of the glucuronides was investigated, HSA solutions

Table 1. *In vitro* net hydrolysis and reversible protein binding of *R*- and *S*-ketoprofen glucuronide in human plasma harvested from four healthy volunteers

Subject (gender)	Age (years)	Serum albumin (g/l)	$T_{1/2,R}$ (hr)	$T_{1/2,S}$ (hr)	% unbound$_R^*$	% unbound$_S^*$
I (f)	28	38	1.77	4.71	11.1	8.59
II (m)	54	39	1.11	2.93	14.4	9.76
III (f)	27	37	1.18	2.99	12.6	9.37
IV (m)	26	42	1.42	3.22	12.3	8.77
Mean			1.37†	3.46	12.6‡	9.12
SD			0.30	0.84	1.4	0.54

* Analysis of variance indicated that there was no difference ($P > 0.05$) in % unbound$_R$ and % unbound$_S$ over the concentration range examined (approximately 1–20 μg/mL of ketoprofen equivalents) and therefore the values for each subject at the various concentrations were averaged.

† Statistically different ($P < 0.005$; unpaired *t*-test) from the corresponding value for *S*-ketoprofen glucuronide.

‡ Statistically different ($P < 0.005$; analysis of variance) from the corresponding value for *S*-ketoprofen glucuronide.

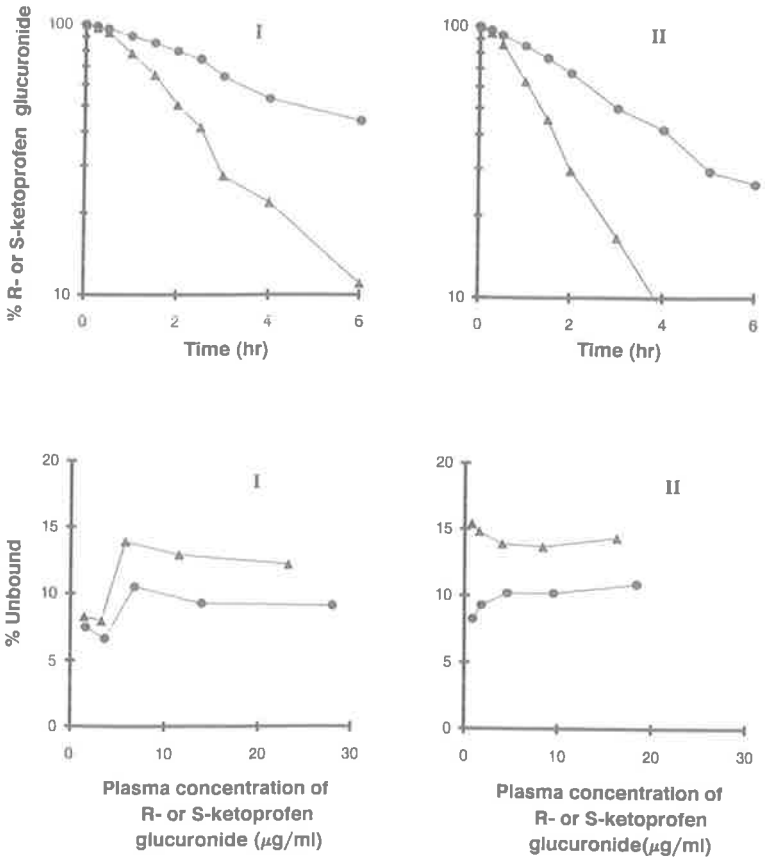

Fig. 2. Net hydrolysis and reversible protein binding at physiological pH and temperature of *R*-ketoprofen glucuronide (▲) and *S*-ketoprofen glucuronide (●) in human plasma from 2 subjects (I and II). The upper panels are semi-logarithmic plots of percentage glucuronide remaining after incubation of 15 μg/mL of glucuronides. The lower panels depict the plasma protein binding data for the glucuronides. Concentrations are expressed as ketoprofen equivalents.

were preincubated (15 min) with warfarin or diazepam prior to addition of the glucuronides.

Analytical methods. In order to quantify the glucuronide or ethyl esters of *R*- and *S*-ketoprofen, samples (hydrolysis incubation media or ultra-filtrate) were alkali-hydrolysed (at time zero only, in the case of hydrolysis experiments) and corresponding matched or timed samples protected against hydrolysis (acid-quenched with 0.025 mL of 2 M sulphuric acid). The differences in the aglycone concentrations between the alkali-treated samples and those of acid-protected samples was taken as a measure of *R*- or *S*-ketoprofen: glucuronide or ethyl ester. The enantioselective HPLC analytical method employed for ketoprofen has been described in detail elsewhere [22]. In outline the method involved rapid solvent extraction of ketoprofen enantiomers together with internal standard (*S*-naproxen) with 10% ethyl acetate in *n*-hexane from acidified (pH 3) matrix. Samples were evaporated to dryness prior to conversion to the intermediate acid chlorides (thionyl chloride) and subsequent derivatization with *S*-1-phenylethylamine (Sigma) generated the diastereomeric *S*-1-phenylethylamides. These diastereomers of ketoprofen and internal standard were resolved on a silica (5 μm) column (250 × 4 mm i.d., SGE, Sydney, Australia) eluted with 8% isopropyl alchol in *n*-heptane at a flow rate of 1 mL/min. Quantification was performed by monitoring the column eluant for UV absorbance (254 nm). Calibration was carried out by spiking drug-free incubation media (hydrolysis experiments) or compound sodium lactate injection B.P. or PBS (binding ultra-filtrate from plasma or HSA, respectively) with racemic ketoprofen in the concentration range 0.31–20.00 μg/mL.

Data analysis. Half-lives ($T_{1/2}$) for the net hydrolysis of glucuronide or ethyl esters of ketoprofen were calculated for the apparent first-order processes in each of the media after performing least-squares linear regression analysis (from time zero) of the logarithmic transform of percentage remaining ester as a function of linear time. The correlation coefficient (r) was calculated for each incubation experiment.

The percentage unbound values of the glucuronides (% unbound) were calculated as the ultra-filtrate (unbound) concentration of *R*- or *S*-ketoprofen

Table 2. Net hydrolysis and reversible protein binding of *R*- and *S*-ketoprofen glucuronide (15 μg/mL of both metabolites: 6.7 of *R*- and 8.3 μg/mL of *S*-ketoprofen glucuronide) upon incubation in fraction V HSA, and net hydrolysis in protein-free PBS

	HSA concentration in PBS (37°, pH 7.4)			Protein-free PBS (37°)	
	40 g/L	4.0 g/L	0.4 g/L	pH 7.4	pH 9.0
$T_{1/2.R}$ (hr)	1.68	2.70	5.47	>24	3.91
Correlation coefficient*	0.986	0.998	0.996	0.998	0.998
% $unbound_R$†	16.9‡	68.0‡	94.5	—	—
SD	0.9	1.5	2.6		
$T_{1/2.S}$ (hr)	6.75	7.34	17.5	>24	3.48
Correlation coefficient*	0.993	0.997	0.999	0.995	0.996
% $unbound_S$†	11.7	60.4	93.4	—	—
SD	0.5	1.7	2.7		

* Correlation coefficient (r) calculated for the linear regression of the logarithmic transformation of percentage glucuronide remaining as a function of time.

† Mean and SD of replicate determinations of HSA binding of each glucuronide (N = 6 for 40 and 4.0 g/L; N = 3 for 0.4 g/L).

‡ Statistically different ($P < 0.0001$; unpaired t-test) from the corresponding value for *S*-ketoprofen glucuronide.

glucuronide divided by the total (bound plus unbound) concentration of the respective glucuronide.

Analysis of variance or the Student's t-test was used, as appropriate, to assess the statistical significance of differences between groups. Differences were considered significant at $P < 0.05$.

RESULTS

Hydrolysis of glucuronides to aglycones

The apparent first-order net hydrolysis $T_{1/2}$ of *R*-ketoprofen glucuronide was less than 50% of the corresponding value for *S*-ketoprofen in human plasma at physiological pH and temperature (Table 1 and Fig. 2, upper panels). This rapid, stereoselective deconjugation reaction was unaffected by the presence of plasma esterase inhibitors (8 mM physostigmine or 120 mM sodium fluoride; data not shown). The $T_{1/2}$ values for the glucuronides following incubation in a physiological concentration of HSA (40 g/L in PBS; Table 2) were similar to those recorded in plasma. Whilst maintaining a constant initial concentration of glucuronides (15 μg/mL) and decreasing the incubate HSA concentration by successive orders of magnitude, $T_{1/2.R}$ and $T_{1/2.S}$ increased to a far lesser extent than the corresponding fall in HSA concentration (Table 2). The marked stereoselective nature of the glucuronoconjugate net hydrolysis was maintained in these less concentrated HSA solutions.

Net hydrolysis of ketoprofen glucuronides in protein-free PBS was minimal at physiological pH and temperature. Rapid and largely non-stereoselective hydrolysis of the conjugates was observed when the pH of this solution increased to pH 9.0 (Table 2).

Results from the co-incubation of either warfarin sodium (100 μM) or diazepam (100 μM) with ketoprofen glucuronides in either fraction V of fatty acid-free HSA (4.0 g/L) are given in Table 3. Warfarin appeared to marginally increase $T_{1/2.S}$ in both HSA-type solutions and $T_{1/2.R}$ in fatty acid-free HSA. Co-incubations of glucuronides with diazepam gave rise to slight changes in $T_{1/2}$ values from control values, notably, an increase in $T_{1/2.R}$ in both HSA-type solutions, an increase in $T_{1/2.S}$ in fatty acid-free HSA and a decrease in $T_{1/2.S}$ in fraction V HSA.

Protein binding of the glucuronides

The reversible binding of *R*- and *S*-ketoprofen glucuronide (present simultaneously) to human plasma protein was independent of glucuronide concentration ($P > 0.05$; analysis of variance) over the range of 1–20 μg/mL (depicted for two representative subjects, Fig. 2, lower panels). The mean (N = 4) % unbound of *R*-ketoprofen glucuronide was approximately 1.4 times that of the corresponding value for its diastereomer ($P < 0.005$; Table 1). When a constant amount of ketoprofen glucuronides was added to HSA solutions of varying strength, the stereoselective nature of the binding observed at 40 and 4.0 g/L was abolished in the most dilute HSA solution (0.4 g/L); the % unbound value for each diastereomer was approximately 95% in this solution (Table 2). Warfarin sodium (100 μM) displaced *S*-ketoprofen glucuronide in both fraction V and fatty acid-free HSA (4.0 g/L, $P < 0.001$) and displaced *R*-ketoprofen glucuronide in fatty acid-free HSA only ($P < 0.01$); diazepam (100 μM) displaced *R*-ketoprofen glucuronide in both types of HSA ($P < 0.05$ for fraction V and $P < 0.001$ for fatty acid-free) and *S*-ketoprofen glucuronide in fatty acid-free HSA only ($P < 0.001$). The magnitude of these changes were small in each case (Table 3).

Hydrolysis of the ethyl esters of ketoprofen

The hydrolysis of the ethyl esters of *R*- and *S*-ketoprofen in human plasma from a single volunteer and the influence thereon of physostigmine (8 mM)

Table 3. Influence of warfarin (100 μM) and diazepam (100 μM) on the net hydrolysis and reversible protein binding (37°, pH 7.4) of *R*- and *S*-ketoprofen glucuronide (15 μg/mL of both metabolites: 6.7 μg/mL of *R*- and 8.3 μg/mL of *S*-ketoprofen glucuronide) in fraction V or fatty acid-free HSA (4.0 g/L)

	Fraction V HSA			Fatty acid-free HSA		
	Control	Warfarin	Diazepam	Control	Warfarin	Diazepam
$T_{1/2,R}$ (hr)	2.70	2.82	3.04	2.86	3.19	3.00
% unbound$_R$*	68.0	69.8	70.4†	55.8	57.7‡	61.0§
SD	1.5	1.4	1.9	1.3	1.1	0.7
$T_{1/2,S}$ (hr)	7.34	9.13	6.21	6.35	8.74	7.64
% unbound$_S$*	60.4	67.2§	61.6	52.5	58.0§	56.4§
SD	1.7	1.3	2.1	1.2	1.1	0.7

* Percentage unbound of R- and S-ketoprofen glucuronide expressed as the mean value and standard deviation from six replicate determinations.

Statistically different (†$P < 0.05$, ‡$P < 0.01$ and §$P < 0.001$: analysis of variance) from the corresponding value for the control sample (no warfarin or diazepam).

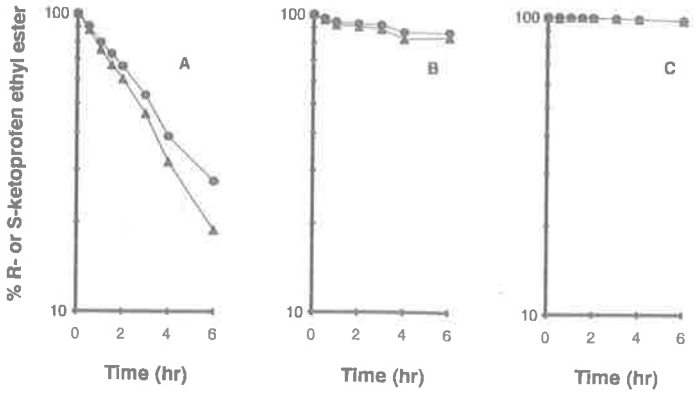

Fig. 3. Semi-logarithmic plots of the hydrolysis (pH 7.4, 37°) of *R*-ketoprofen ethyl ester (▲) and *S*-ketoprofen ethyl ester (●) in human plasma (expressed as percentage remaining ethyl ester) upon incubation of 15 μg/mL of racemic compound in each of: plasma (panel A), plasma spiked with 8 mM physostigmine (panel B) and plasma spiked with 120 mM sodium fluoride (panel C).

or sodium fluoride (120 mM) are depicted in Fig. 3. In the absence of esterase inhibitors the apparent first-order hydrolysis $T_{1/2}$ values for the *R*- and *S*-enantiomers were 2.48 hr ($r = 0.999$) and 3.14 hr ($r = 0.999$), respectively (Fig. 3, panel A). Both physostigmine and fluoride were effective in blocking ethyl ester hydrolysis (Fig. 3, panels B & C). There was negligible hydrolysis of these esters at physiological pH and temperature in either HSA solutions or in protein-free PBS solutions (data not shown).

DISCUSSION

These data demonstrate the pivotal role of albumin in the rapid, stereoselective net hydrolysis of the glucuronoconjugates of ketoprofen *in vitro*. The similarity between plasma and HSA solution (40 g/L) suggests that albumin may be the major source of hydrolytic activity in plasma (Tables 1 and 2). Recent studies examining the hydrolysis of conjugates of fenoprofen [16], flurbiprofen [23] and carprofen [24] attributed similar importance to albumin. Of interest, Knadler and Hall [23] noted preferential hydrolysis of the conjugate of *S*-flurbiprofen in contrast to our studies with ketoprofen and previous studies with fenoprofen [16] and carprofen [24] where the rate of conjugate hydrolysis was faster for the respective *R*-acyl glucuronide. In contrast to glucuronide hydrolysis, HSA solutions (40 g/L) did not effect detectable ketoprofen ethyl ester hydrolysis after 6-hr incubations at physiological pH and temperature (data not shown). However, incubations of ethyl ester with plasma led to significant hydrolysis (Fig. 3, panel A). The marked degree of stereoselectivity elicited by plasma on ketoprofen glucuronide hydrolysis (Fig. 2, upper panels) was in contrast to our studies with ketoprofen ethyl esters. In the latter case, this plasma-derived esterase activity was largely indiscriminant of the stereochemical configuration of the acyl moiety.

Human serum albumin has been shown to exhibit *in vitro* hydrolytic activity towards the acyl glucuronides of fenoprofen [16], oxaprozin [17, 25]

and carprofen [24], and towards cinnamoylimidazoles [26] and phenyl esters [27]. In contrast, an *in vivo* study by Rowe and Meffin [28] suggested that plasma esterases were responsible for the hydrolysis of clofibric acid glucuronide based on experiments with the irreversible esterase inhibitor, diisopropylfluorophosphate. These workers administered very high doses of the inhibitor to anaesthetized rabbits (approximately 30-fold that of the LD_{50} in conscious animals) and observed an increase in clearance of clofibric acid, presumed to be due to blockade of esterase-mediated hydrolysis of the glucuronide. Such a large dose of diisopropylfluorophosphate could conceivably lead to alkylphosphorylation of albumin, as observed *in vitro* by Wells *et al.* [25]. This might implicate albumin as the macromolecule responsible for deconjugating clofibric acid in the above study [28]. Further, Lin *et al.* [29] observed a lack of effect of an alternative esterase inhibitor (phenylmethylsulphonyl fluoride) on the clearance of diflunisal (subject to significant acyl glucuronide formation) in rats with experimentally induced renal failure. Moreover, when we incubated ketoprofen glucuronides in plasma spiked with classical esterase inhibitors (physostigmine and sodium fluoride) the rates of net hydrolysis of ketoprofen glucurononconjugates were unaffected (data not shown). Indeed we verified that these agents were potent inhibitors of plasma esterases by observing a marked reduction in the rate of hydrolysis of ketoprofen ethyl ester (Fig. 3).

It is interesting to note that the reversible binding of the glucuronide conjugates of ketoprofen to HSA and plasma exhibited significant stereoselectivity, while in a recent study [21] we observed a lack of stereoselectivity in the binding of ketoprofen aglycones to plasma. In the case of carprofen, stereoselectivity was observed, with respect to HSA binding, with both the carprofen glucuronide conjugates and the progenitive aglycones [30]. It is likely that incubations of ketoprofen glucuronides in protein solutions leads to the generation of covalent ketoprofen–protein adducts (irreversibly bound drug) based on previous *in vitro* studies with acyl glucuronides of other NSAIDs including fenoprofen [16], diflunisal [18] and zomepirac [31]. The degree to which acyl glucuronides form covalent adducts with proteins is quantitatively minor (1% or less for the three examples cited above) and time dependent. We determined the degree of reversible protein binding of ketoprofen glucuronides in solutions which had been incubated for less than 15 min. Thus our estimates of reversible binding would be unlikely to be influenced by covalent drug–protein adduct generation (presumptively) following *in vitro* incubation of ketoprofen glucuronides.

We were initially led to suspect that the site on the albumin molecule at which ketoprofen glucuronide hydrolysis was taking place might be the same site at which the glucuronides bind, as suggested for oxaprozin glucuronide [17, 25]. However, we observed significant and stereoselective binding of ketoprofen glucuronides to human plasma (Table 1 and Fig. 2) and physiological concentrations of HSA (Table 2); the eudismic ratio for the % unbound values was opposite to that for the $T_{1/2}$ values for the hydrolysis of the conjugates. This suggested to us the possible existence of a binding site distinct from the hydrolytic or catalytic site on the albumin molecule with respect to ketoprofen glucuronides. Indeed, incubations of ketoprofen glucuronides (15 μg/mL) in very dilute HSA solutions (0.4 g/L or 6 μM) where the ligand to albumin molar ratio was approximately 5:1 for each diastereomer, showed low, non-stereoselective binding yet markedly stereoselective conjugate hydrolysis (Table 2). It was also evident from these data (Table 2) that as binding of the glucuronides to HSA decreased (in successively more dilute protein solutions), more of these metabolites became available (as unbound glucuronides) for engagement with the catalytic site(s) on HSA molecules (and subsequent hydrolysis to their respective aglycones). A recent study with diflunisal acyl glucuronide [18] supports the alternative hypothesis of separate catalytic and binding sites on the albumin molecule. It is apparent that diflunisal acyl glucuronide, an extremely highly bound conjugate, is partially protected from hydrolysis in HSA solutions by virtue of this binding site being distinct (and a significant sink for glucuronide) from the catalytic site. Furthermore, the preferential deconjugation of *R*-carprofen glucuronide by HSA [24] and the greater percentage unbound value for this diastereomer compared to *S*-carprofen glucuronide [30] provides additional support for the hypothesis. It is possible that oxaprozin glucuronide binds to, and is degraded at, a common site on the albumin molecule. Wells *et al.* [25] reported this to be the benzodiazepine site, or Site II as classified by Sudlow *et al.* [32]. In contrast, carprofen glucuronides have been reported [30] to bind preferentially to the warfarin site (Site I).

We observed minor displacement of *R*- and *S*-ketoprofen glucuronide (*ca* 30 μM) in fraction V and fatty acid-free HSA (4.0 g/L or 60 μM) by warfarin (100 μM) or diazepam (100 μM) (Table 3). In parallel, we recorded slight increases in the corresponding conjugate hydrolysis $T_{1/2}$ values in those solutions where displacement had taken place. While these changes were subtle they are still consistent with the concept of separate hydrolytic and binding sites on the albumin molecule. It is possible that warfarin and diazepam exert an allosteric effect on the hypothesized separate catalytic site(s). Alternatively, these competing ligands might bind to more than one site on the albumin molecule, including the catalytic site(s).

The rearrangement of acyl glucuronides from the biosynthetic 1-*O*-β-isomer to other positional isomers has been demonstrated for a number of NSAIDs and other carboxylic acids including clofibric acid [33], valproic acid [34], oxaprozin [25], fenoprofen (Ref. 16 and references to other NSAIDs therein). While there are no reports in the literature, presumptively, ketoprofen glucuronides undergo such reactions. Thus, the part of our study concerned with glucuronide hydrolysis (analysis by the difference method) describes this process as the net result of hydrolysis of all possible glucuronide isomers. Caution is required in the interpretation of, for instance, the very low net hydrolysis of

conjugates in protein-free PBS at physiological temperature and pH (Table 2). These data might belie rapid 1-*O*-β-glucuronide degradation to other regioisomers. Fundamentally, we have attempted to describe the hydrolysis of ketoprofen glucuronides to the enantiomeric aglycones (pharmacologically active in the case of the *S*-enantiomer). This process potentially incorporates as yet unidentified intermediate pathways of glucuronide transacylation reactions, mutarotation and lactonization [14, 15].

In summary, acyl glucuronides are polar compounds generally restricted to the vascular and interstitial fluid compartments; regions containing significant concentrations of albumin. Thus, interactions of such metabolites with individual components of these compartments are of particular importance in determining the disposition of both metabolite and pharmacologically active aglycone. Our study implicates albumin to be of fundamental importance in the disposition of ketoprofen. Moreover, we suggest that this macromolecule possesses separate binding and catalytic sites for the acyl glucuronides of ketoprofen. The significant renal clearance of ketoprofen glucuronides [20] implies that in subjects with diminished renal function, the interaction of these accumulated glucuronides with albumin becomes of major pharmacological significance.

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