CHARACTERIZATION OF THE PROTEIN BINDING OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN SYNOVIAL FLUID

by

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SUMMARY

The clinical pharmacokinetic properties of NSAID's are markedly influenced by the affinity and extent of their protein binding properties in plasma and synovial fluid. Whilst the total level of NSAID is dependent upon concentration and affinity of local protein, it is the unbound / free fraction that is the species which readily diffuses between plasma and synovial fluid. The synovial fluid kinetics of the NSAID's have received remarkably little attention until recent years, despite the increasing accumulation of evidence casting doubt on a consistent pharmacokinetic correlation between plasma concentrations of NSAID's and respective analgesic / anti - inflammatory activity. This study examined the effect of some physical and environmental factors on the protein binding of piroxicam and ibuprofen in synovial fluid.

In this current work, the Amicon MPS-1 ultrafiltration device provided rapid and consistent separation of unbound NSAID in synovial fluids and HSA solutions. Radiolabelled piroxicam provided a convenient and relatively simple means of measuring the free fraction of this drug in synovial fluid using this device, with a coefficient of variation of 9.8%.

The 2-arylpropionic acids include some marketed and commonly prescribed NSAID's. They possess a chiral centre, are stereospecific in action and may undergo metabolic inversion from the inactive R-(-) to the active S-(+) enantiomer. The prostaglandin inhibitory effect resides with the S-(+) enantiomer, yet with the exception of S-(+) naproxen, these agents are generally administered as racemic

mixtures. The extent of metabolic chiral inversion in man is variable and may be one very important factor in explaining the variability in response to these agents.Despite the fact that the enantiomers of the 2-arylpropionic acid NSAID's differ in pharmacological properties, we are still witnessing articles in the literature that generate data of a non stereospecific nature.

In order to characterize the protein binding of the enantiomers of the chiral NSAID's (ibuprofen and ketoprofen) in synovial fluid, a GC/MS assay procedure was developed that was capable of determining ibuprofen enantiomer concentrations of 2ng/mL with a coefficient of variation of $\leq 10.8\%$ and $\leq 7.4\%$ for s-ibuprofen and r-ibuprofen respectively. The entire procedure (complete in a matter of minutes), was in marked contrast to the more lengthy methods found in the literature. The procedure was readily adaptable to allow resolution of ketoprofen enantiomers with a similar degree of sensitivity.

The free fractions of piroxicam and ibuprofen enantiomers were found to be markedly dependent upon their respective drug and associated albumin concentrations in synovial fluid according to pre-determined physical conditions. Binding of piroxicam was found to be independent of pH at concentrations of drug and albumin typically found in synovial fluid. There were marked differences in the extent of binding of ibuprofen enantiomers to human serum albumin (HSA) solutions, "fatty acid - stripped" HSA solutions, and synovial fluid. The current studies suggest that caution with interpretation of binding constants determined from experimental data (in these and similar studies), is essential, as most of the commonly calculated values are based on saturation functions such as those described by Scatchard and Klotz. Consideration should also be given to alternative mathematical functions when interpreting the results of non-saturated binding studies.

As synovial fluid is close to the target tissue where a NSAID's effect is exerted, it is reasonable to expect its concentration in this fluid to be a better indicator of drug activity in rheumatic disease than in plasma. However, before an attempt to correlate synovial fluid NSAID concentration with clinical response can be made, consideration of the physical and environmental parameters affecting the protein binding of the whole class of these drugs in synovial fluid may be beneficial.

This thesis contains no material which has been accepted for the award of any degree or diploma in any University or College.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except when due reference is made in the text of this thesis.

Damon S. Jack

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 NON STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAID's)

1.1.1 Background

These are amongst the most commonly prescribed drugs in our community[8]. Phenylbutazone, indomethacin and later ibuprofen, were the first of this "new" class of drugs of the early 1960's to compete with aspirin in the treatment of rheumatic , traumatic and other related disorders. In more recent times, a multiplicity of other "me too" NSAID's have evolved - essentially with promise of improved efficacy and/or tolerability. Most of these later drugs are systematic developments of earlier NSAID's following careful consideration of such factors as drug transport through biological membranes, atomic/spatial molecular structure determining receptor "fit", etc. Many of these criteria contribute to the development of what the individual pharmaceutical companies contend to be a unique drug species, representing a major advance towards the relief of the symptoms of rheumatic disease. The reality is that aside from a few potentially notable exceptions*, there is essentially little between them in terms of spectral activity. This skepticism is further expressed by bodies such as the Australian regulatory authorities who on many occasions have either rejected or suspended judgment on a number of newer NSAID's (though marketed in numerous other countries), on the grounds of lack of convincing evidence of efficacy or safety and demonstrable advantages over currently registered NSAID's.

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* A recent symposium reviewed the effects of NSAID's on cartilage destruction,neutrophil activation and the immune system. Many NSAID's exhibited important differences concerning their respective influence on these parameters.[9]

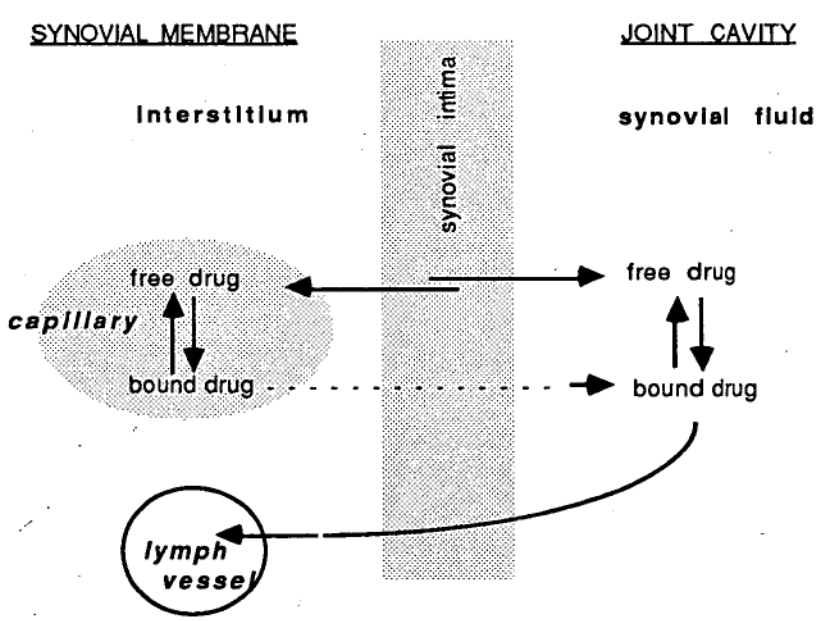
It is therefore not surprising to learn of an increased interest in the physical and pharmacokinetic parameters of these drugs in an attempt to shed further light on why some NSAID's appear to work better than others in certain patients and disease states. Few studies, for example, have tried to assess the possible effects of rheumatoid arthritis on drug disposition in patients[59].

Clearly, a closer examination of these drugs at or near their assumed site of action is warranted, (Fig 1.1).

1.1.2 Synovial Distribution of NSAID's

Most NSAID's (except aspirin) are extensively protein bound (Table 1.1). When tissues are inflamed, proteins extravasate from capillaries into adjacent tissues. Free or unbound NSAID tends towards diffusional equilibrium across the synovium, whilst larger solutes pass unidirectionally from plasma to synovial fluid and finally to lymphatic vessels, (Fig 1.1):

Fig 1.1 NSAID DIFFUSION THROUGH THE SYNOVIAL MEMBRANE*



* adapted from Netter et al[70]

Synovial tissue would appear to be the ideal point at which to study the effects of anti-rheumatic drugs on target organs as data in this area have been amazingly scant. This may in fact be partly due to the obvious practical limitations in obtaining sufficient,consistent and uniform samples for analysis. Synovial fluid on the other hand is close to the target organ and provides a clearly more practical means of monitoring drug activity (using intra-articular catheters or repeated athrocentesis) than does analysis of tissue specimens taken by percutaneous synovial biopsy or surgical synovectomy.

The synovial fluid kinetics of the NSAID's have received remarkably little attention until recent years, despite the increasing accumulation of evidence casting doubt on a consistent pharmacokinetic correlation between plasma concentrations of NSAID's and respective analgesic / anti-inflammatory activity[12].

In general, NSAID synovial fluid kinetics are influenced by the physico-chemical properties of the individual drug concerned. For example, the larger the molecule, the slower the rate of diffusion - salicylates for instance are said to diffuse more readily than larger NSAID molecules such as indomethacin[12]. As most of the clinically available NSAID's are acidic with pk_a's between 3.5 and 6.5 (see Table 1.1), an increase in synovial fluid acidity means that these weak acids become progressively less ionised and therefore more readily cross membranes and accumulate in inflamed tissue by the diffusional process[60]. This has been exquisitely demonstrated by Gray and co-workers using radio-labelled phenylbutazone and antipyrine in the carageenan rat paw inflammation model[11].

Whilst the fraction of free or unbound anti-rheumatic drug remains fairly constant in plasma, it would seem unreasonable to attempt extrapolation of such data to synovial fluid. Factors such as variable total protein content, individual protein fraction and exudate/transudate may cause respective free drug fractions to vary disproportionately with those in the plasma. In addition, NSAID's take longer to reach peak levels in synovial fluid as compared to plasma, as illustrated in Table 1.1:

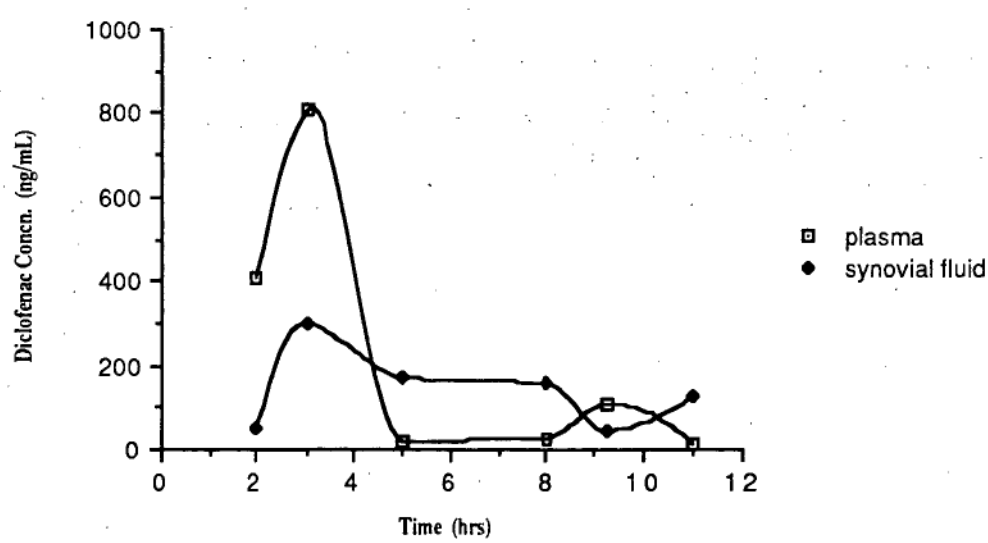
Table 1.1: PROPERTIES OF SOME ORAL ANTI-RHEUMATICS[#]

	Mol. Wt.	pK_a	$t_{1/2}$ (hrs)	Protein Binding (%)	Serum/S.F Time to Peak (hours)	S.F/Serum Mean Conc^n Ratio	Solubility (water)
Indole derivatives							
Indomethacin	358	4.5	2.4	99	1 / 2	4.00	
Oxicams							
Piroxicam	331	5.1	36-45	99		0.43	virt.insol
Tenoxicam					1.6 / 10.1		
Phenylacetic acids							
Diclofenac (im)	318	3.9	1.8	99			soluble
Proplonic Acids							
Flurbiprofen	244	4.2	3.8	99	1.5 / 3-9	0.44	
Ibuprofen	206	5.2	2.5	99	1.5 / 3-4	0.41	virt. insol
Ketoprofen	254		1.8	60-95	1-3 / 5-6	0.61	
Naproxen	230	4.2	13	99		0.74	virt. insol
Pyrazoles							
Phenylbutazone	308	4.5	70	95			virt. insol
Salicylates							
Aspirin	180	3.5	0.25	49	0.55 / 1.15	0.04	soluble
Salicylate	138	3	3-5	87	1.5 / 2.2		soluble

# adapted from Wallis & Simkin[12] and Furst[5]
S.F = synovial fluid, $t_{1/2}$ = plasma half life

Whilst most NSAID's have similar pK_a' s and protein binding properties, there are considerable differences in plasma $t_{1/2}$'s , (the majority being relatively short). For example, diclofenac reaches maximal plasma levels within an hour or so (roughly in agreement with the perceived onset of analgesia), then steadily declines whilst simultaneous synovial fluid concentrations (essentially responsible for the anti-inflammatory effect), take about four hours to reach maxima. Whilst drug levels are relatively lower in synovial fluid compared to plasma, they persist at a substantially higher concentration for a longer period of time after dosing, and beyond, (Fig 1.2). Such observations help to explain why this agent, despite a short half life, may often be justifiably given to patients in a twice daily dosage, yet still maintain an extended therapeutic effect.

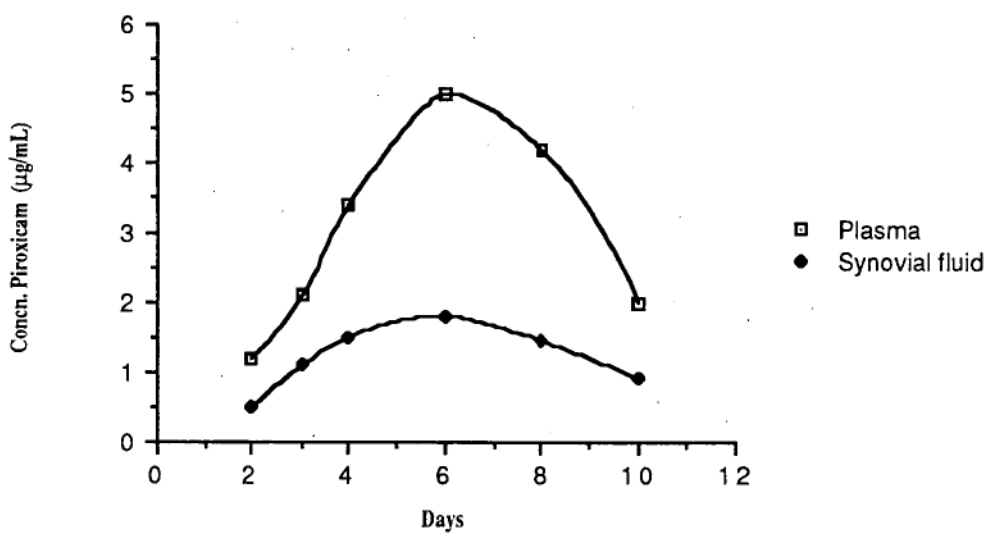
Fig 1.2 : **MEAN PLASMA AND SYNOVIAL FLUID CONCENTRATIONS IN 16 PATIENTS ON CHRONIC DICLOFENAC THERAPY FOR 11 HRS POST DOSE***



* adapted from Fowler [72]

By contrast, the oxicams have much longer $t_{1/2}$'s and generally take a week or longer to reach steady state plasma and synovial fluid concentrations following chronic administration at normal therapeutic dosage. Unlike the shorter $t_{1/2}$ NSAID's, synovial fluid concentrations increase with time in a manner similar to those in plasma with mean concentrations approximately 40% of plasma[7], (as illustrated below in Figure 1.3 for Piroxicam:

Fig 1.3 : **PIROXICAM DISPOSITION FOLLOWING 20mg ORALLY FOR 7 DAYS#**



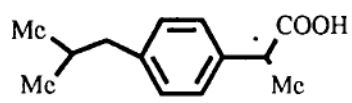
adapted from Fenner[7]

1.2 CHIRAL NSAID'S

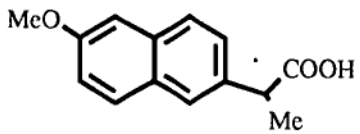
A significant proportion of synthetic drugs, (including some of the NSAID's) exhibit chirality[108]. In other words, they exist as optical / mirror image isomers or

enantiomers. The 2-arylpropionic acids include some marketed and commonly prescribed NSAID's (Fig 1.4):

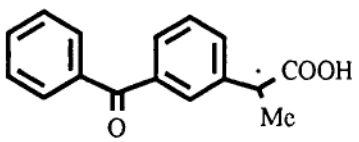
Fig 1.4: **2 - ARYLPROPIONIC NSAID'S**



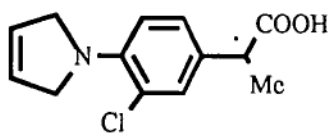
Ibuprofen



Naproxen



Ketoprofen



Pirprofen

. denotes chiral centre

The propionic acid side-chain possesses an asymmetric centre, the alpha carbon atom, so that two optical isomers (ie: enantiomers) exist.

From a pharmacological viewpoint, the prostaglandin synthetase inhibiting effect of these NSAID's is attributable to the S(+) enantiomers[79]. Furthermore, these compounds undergo metabolic inversion from the R(-) enantiomer to the corresponding S(+) form by an unproven mechanism[31]. The reverse reaction has never been detected[32]. There appears to be considerable variability amongst the chiral NSAID's in terms of the extent of inversion. Ibuprofen, for example, has been demonstrated to undergo approximately 2/3 conversion of a dose of r(-) to s(+)

ibuprofen[33], whilst ketoprofen to a significantly lesser extent[81]. Moreover, it has been claimed that the pharmacokinetic behaviour of the two enantiomers in humans may be different[31,109], so the stereoselective investigation of the pharmacokinetic parameters of these agents would appear to be of scientific and medical relevance.
The disposition of the enantiomers has received little attention[78], as much of the earlier analytical methodology did not discriminate the individual optical isomers[71].

1.3 CHARACTERISTICS OF SYNOVIAL FLUID

1.3.1 The Nature of Synovial Fluid

Synovial fluid is a clear, pale yellow or straw coloured viscous liquid consisting of serum ultrafiltrate and mucopolysaccharide secretions produced exclusively by the synovial membrane. This thin film of fluid between the joints, aside from offering mechanical protection against joint " wear and tear ", permits nutrient and metabolic waste exchange for articular cartilage. Numerous studies[1], many dating back to the early 1940's support the general concept that electrolytes and non-electrolytes are distributed in synovial fluid as a plasma dialysate to which hyaluronate (mucin) is added. However, it is of interest to note that Cl^- and HCO_3^- are present in synovial fluid in high concentrations relative to serum whilst Na^+, K^+, Ca^{2+} and Mg^{2+} are relatively lower[2].
Water and small solutes (including the vast majority of anti-rheumatic drugs) move readily between synovial fluid and plasma via the synovial interstitium and are diffusion rate limited[12].
Hyaluronic acid (a non sulphated polysaccharide composed of equimolar quantities of d-glucuronic acid/n-acetyl-d-glucosamine residues) represents about 0.3% of the composition of synovial fluid[1]. It is this substance in particular which is responsible for some of the unique properties of synovial fluid, including its viscosity characteristics. In vitro work by Ogston and Phelps[3] demonstrated that the presence

of this physically large, asymmetric molecule can significantly obstruct the passage of some solutes. This concept enables one to gain an appreciation as to why larger molecules such as fibrinogen are excluded whilst smaller molecules are not. One may therefore be led to suspect that quantitative/qualitative variations in hyaluronate produced under varying rheumatic conditions could make significant contributions to the nature of synovial fluid.

Transynovial exchange of plasma proteins on the other hand is significantly different from that of small solutes and is monodirectional into the joint space. Protein levels are always lower in synovial fluid compared to plasma. Typical total protein levels are around 18g/L[4], however this may increase in trauma and rheumatoid states (see Table 1.2).

1.3.2 Protein Binding of NSAID's in Synovial Fluid

1.3.2.1 *Proteins in synovial fluid*

As previously discussed, most of the NSAID's are acidic and bind principally to albumin. As such, drug levels in synovial fluid are usually a reflection upon the fluid's corresponding albumin concentration.Table 1.1 shows the commonly accepted values for protein binding of some commonly prescribed NSAID's. A close examination of the available data reveals that little emphasis has been placed upon measurement of the free or unbound levels of NSAID's in blood(plasma,serum) at commonly used therapeutic dosages in clinical practice, let alone in synovial fluid. Presumably, this is due to the difficulties involved in accurate and reliable measurement of such low levels in the laboratory.

The albumin concentration in synovial fluid, (whilst lower than the corresponding concentration in plasma) can also vary dramatically under various pathological situations as Table 1.2 also illustrates. In addition, albumin concentrations tend to fall with age, resulting in higher free NSAID fractions compared to younger patients[69].

Table 1.2 : **SYNOVIAL FLUID ANALYSIS UNDER VARIOUS PATHOLOGICAL CONDITIONS#**

	Appearance	ρ	WBC	P_t g/L	Alb %	α1 %	α2 %	β %	γ %	[HA] mg/100mL
Non Inflam. Effusion										
Normal	clear,yellow	0	200	18	63	7	7	9	14	300
Trauma	clear+	<	1500	33	58	6	8	12	16	170
D.J.D.	clear++	<	600	30	57	6	8	12	16	190
Non Inf.Inflam. Effusion(severe)										
R.A.	turbid	<	15500	42	42	8	11	14	25	90
Gout	turbid	<	13500	49	70	3	6	12	9	50
Non Infectious Inflam. Effusions(mild)										
S.L.E.	clear/turbid	<	2860	32	60	4	6	15	15	180
Inflam.Effusions Infectious										
Acute Bacterial	very turbid	<	73000	44	33	8	10	11	37	40

adapted from Cohen[1]
< decreased
\+ occasionally bloody
++ occasionally slightly turbid
ρ viscosity : (0 = normal, < = decreased)

DJD degenerative joint disease
RA rheumatoid arthritis
WBC white blood cell count
HA hyaluronic acid
P_t total protein
Alb albumin

Whilst there can be up to a three fold increase in albumin concentration in the presence of inflammation, Ballantyne and co-workers[13] demonstrated lower synovial fluid albumin levels in patients with chronic inflammatory joint disease. They maintain this is principally a result of increased albumin catabolism. Numerous attempts have been made to demonstrate a quantifiable difference between albumins in normal and rheumatoid patients, but with mixed success[14 15], whilst others have attempted to demonstrate albumin drug binding inhibition via endogenous binding antagonists, such as free fatty acids[16].

The albumin concentration in synovial fluid generally ranges from 11-12g/L (normal subjects) through 17-20g/L (rheumatoid arthritis, trauma and degenerative joint disease) to over 30g/L (gouty arthritis).

1.3.2.2 *Binding in Synovial Fluid*

Unbound drug equilibrates between plasma and synovial fluid so that at steady state, the average concentrations of drug in each compartment are the same. As the albumin concentration in synovial fluid is lower than plasma, the binding is also lower. For an NSAID with low hepatic extraction, this results in the following changes (Table 1.3):

Table 1.3: **EFFECT OF BINDING CHANGES ON KINETICS***

Pharmacokinetic Parameter	**Effect of Reduced Binding for:**	
	Total Drug	**Unbound Drug**
Clearance (Cl)	increased	unchanged
Volume of Distribution (V_d)	increased	unchanged
Half Life ($t_{1/2}$)	unchanged	unchanged
Plasma Concn. at steady state (C_{ss})	decreased	unchanged

* adapted from Birkett[73]

Bound to free ratios of drug concentration in the extravascular compartment are dependent upon a number of factors including the presence of other exogenous substances (including other drugs) and non specific tissue protein binding, which all have the potential to inhibit NSAID binding to albumin. These factors have received little attention over the years[12].

1.3.2.3 *Binding Sites*

The primary structure of Human Serum Albumin is now well established[17,18] and it has been demonstrated that many substances, (aside from NSAID's) bind with high affinity, including fatty acids,bilirubin,tryptophan and numerous drugs. In addition, some NSAID's are capable of binding to multiple sites on albumin and with varying degrees of affinity[48], (Table 1.4):

Table 1.4 : **HSA BINDING SITES OF SOME COMMON NSAID'S** #

SITE 1 (WARFARIN SITE)	**SITE 2** (DIAZEPAM SITE)	**SITE 3**	**SITE 4**
Phenylbutazone	Flurbiprofen (1)+	Indomethacin	Tolmetin
Sulphinpyrazone	Ibuprofen (1)+		
Flurbiprofen (2)+	Indomethacin		
Indomethacin	Ketoprofen (1)+		
Ketoprofen (2)+	Naproxen		
Naproxen	Mefenamic acid		
Salicylamide			
Salicylazosalicylic acid			
Salicylosalicylic acid			

Adapted from Sjoholm[19], Sudlow et al[20] and Wanwimolruk et al[21].
+ These substances can bind to multiple sites, [primary (1), secondary (2)].

Binding sites should not be perceived as preformed locations or zones on the protein surface, but more flexibly as areas of binding which are principally determined by the bound drug itself and are able to be altered according to general thermodynamic principles. Energetic coupling between these binding sites may incite a ligand bound to one site to impair or improve the binding of a second ligand at another binding site. However, the pharmacological dosage of the currently available NSAID's is relatively small so that plasma / synovial fluid concentrations never reach equimolar HSA concentrations.Essentially therefore, only one site is active with very little influence from secondary sites[19].

1.4 MEASUREMENT OF FREE / UNBOUND NSAID

The direct methods of spectrophotometry, NMR, ESR and optical rotatory dispersion, whilst allowing measurement of rapid binding changes between drug molecules and proteins, are limited to special applications for which values are primarily relative and unsuitable for routine binding estimations.

The indirect methods of equilibrium dialysis, ultrafiltration, ultracentrifugation, gel filtration and electrophoresis are generally perceived as more adaptable methods. Complete separation of drug and protein is unnecessary as only a small portion of protein - free solution need be collected in order to ascertain the free drug concentration in the original solution. Binding is indirectly calculated as:

$$C_{bound} = C_{total} - C_{free} \qquad \textbf{Equation 1.1}$$

The use of one or more of these particular methods requires consideration of the physico - chemical properties of both the drug and the protein, (Table 1.5):

Table 1.5 COMPARISON OF INDIRECT METHODS FOR BINDING STUDIES[#]

	Advantages	Disadvantages
Equilibrium Dialysis	• minimal disturbance to binding equilibrium. • suitable for high molecular wt. drugs. • relatively reliable method.	• adsorption to membrane. • Donnan effect? • altered drug concentration, as determined by binding rate. • time consuming, especially with high molecular wt. drugs.
Ultrafiltration	• relatively insignificant changes in drug concentration. • suitable for high concentration protein solutions. • rapid method.	• error increases with sieve effect in drugs with molecular wt.>300. • protein concentration increases during filtration. • adsorption to membrane material. • Donnan effect?
Ultracentrifugation	• minimal adsorption of drugs to centrifuge tubes.	• drug sedimentation a problem with high molecular weight drugs. • relatively slow (12 - 15 hours). • complex technique, leads to increased variability.
Gel Filtration	• especially suitable for drugs with high molecular wt. • can be less time consuming if preconditions known.	• inaccurate for drugs with low protein affinity. • relatively large volumes of drug/protein required. • unsuitable for drugs strongly bound to gel.
Electrophoresis	• simple qualitative and quantitative estimations.	• variable effect on binding equilibria. • buffer/current combinations often tricky and time consuming.

adapted from Kurz[22] and Svensson[74]

1.5 GENERAL OBJECTIVES OF THIS STUDY

The above examples highlight the relative importance of monitoring synovial fluid NSAID levels as an aid in characterising drug behaviour. An appreciation of the unbound drug level is particularly appropriate, as it has been well established that it is free drug (the active species), which is in equilibrium between plasma and synovial fluid. Understandably, recent advances in techniques to determine free drug concentrations have lead to a substantial increase in the monitoring of this parameter in clinical practice[68].

The general objectives of this study were to examine the influence of endogenous and physical factors on this equilibrium, and to establish the possible significance (if any), that fluctuations in their serum concentrations may have on NSAID disposition.

The research was undertaken in two distinct parts:

a) The development and establishment of procedures to separate the free NSAID fraction in synovial fluid and assay the extremely low levels of unbound drug therein.

and

b) The use of these procedures to examine some of the endogenous and physical factors which affect the protein binding and consequential free fraction of selected NSAID's in both synovial fluid and plasma.

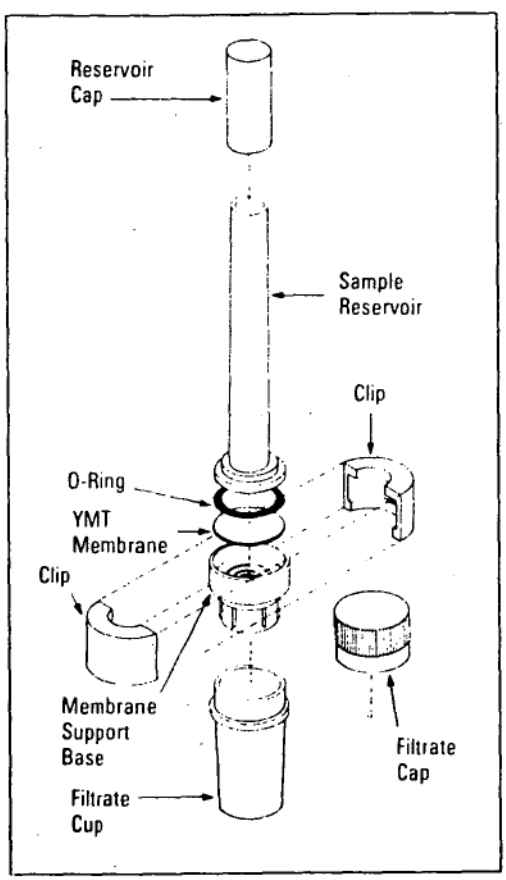
CHAPTER 2

PROTEIN BINDING DETERMINATIONS

2.1 THE AMICON ULTRAFILTRATION SYSTEM

For the following studies, the Amicon MPS-1 ultrafiltration apparatus was employed, (Fig 2.1):

Fig 2.1 : THE AMICON MPS-1 ULTRAFILTRATION DEVICE



The device rapidly and efficiently separates free/unbound drug from protein bound microsolute in small volumes (0.15-1.0mL). Convenient separation of a portion of unbound drug is achieved by convective filtration of unbound material through the semi - permeable YMT ultrafiltration membrane. Extremely high YMT membrane permselectivity is demonstrated by retention of > 99.9% serum protein and < 5% l-thyroxine[99]. Centrifugation (1000-2000g) provides sufficient driving force for the samples of synovial fluid and albumin used. Ultrafiltrate (containing unbound drug) collects in the filtrate cup and as the total drug volume is unchanged, unbound drug concentration remains unchanged on both sides of the membrane. This has been amply demonstrated by Whitlam and Brown[24]. Using a similar device, they showed that during the ultrafiltration procedure, the concentration remained undisturbed and that the fraction of fluid filtered is therefore unimportant .

2.2 MEASUREMENT OF UNBOUND NSAID LEVEL

2.2.1 Ultrafiltration in Conjunction with Radiolabelling

NSAID's are very highly protein bound. For example, at normal therapeutic concentrations of piroxicam (synovial fluid concentration of approx. 2μg/mL), this NSAID is about 99% bound[7,67]. This relates to a free / unbound fraction of 1% or less. A procedure for measuring free levels of the order of 20ng/mL or lower, with consistency and precision is required. The use of radiolabelled NSAID (usually ^{3}H or ^{14}C), offers an exquisitely suitable solution to this problem. Synovial fluid may be spiked with the radio-labelled NSAID and a portion of the free fraction collected by ultrafiltration. Scintillation counting of samples before and after filtration allow for the determination of free fraction at these low levels. This method is relatively fast,

convenient and in many instances, sufficiently sensitive to determine unbound drug concentrations in normal clinical practice.

2.2.2 Ultrafiltration in Conjunction with Chromatography

However, in many instances, the specific activity of the radiolabelled NSAID is too low to enable measurement of the free fraction at normal therapeutic levels. In fact, in many instances when working at normal therapeutic concentrations of the NSAID in question, the operator may have difficulty in resolving drug radioactivity from background radiation. In these instances, radiolabelling is inappropriate and alternative procedures need to be considered.These include HPLC, GC or GC/MS assays. With some notable exceptions, HPLC assays are generally unsuitable for measuring free levels of such highly bound drugs due to sensitivity limitations[40]. GC assays, though often involving lengthy and sometimes complex derivatizations, usually offer enhanced sensitivity compared to other methods. Futhermore, use of GC/MS techniques enable an even greater opportunity for further sensitivity and have the advantage of permitting confirmation of structure of derivatized drug.

CHAPTER 3

METHODS

3.1 SYNOVIAL FLUID

3.1.1 Collection Procedure

Following receipt of Ethics Committee approval from the institutions concerned, samples of synovial fluid were collected from patients in accordance with protocol (see Appendix 1). It is usual to collect fluid from effusions into the knee, as this is the most accessible joint. Synovial fluid aspirations for this study were only performed as part of their normal therapeutic treatment regimen. In many instances, aspiration serves to provide a substantial therapeutic effect for these patients - some by relieving pressure in the joint, others by permitting the instillation of medication (eg: antibiotics) in certain infected joint spaces. Following intracutaneous injection of local anesthetic (lignocaine or procaine 1%), the aspiration needle is progressively passed through the synovial membrane and into the joint cavity whereupon the fluid is withdrawn into the syringe. All fluids used in these studies, unless otherwise stated, were collected via aspiration of the knee joint.

3.1.2 Sample Classification

In accordance with guidelines set by Schumacher[66], this analysis commenced

literally the moment the specimen appeared in the syringe following joint aspiration. The colour was noted and particular attention given to the presence or absence of blood, (due to trauma of the surrounding tissue structures). In addition to the colour of the fluid, note was made of its visual clarity - clear, turbid or sometimes grossly purulent. Routine WBC counts were performed in most instances as an objective measure of inflammation. Although the WBC count is a non specific diagnostic procedure, it is an important laboratory aid in distinguishing different types of synovial fluid - at least within broad groupings (Table 1.2). For WBC analysis, synovial fluid was placed in a glass sample vial containing an anticoagulant (EDTA or heparin) and analysed* within 2 hours of joint aspiration. A portion of this sample was also used to establish albumin and total protein concentrations.

3.1.3 **Protein Determinations***

3.1.3.1 Albumin and Total Protein

Albumin was determined by colourimetric determination with bromocresol green[75] using an automatic stat random analyser (ASTRA®)[76]. The usable range for serum / synovial fluid albumin determination on the ASTRA® is 10-70g/L with an imprecision of 3.0%.

Total protein was determined by the classical biuret method[75] on the ASTRA® by injection of the sample into the alkaline copper reagent and measurement of the rate of change of absorption (wavelength = 545nm) at 11 sec by a microprocessor - linked optical system. The usable range is 30-120g/L for plasma or synovial fluid with an imprecision of 3.0%.

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* By courteous assistance of the Repatriation General Hospital, Hobart, Tasmania.

3.1.3.2 Electrophoresis

The synovial fluid must be "fresh" (less than 24 hours since collection) as even a small decrease in dispersion of its colloidal components can create undesirable "tailing" across the strips due to coarsely dispersed protein. Following collection, the synovial fluid was subjected to centrifugation at 2500g for 40 minutes at 37^0C, and a 5µL portion of the supernatant transferred to a glass slide. Cellulose acetate electrophoretic strips (Gellman Sciences Inc, Arbor, Michigan, USA), were saturated in tris barbital buffer (Gellman Sciences) for 15 minutes and then removed and blotted of excess liquid. 1µL of the clarified synovial fluid was applied to a marked position on the first quartile segment of the strips via a fine, twin wire applicator(Gellman Sciences). The strip was immediately suspended between the two electrode - containing buffer cells of the electrophoresis tank (Gellman Sciences), and the entire tank sealed to minimise evaporation from the surface of the strip. The electrodes from each cell in the tank were connected to a Vokam SAE 2761 constant voltage power supply (Shandon Sci, London, England), and a voltage of 150V maintained for 90 minutes*. This allowed a current (equating to approximately 1.5mV) to flow per strip. The strip was then removed from the tank and placed in a 0.2% w/v ponceau S dye solution (Gellman Sciences) for 10 minutes. The strip was then removed, rinsed repeatedly in 10% v/v acetic acid solution (BDH, Port Fairy, Vic, Australia - analytical reagent grade) to remove excess dye,and then dried in an air oven at 70^oC for 30 minutes. Dried strips were soaked in Whitemor Oil (Gurr's, London, England) prior to analysis on an ACD-18

*The albumin "front" may be readily checked or qualified by the addition of bromophenol blue to the original protein solution prior to electrophoresis.

Automatic Computing Densitometer (Gellman Sciences), at a wavelength of 525nm.

3.1.4 **Practical Considerations**

During the initial investigation of the characteristics of synovial fluid, there was concern that formation of a mucin clot may hinder subsequent binding studies (perhaps by altering albumin concentration). To investigate this possibility, the initial synovial fluid samples collected were analysed for their respective albumin concentrations at 2 hours and again after 36 hours storage, (by which time a clot had formed).

3.1.5 **Storage**

3.1.5.1 General

All synovial fluid samples were transferred to glass tapered tubes, centrifuged at 1000g for 3 minutes (as soon as practically possible following collection), decanted, dead space in the containers flushed with a stream of nitrogen, stoppered and stored at 4°C until required.

3.1.5. Stability Studies Using Piroxicam

1mL aliquots of blank synovial fluid were transferred to glass tubes and the pH measured (Beckman Model 12 pH/ISE - c/- Activon Sci, Thornleigh, NSW, Australia). Samples were then spiked with 2μg radiolabelled ^{3}H piroxicam stock solution and the free or unbound fraction (F_u) calculated in accordance with

procedure 3.2.2. The procedure was performed initially, and after 36 hours storage at 4°C.

3.2 PIROXICAM FREE FRACTION

3.2.1 **Radiolabelled Piroxicam**

3.2.1.1 Background

Protein binding studies of piroxicam in synovial fluid and Human Serum Albumin (HSA) were greatly facilitated by the availability of radiolabelled piroxicam. Radioactive ^{3}H Piroxicam was a generous gift from Pfizer Ltd (West Ryde, NSW, Australia).

3.2.1.2 Purity

The radiochemical purity (>90%) was verified at 6 monthly intervals by thin layer chromatography using the solvent mixture - chloroform : ethyl acetate : acetic acid : water (60:38:1.5:0.5). The specific activity was 29.98 μCi/mg.

3.2.1.3 Stock Solution

A radiolabelled ^{3}H piroxicam solution (1mg/mL in methanol) was prepared monthly and stored at 4°C.

3.2.1.3.1 *Assay of Stock Solution*

Freshly prepared stock solutions were verified by HPLC. Whilst numerous assays for this drug have been published[50-52], an assay following guidelines by Owen[46] was chosen due to its extreme simplicity and speed.
Stock solutions (prepared monthly) were verified by this method, and where necessary, adjusted to the required concentration (1mg/mL) with methanol.

3.2.1.3.2 *Instrumentation*

The HPLC system consisted of a Waters M45 solvent delivery system (Waters & Associates, Milford, MA, USA), which delivered the mobile phase at a flow rate of 1mL/min to a Waters C_{18} μBondapak column (steel - 300 x 3.9 mm ID, 10μm average particle size) fitted with a guard column (23 x 3.9 mm ID) packed with Waters μBondapak C_{18}/Corasil®, thence to a Waters model 441 fixed wavelength UV absorbance detector set at 254nm. Data were recorded on a LDC/Milton Roy C1-10 Integrator and SEK printer / plotter (LDC / Milton Roy Co, Clare, Ireland). The mobile phase [acetonitrile : 0.03% phosphoric acid (pH 2.5) - 45:55] was filtered and degassed under reduced pressure before use.

3.2.1.3.3 *Procedure*

A standard curve was prepared by serial dilution of piroxicam (Pfizer Pty Ltd) with methanol, to produce a range of concentrations around the expected concentration of radiolabelled stock solution. 10μL of p-toluic acid (Sigma, St Louis, MO, USA) solution (1μg/mL) was added as an internal standard. After vortex mixing for 10s, 20μL of each sample was injected into the HPLC system.

3.2.2 **Ultrafiltration Procedure**

The binding of piroxicam to synovial fluid proteins was measured using ultrafiltration, facilitated by the Amicon MPS-1 micropartition system using YMT ultrafiltration membranes, (Chapter 2).

A known quantity of radiolabelled ^{3}H piroxicam stock solution (1mg/mL) was added to 1mL of synovial fluid or albumin solution in a glass vial. The vial was then capped, inverted three times to facilitate mixing and incubated at 37°C for 5 min. A 100µL aliquot was transferred to a glass scintillation vial (Packard Instrument Co, Downers Grove, Ill, USA) and 5mL liquid scintillation fluid added (Insta-Gel : Packard Instrument Co). The mixture was counted by liquid scintillation spectrometry (LKB Wallac - Rack 1215 Beta 2). The remainder of the incubated mixture was transferred to an Amicon MPS-1 unit (that had previously been warmed to 37°C) and placed in a small swinging bucket rotor centrifuge (Jouan CT1000 - Jouan SA-BP, St Nazaire, Ce´dex, France) prewarmed to 37°C. The sample was centrifuged at 1500g for 20 min to produce approximately 200µL of ultrafiltrate. A 100µL portion of this ultrafiltrate was analysed by liquid scintillation spectrometry as above. Successive channel ratio readings were similar over the range of concentrations used { coeff.var. ≤ 1.8%, (n=20)} and obviated the need for correction due to quenching. Disintegrations per minute (dpm) were corrected for background noise and the fraction of unbound piroxicam (F_u) calculated as:

$$F_u = \frac{\text{dpm ultrafiltrate}}{\text{dpm prefiltrate}} \qquad \textbf{Equation 3.1}$$

After use, the Amicon MPS-1 unit was disassembled, filter and collection cup

discarded and the remaining components rinsed in hot tap water, soaked overnight in Decon®, then rinsed in tap water, followed by distilled water and then dried in an air oven at 37°C.

3.2.2.1 Reproducibility

The precision of the ultrafiltration procedure was determined in freshly prepared HSA (fraction V - CSL, Melbourne, Australia) 30g/L in water at a piroxicam concentration of 2μg/mL.

3.3 IBUPROFEN / KETOPROFEN FREE FRACTION

3.3.1 Ketoprofen HPLC Enantiomeric Assay

3.3.1.1 Chemicals and Reagents

Standard stock solutions (2μg/mL) of racemic ketoprofen (May and Baker, Melbourne, Australia) were prepared in methanol and stored at 4°C. The l - leucinamide (Sigma, St Louis, MO, USA) derivatizing reagent was prepared fresh weekly. All solvents were HPLC grade (Waters & Associates, Melbourne, Australia).

3.3.1.2 Instrumentation

The instrumentation was a modification of that used by Foster and Jamali[41]. The

HPLC system in the current studies consisted of a Waters 6000A solvent delivery system (Waters & Associates,Milford,MA,USA) and a Waters model 450 UV variable wavelength spectrophotometer. At ambient temperature, a Waters Novapak C_{18} Reversed Phase Column (3.9mm x 15cm - steel) fitted with a guard column (23 x 3.9 mm ID) packed with μBondapak C_{18}/Corasil® (Waters) was utilised throughout. The mobile phase 0.06M potassium dihydrogen phosphate : acetonitrile : triethylamine (64:36:0.02) was filtered, degassed under reduced pressure and then pumped at a flow rate of 1mL/min. The absorbance of the eluent was measured at 275nm and recorded on a dual-channel Omniscribe recorder (Houston Instruments, Austin, Texas, USA) tracking at 0.25cm/min.

3.3.1.3 Sample Preparation

A 0.5mL sample of "drug free" synovial fluid was spiked with a known amount of r,s - ketoprofen then acidified with 100μL of 0.6M sulphuric acid, and the mixture extracted with 3mL 5% isopropyl alcohol in isooctane (after vortex mixing for 30s and centrifuging at 1500g for 5 min). The organic layer was transferred to a clean glass tube and evaporated to dryness under a gentle stream of nitrogen. The residue was re-constituted in 100μL of 50mM triethylamine in acetonitrile. To this mixture, at 30s intervals were added 50μL of 60mM ethylchloroformate in acetonitrile and 50μL of a mixture of 1M l - leucinamide hydrochloride and 1M triethylamine in methanol. After 2 min, 50μL of water was added and 40μL of this solution injected into the HPLC system.

3.3.2 **Ibuprofen/Ketoprofen Enantiomeric GC/MS Assays**

3.3.2.1 Chemicals and Reagents

Samples of r (-) , s (+) and r,s- ibuprofen were the generous gift of The Boots Company (Boots, Nottingham UK). Standard stock solutions of ibuprofen (1mg/mL) were prepared in methanol and stored at 4°C .
A stock solution of r,s-ketoprofen (Sigma, St Louis, MO, USA) 1µg/mL was prepared in methanol and stored at 4°C.
1,1'-carbonyldiimidazole (CDI) was purchased from Aldrich (Steinheim, West Germany). Dexamphetamine Sulphate was a gift from the Department of Community Services and Health (Hobart, Tasmania, Australia). All solvents were HPLC grade (Waters and Associates) and glass distilled prior to use. Water was deionised and glass distilled. All non-disposable glassware was washed with Pyroneg, rinsed with tap water, soaked overnight in Decon®, then rinsed with distilled water before drying in an air oven over silica gel.

3.3.2.2 Optical Purity of Dexamphetamine

The purity of dexamphetamine sulphate was estimated by optical rotation to be approximately 90%, (the 10% discrepancy attributable to contamination with its optical counterpart, l - amphetamine). On this basis, derivatization with ibuprofen {R (-) and S(+) }, will produce four diastereomers (Rd, Rl, Sl, & Sd) - Fig 6.3; resolvable as two chromatographic peaks. A correction factor for this impurity can be made (via simultaneous equations) in subsequent quantitation of drug enantiomers.

3.3.2.3 Preparation of Stock Solutions

Standard solutions of CDI (10µg/mL) were prepared fresh daily in chloroform. Dexamphetamine sulphate (equivalent to 500µg free base per mL) was prepared in water and stored at 4°C - stock solution A.

A stock solution of dexamphetamine in methanol (stock solution B) was prepared as follows: 10mL of dexamphetamine sulphate aqueous stock solution was basified to pH 11 by the addition of 100µL 2M sodium hydroxide and extracted with 25mL diethyl ether. The organic phase was carefully evaporated to near dryness under a gentle stream of nitrogen gas. The residue was reconstituted with 10mL methanol, stoppered and stored at 4°C.

3.3.2.4 Preparation of Test Samples

3.3.2.4.1 *Using CDI*

1mL Sorensens Phosphate Buffer pH7.4 was spiked with racemic ibuprofen, acidified to pH1 with 100µL 5M hydrochloric acid and then extracted with 4mL toluene. Meanwhile,100µL of dexamphetamine stock solution A was basified with 100µL 1M sodium hydroxide and extracted with 4mL toluene. The two extracts were combined in a 10mL reaction vial (Alltech,Deerfield,Il,USA), evaporated to dryness and reconstituted with 1mL of CDI stock solution. The tube was screw capped and sealed under an atmosphere of nitrogen gas and then heated for 2 hours at 85°C in a GC oven (Pye 104: Pye,Cambridge,UK). After cooling to room temperature, the reaction mixture was washed with 1mL 1M hydrochloric acid and the organic phase dried over magnesium sulphate and evaporated to dryness. The residue was reconstituted with 100µL toluene and 1µL of this solution was then

injected into the GC/MS system.

3.3.2.4.2 *Using Ethylchloroformate*

A 200µL portion of ibuprofen stock solution (containing 20µg ibuprofen) was transferred to a tapered glass centrifuge tube and carefully evaporated to dryness under a gentle stream of nitrogen gas. The residue was reconstituted with 100µL of 50mM triethylamine in acetonitrile. To this mixture at 30s intervals were added 50µL of 60mM ethylchloroformate in acetonitrile and 50µL of dexamphetamine stock solution B. After 1 min, 0.5mL of chilled water was added followed by 3mL dichloromethane. The mixture was vortexed for 30s, centrifuged, (Jouan CT1000 - Jouan SA-BP, St Nazaire,Ce´dex,France) for 5min at 1500g and 4°C. The organic layer was transferred to a clean tapered glass tube and carefully evaporated to dryness under a stream of nitrogen. The residue was then reconstituted in 20µL toluene and 1µL of this sample was injected into the GC/MS system.

3.3.2.5 Instrumentation

The GC/MS system comprised a Hewlett Packard 5890 GC coupled with an HP5970 mass selective detector and an HP59970A data system. A 25 x 0.32mm internal diameter fused silica capillary column with a cross-linked methyl silicone bonded stationary phase of film thickness 0.17µ (HP-1) was used with helium carrier gas at a linear velocity of 55 cm/sec at 50°C. The oven temperature was programmed from 50°C (for 1min) to 220°C at 30°C/min and from 220°C to 270°C at 10°C/min. The injector temperature was 250°C and the transfer line temperature was 290°C. Injection (1µL) was by means of a Hewlett Packard 7673A autosampler. Structures of the diastereoisomeric amides were to be

confirmed by electron impact MS. Full scan reference spectra of the amphetamine derivatives of both enantiomers[40] were used to select appropriate ions for quantitative selected ion monitoring (SIM) during analytical runs.

3.3.2.6 Internal Standards

3.3.2.6.1 *O - Toluic Acid*

1µg samples (from a 1µg/mL stock solution) of o-toluic acid (Sigma, St Louis, MO, USA) were prepared, derivatized (3.3.2.4.2) and chromatographed (3.3.2.5):

Sample 1:	200µL water spiked with 1µg r,s-ibuprofen and then extracted as above. 1µg o-toluic acid was then added (as an internal standard) and the mixture then derivatized and chromatographed.
Sample 2:	200µL water spiked with 1µg o-toluic acid and then extracted as above. 1µg r,s-ibuprofen was then added (as an internal standard) and the mixture then derivatized and chromatographed.
Sample 3:	200µL water spiked with 1µg o-toluic acid and 1µg r,s-ibuprofen and then extracted, derivatized and chromatographed.
Sample 4:	1µg o-toluic acid and 1µg r,s-ibuprofen mixed together,evaporated to near dryness and then derivatized and chromatographed.
Sample 5:	Repeat of sample 4.
Sample 6:	1µg o-toluic acid,1µg r,s-ibuprofen and 3mL of the extraction solvent were mixed,evaporated to near dryness and then derivatized and chromatographed.

Peak area ratios of o-toluic acid to r,s-ibuprofen were then calculated.

3.3.2.6.2 Naproxen

A 1μg/mL stock solution of Naproxen (Syntex,Sydney,Australia) in methanol was prepared and stored at 4°C. A 1mL portion of this stock solution was carefully evaporated to near dryness under a gentle stream of nitrogen and then derivatized (3.3.2.4.2) and chromatographed (3.3.2.5) in accordance with fore mentioned procedures.

3.3.2.7 Effect of Reaction Time on Derivatization

To ascertain optimum times for this assay, 40ng s-ibuprofen and 1μg p-toluic acid from their respective stock solutions were derivatized together according to 3.3.2.4.2, except a series of samples were prepared by varying the reaction time from 0.25 to 10 minutes.

3.3.2.8 Effect of Varied Amount of Dexamphetamine Reagent

Samples of 40ng s-ibuprofen and 1μg p-toluic acid were prepared (3.3.2.4.2), except the amount of dexamphetamine derivatizing reagent was varied between 20 - 300μL.

3.3.2.9 Extraction Solvent

0.1% iso-propyl alcohol in toluene (Waters and Associates), were both HPLC grade and freshly glass distilled prior to use.

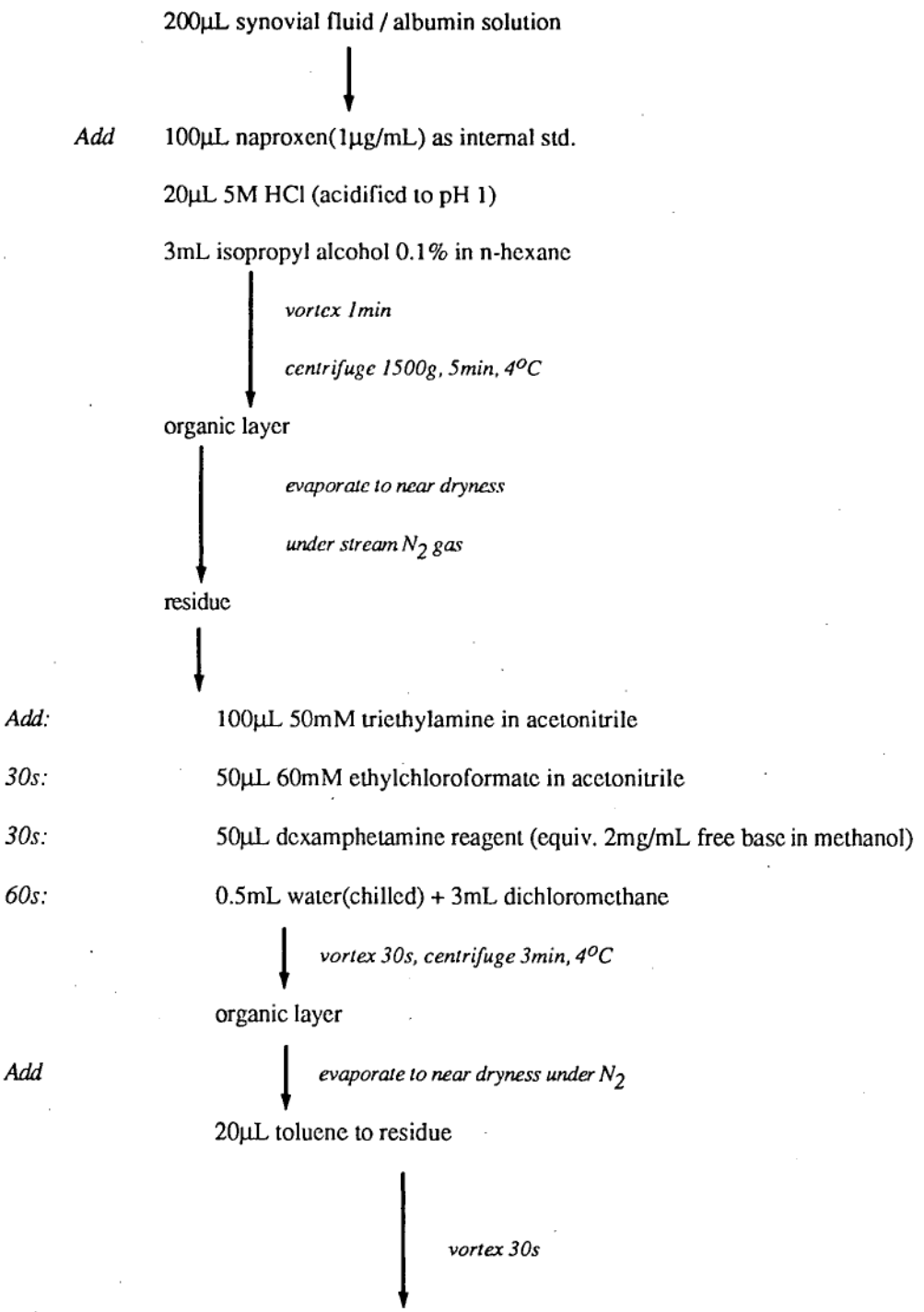
3.3.2.10 Contamination

"Blank" (or NSAID free) samples were run in conjunction with all GC/MS sample runs, to monitor for accidentally introduced contaminants in the assay system.

3.3.2.11 Preparation of Biological Samples:

All biological samples were prepared according to procedure depicted in Fig 3.1:

Fig 3.1: DERIVATIZATION OF BIOLOGICAL SAMPLES



3.3.2.12 Preparation of Standard Curves

200μL samples of "blank" albumin (Fraction V - CSL, Melbourne, Australia) 18g/L in Sorensen's phosphate buffer pH 7.4, were spiked with predetermined amounts of s(+) and r(-) ibuprofen or ketoprofen, and derivatized(3.3.2.11) and chromatographed (3.3.2.5) as before.
Calibration curves were prepared by plotting the ratio of peak area of the drug enantiomer to the peak area of the internal standard versus concentration of the drug.Standards were prepared fortnightly and stored at 4°C.

3.3.2.13 Assay Reproducibility and Precision

1mL samples of Sorensens phosphate buffer pH 7.4, were spiked with pre-determined amounts of s(+)- and r(-)- ibuprofen and ultrafiltrated using the Amicon MPS-1 device (Chapter 2). 200μL aliquots of ultrafiltrate were derivatized (3.3.2.11) and chromatographed (3.3.2.5).

3.3.2.14 Sample Stability

Two sample sets of ibuprofen standard solutions (3.3.2.12), were re-chromatographed 48 hours after derivatization.

3.3.2.15 Ultrafiltration Procedure

1mL samples of synovial fluid / albumin solutions were ultrafiltrated using the Amicon MPS-1 ultrafiltration device. The ultrafiltrate was derivatized (3.3.2.11) and chromatographed (3.3.2.5) and the free fraction calculated (Equation 3.1).

3.4 METHODS FOR NSAID BINDING STUDIES

3.4.1 Effect of NSAID Concentration

3.4.1.1 Piroxicam

Radiolabelled ^{3}H piroxicam at a range of concentrations between 1 and 6μg/mL was incubated at 37°C for 5 min in the presence of HSA* (Fraction V - CSL, Melbourne, Australia) 18g/L in 0.067M Sorensen's phosphate buffer pH7.4, and ultrafiltrated using the Amicon MPS-1 device (3.2.2).

3.4.1.2 Ibuprofen

Experiments were conducted using racemic ibuprofen concentrations expected within the normal therapeutic range in synovial fluid (1-20μg/mL)[39]. 1mL portions of blank HSA (Fraction V - CSL, Melbourne, Australia) 18g/L in 0.067M Sorensen's phosphate buffer were transferred to glass vials and spiked with pre - determined amounts of r(-)- and s(+)- ibuprofen stock solutions (1mg/mL in methanol), to produce a range of pseudo-racemic ibuprofen concentrations within the desired range. The mixtures were inverted to facilitate mixing, incubated in an air oven at 37°C for 5 min, then transferred to the Amicon MPS-1 devices and subject to ultrafiltration (3.3.2.15). 200μL portions of ultrafiltrate were derivatized (3.3.2.11) and chromatographed (3.3.2.5) as previously outlined.

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* HSA = Human Serum Albumin

3.4.2 **Effect of Protein Concentration**

3.4.2.1 Piroxicam

Radiolabelled ^{3}H piroxicam (at a concentration of 2µg/mL) was incubated for 5min at 37°C in 0.067M Sorensen's phosphate buffer pH7.4, in the presence of HSA(Fraction V - CSL, Melbourne, Australia) within the concentration range 12 to 30g/L. The incubation mixture was subject to ultrafiltration via the Amicon MPS-1 device (3.2.2). The experiment was repeated with two samples of synovial fluid {previously assayed (3.1.3) and found to contain 24g/L and 21g/L albumin respectively} which were serially diluted with phosphate buffer.

3.4.2.2 Ibuprofen

A series of 1mL synovial fluid / albumin solutions were prepared by dilution with pre-determined quantities of 0.067M Sorensen's phosphate buffer pH 7.40. The 1mL samples were then spiked with 20µg r,s-ibuprofen, incubated for 5 minutes at 37°C, ultrafiltrated (3.3.2.15), derivatized (3.3.2.11) and chromatographed (3.3.2.5).

3.4.3 **Chiral NSAID Enantiomer Ratio**

The effect of varying this ratio by increasing the proportion of s(+)-ibuprofen was investigated, commencing with a pseudo-racemic ibuprofen concentration of 12µg/mL, (ie: 6µg/mL of r(-)-ibuprofen plus 6µg/mL of s(+)-ibuprofen enantiomers). 1mL HSA (Fraction V - CSL) 18g/L in 0.067M isotonic phosphate buffer pH 7.37 was spiked with 12µg r,s-ibuprofen. A series of similar samples were prepared, maintaining r - ibuprofen at 6µg/mL but with incremental quantities of

s-ibuprofen up to 40µg/mL. The samples were incubated for 5 minutes at 37°C, ultrafiltrated (3.3.2.15), derivatized (3.3.2.11) and chromatographed (3.3.2.5).

3.4.4 **Effects of Fatty Acids**

3.4.4.1 Binding in "Stripped" Albumin

1mL samples of HSA (Fraction V - Sigma, St Louis, Mo, USA) 18g/L (that had previously been stripped of fatty acids by the method of Chen[49]), in 0.067M Sorensen's phosphate buffer pH7.4, were transferred to glass vials. Pre-determined quantities of r(-) - and s(+) - ibuprofen stock solutions (1mg/mL in methanol) were added to these solutions in the range 2 - 20µg/mL for each enantiomer. The samples were incubated for 5 minutes at 37°C, ultrafiltrated (3.3.2.15), derivatized (3.3.2.11), chromatographed (3.3.2.5), and the free fractions subsequently calculated.

3.4.4.2 Addition of Fatty Acid

1mL samples of 'stripped' albumin solutions were transferred to glass vials. Pre-determined quantities of sodium oleate (1mg/mL) stock solution were added to these tubes to produce a range of albumin solutions from 0.5mM to 1mM with respect to oleic acid. Varying amounts of r(-)- or s(+)- ibuprofen stock solutions (1mg/mL in methanol) were added to these solutions in the range 2 - 20µg/mL for each enantiomer. The samples were then incubated, ultrafiltrated, derivatized and chromatographed according to procedure 3.4.4.1.

3.4.5 **Effect of pH**

For each sample, 18mg HSA (fraction V - CSL) was reconstituted in 1mL of Sorensen's phosphate buffer solution, which had been carefully adjusted to the required pH[77]. After gentle mixing, 2µg of radiolabelled ^{3}H piroxicam stock solution (1mg/mL in methanol) was added, mixed by inversion and pH of the resultant solution reconfirmed, (Beckman model 12 pH/ISE, Activon Sci.). The samples were incubated for 5 minutes at 37°C, ultrafiltrated (3.3.2.15), derivatized (3.3.2.11) and chromatographed (3.3.2.5).

3.5 DATA INTERPRETATION

Piroxicam and ibuprofen binding data (3.4.1) were compared to the following mathematical models for goodness of fit, using FUNFIT®, an interactive program for non-linear regression[100]:

(i) Scatchard Model[88]: $r = (n_1 k_1 C / 1 + k_1 C) + (n_2 k_2 C / 1 + k_2 C)$

(iii) Klotz Model[85]: $r = (k_1C + 2k_1k_2C^2) / (1 + k_1C + k_1k_2C^2)$

(iii) Larsen Model[82]: $r = b_1 \ln (b_2 C + 1)$

(iv) Larsen / Klotz Model[82]: $r = k_1 \ln [2 (k_1 - 2k_2) C + 1] / 2 (k_1 - k_2)$

(v) Trigonometric Model[86]: $B = a_1 \arctan (a_2 T)$

(vi) Exponential Model[86]: $B = b_1 (1 - \exp (- b_2 T))$

where: r is the average number of bound drug molecules per molecule of albumin; C is the free drug concentration; k_1 and k_2 are the association / binding constants; n_1,n_2 are the number of class binding sites; a_1 and a_2 and b_1 and b_2 are the parameters of the models as determined from the experimental data; B is the concentration of bound drug; T is the concentration of total drug.

CHAPTER 4

RESULTS: ASSAY OF UNBOUND DRUG

4.1 SYNOVIAL FLUID SAMPLES

4.1.1 Sample Stability

The albumin concentrations of three samples of synovial fluid were calculated immediately after joint aspiration and after 36 hours storage at 4°C, (Table 4.1):

Table 4.1 : EFFECT OF CLOT FORMATION ON ALBUMIN CONCENTRATION IN SYNOVIAL FLUID

Patient No.	Patient I.D.	[Albumin] 0-2hrs (g/L)	[Albumin] 36hrs (g/L)
1	AA	17.2	17.0
2	GY	20.7	21.0
3	CC	18.0	17.6

There was an insignificant (< 3%) alteration to albumin concentration following clot

formation. For sampling purposes it was therefore considered reasonable that when a large clot formed in subsequent samples, fluid would be withdrawn from the collection vessel using the tip of a micropipette positioned between the clot and the wall of the container so as to minimise volumetric error.

4.1.2 **Electrophoresis**

A sample of non-inflammatory synovial fluid (white cell count <100) was subjected to the procedure outlined in 3.1.3.2, and the individual protein fractions resolved, (Fig 4.1):

Fig 4.1: **Electrophoretic strip showing component proteins* in synovial fluid from a rheumatoid arthritic patient (non-inflammatory).**

γ	β	α_2	α_1	albumin
(1.7g/L)	(2.2g/L)	(3.8g/L)	(4.2g/L)	(18g/L)

* Total Protein (method 3.1.3.1) = 30g/L

4.1.3 Sample Storage

The multiplicity of tests conducted on each synovial fluid sample were foreseen to be very time consuming events and therefore stability tests under varying environmental conditions were deemed necessary. It was considered feasable that denaturation of a major binding component during storage could influence free NSAID fraction in the samples. To investigate this possibility, the appearance,pH and protein binding characteristics of numerous samples were investigated whilst being stored at 4°C in stoppered glass tubes with minimal dead space. The data are illustrated in table 4.2.

Table 4.2: EFFECT OF STORAGE ON SYNOVIAL FLUID

Sample	Day 0		Day 2		Day 10		Day 30	
	pH°	F_u*	pH°	F_u*	pH°	F_u*	pH°	F_u*
1	7.51	2.11	7.62	2.15	7.67	2.15	7.95	2.25
2	7.56	2.40	-	-	7.61	2.47	7.76	2.21
3	7.45	2.62	-	-	7.50	2.38	7.55	2.40

F_u = fraction unbound of piroxicam:
* coefficient of variation = 9.8%
° coefficient of variation = 0.3%

4.2 PIROXICAM

4.2.1 HPLC Assay

4.2.1.1 Identification

The internal standard (p-toluic acid), and piroxicam were eluted at 5.89 and 7.73 minutes respectively, (Fig 4.2):

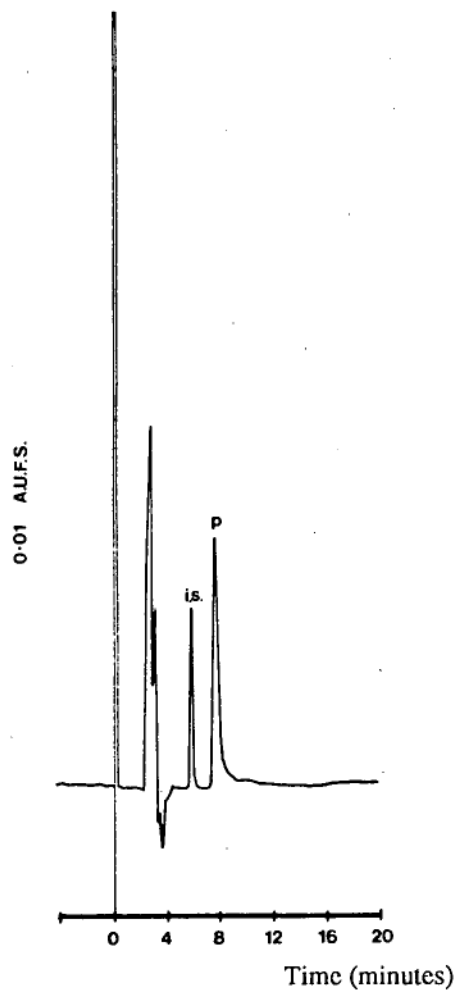
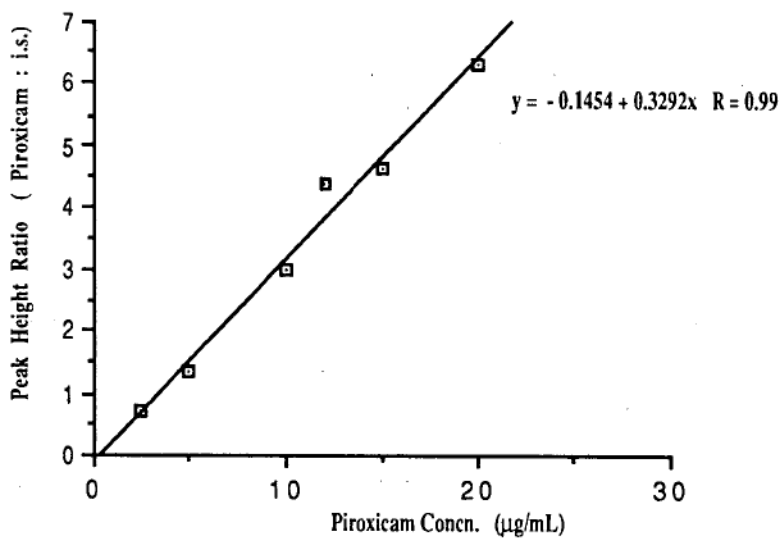


Fig 4.2 **HPLC profile of piroxicam in methanol. Peaks: i.s. = internal standard; p = piroxicam.**

4.2.1.2 Standard Curve

A standard curve was prepared by plotting the ratio of peak height of piroxicam to the peak height of the internal standard versus concentration of piroxicam (Fig 4.3):

Fig 4.3: PIROXICAM STANDARD CURVE



The precision of the assay has been previously assessed[46], and the coefficient of variation for piroxicam at 5µg/mL calculated to be 4.1%.

4.2.2 Reproducibility of Ultrafiltration Procedure

At a piroxicam concentration of 2µg/mL, the free/unbound fraction (F_u) was calculated in five samples, (Table 4.3):

Table 4.3 **PIROXICAM FREE FRACTION BY ULTRAFILTRATION**

Sample	F_u
1	0.0125
2	0.0101
3	0.0102
4	0.0123
5	0.0109
Mean (± SD)	0.0122 (0.0011)

Coefficient of variation = 9.8%

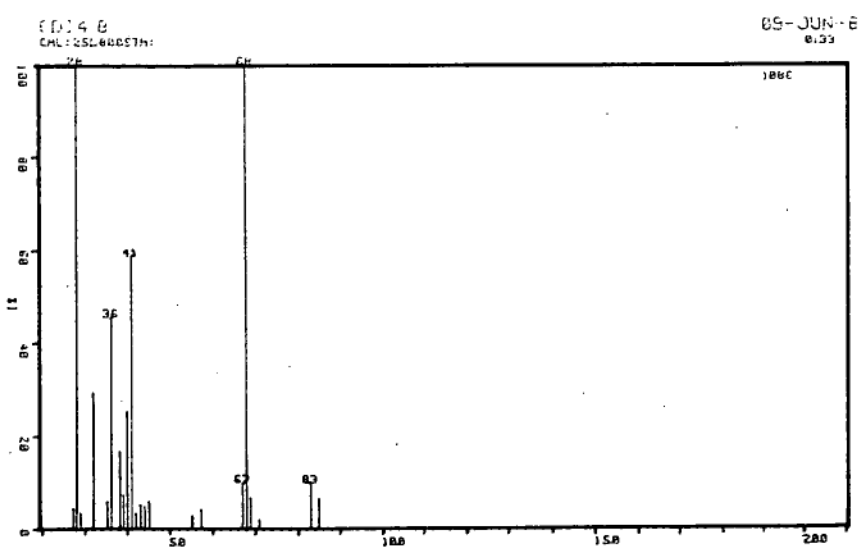
Recovery of radiolabelled ^{3}H piroxicam from the Amicon MPS-1 device was determined in 4 samples of HSA (fraction V -CSL) 18g/L in isotonic phosphate buffer which had been spiked with radiolabelled drug. The mean recovery (2.55µg/mL piroxicam) was 92.7% (SD 8.1%). The difference may be attributed to binding associated with the Amicon device and / or YMT membrane. According to previous studies by Whitlam and Brown[24], this is unlikely to be significant.

4.3 IBUPROFEN

4.3.1 GC/MS Assay Using Dexamphetamine / CDI

With reference to retention times and mass spectra undertaken by previous researchers[40], the enantiomers in their reaction sequence were either undetected or unresolved. A 1μL portion of the sample was transferred to a direct insertion probe on a VG 7070F mass spectrometer for confirmation of structure by electron impact MS. The spectra did not show the characteristic $[M-117]^+$ fragment which is characteristic of the amphetamine portion of the molecular ion, nor did it show other characteristic fragments,(m/z 91,119,118) previously investigated[40]. As there was no evidence of formation of amphetamine derivatives based on the mass spectra subsequently obtained, it was hypothesised that CDI was not acting as the intended coupling agent in the derivatization reaction. A 1μL sample of freshly prepared CDI reagent was transferred to the insertion probe and mass spectra obtained, (Fig 4.4).

Fig 4.4: MASS SPECTRA OF FRESHLY PREPARED CDI REAGENT

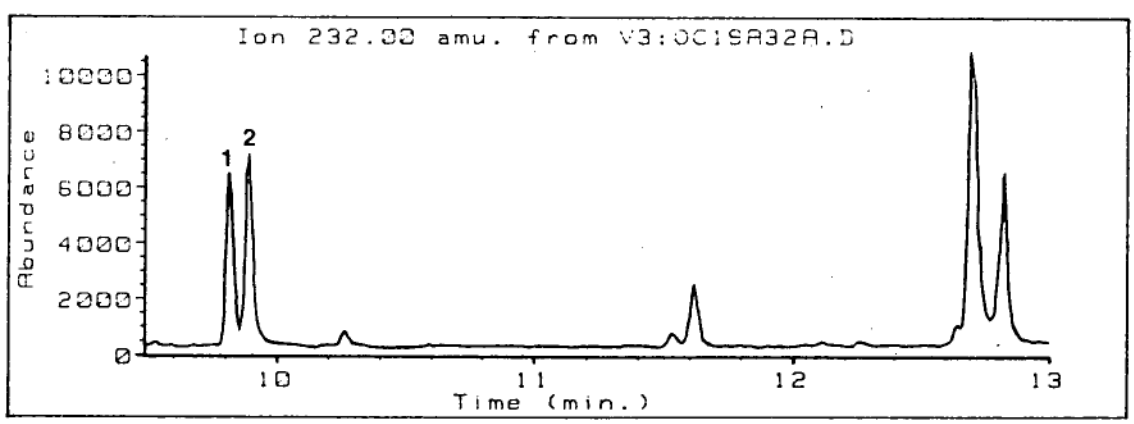


4.3.2 GC/MS Assay Using Dexamphetamine / Ethylchloroformate

4.3.2.1 Identification of Enantiomers

Two peaks were resolved at 9.82 and 9.89 min, and mass spectra published by previous investigators[40] confirmed these peaks to be the required derivatives. SIM of m/z 232 gave the cleanest trace, with freedom from interfering peaks. Fig 4.5 depicts a chromatographic trace originating from a 250ng test sample of ibuprofen in methanol:

Fig 4.5: GC TRACE OF IBUPROFEN DIASTEREOMERS



Peaks: 1 = s(+)- ibuprofen; 2 = r(-)- ibuprofen diastereomers

4.3.2.2 Selection of Internal Standard

4.3.2.2.1 *p - toluic acid*

The amphetamine derivative of p-toluic acid was confirmed to elute at 8.61min, with SIM of m/z 119 providing the best chromatographic trace. However, there were numerous interfering peaks which occured in conjunction with all p-toluic acid samples available in these laboratories, and an alternative was therefore investigated.

4.3.2.2.2 *o - toluic acid*

SIM of m/z 162 provided the 'cleanest' chromatographic trace. Inconsistencies with repetitive derivatizations of some samples were investigated (method 3.3.2.6.1) and summarised by Table 4.4:

Table 4.4: **o-TOLUIC ACID / R,S-IBUPROFEN ASSAY**

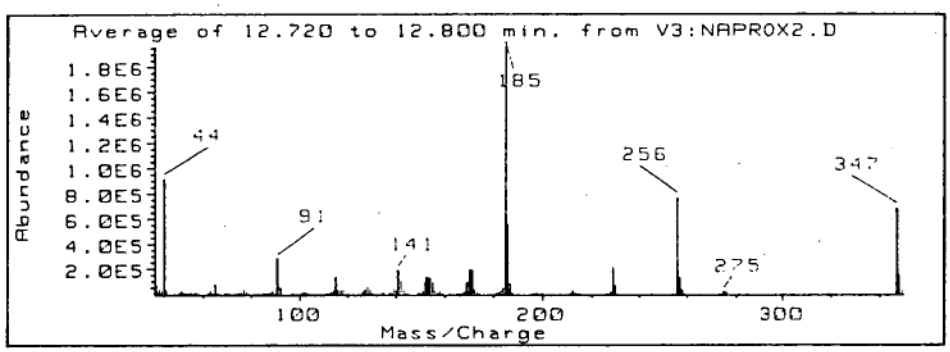
Sample Number	Peak Area Ratio (o-toluic acid : r,s- ibuprofen)
1	1.26
2	0.15
3	0.26
4	0.19
5	0.16
6	0.13

4.3.2.2.3 *Naproxen*

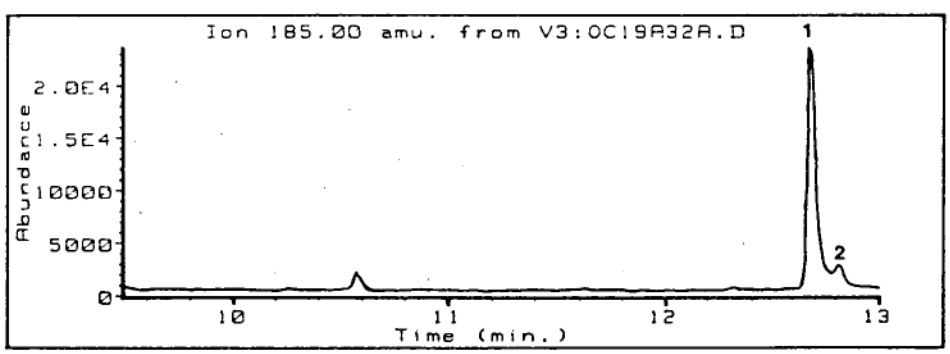
Using SIM m/z 185, two peaks were eluted at 12.68 and 12.82 min, with mass spectra characteristic of the amphetamine derivatives of naproxen. SIM of m/z 185 provided the best chromatographic trace, free from interfering peaks, (Fig 4.6):

Fig 4.6: **GC & MASS SPECTRA OF NAPROXEN DIASTEREOMERS**

A



B

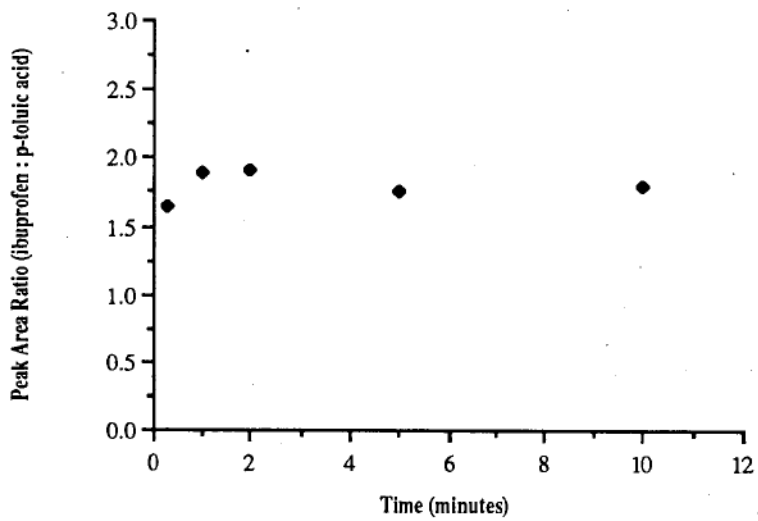


A = mass spectra of naproxen-dexamphetamine diastereomers.
B = gc/ms chromatographic resolution of 500ng/mL naproxen(1); peak(2) is a minor contaminant resulting from naproxen and/or dexamphetamine contamination[40].

4.3.2.3 Reaction Time

The optimum time interval for the coupling reaction was assessed (method 3.3.2.7), and represented by Fig 4.7:

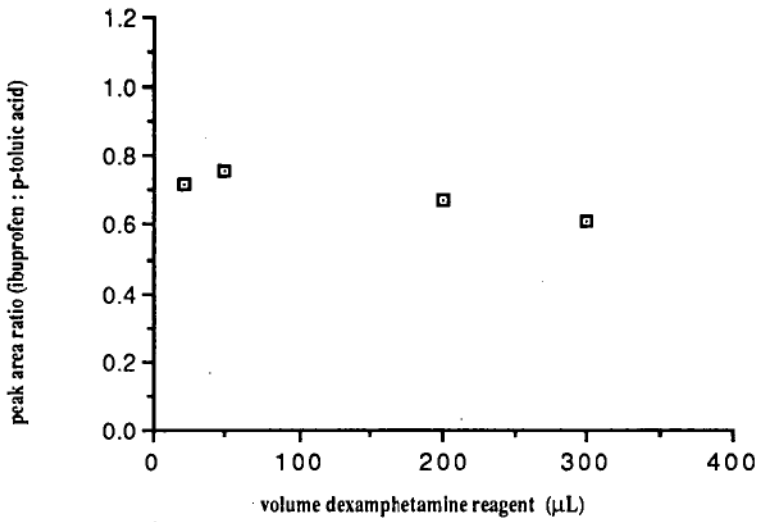
Fig 4.7: **EFFECT OF REACTION TIME**



4.3.2.4 Derivatizing Reagent

The optimum quantity of derivatizing reagent was assessed (method 3.3.2.8), and represented by Fig 4.8:

Fig 4.8: EFFECT OF AMOUNT DEXAMPHETAMINE REAGENT ADDED

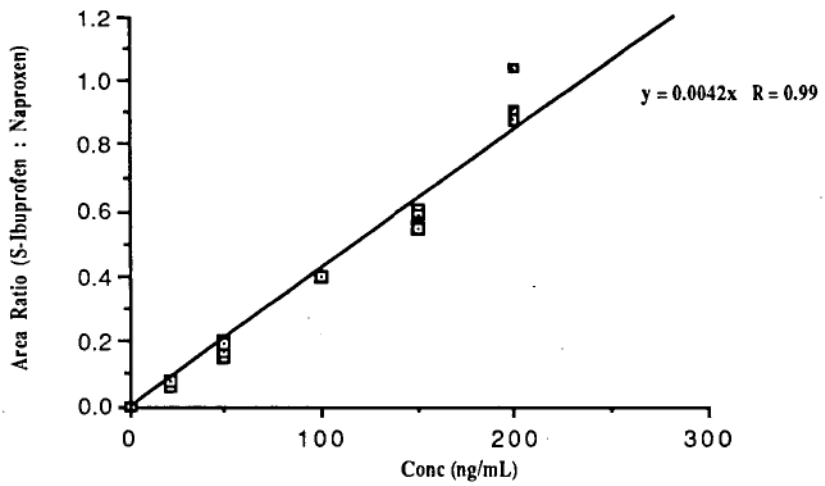


4.3.2.5 Standard Curves

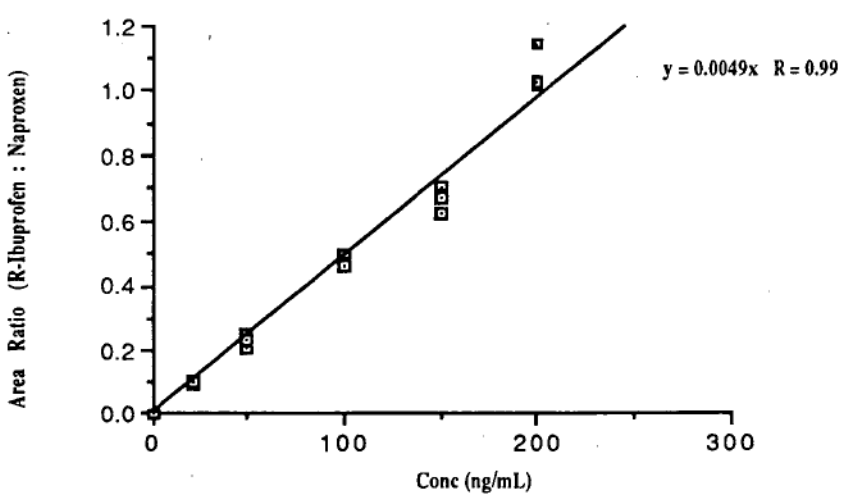
The calibration curves (Fig 4.9) were linear over the concentration range 10-200ng/mL. The minimum quantifiable concentration was 2.5ng/mL, using the ultrafiltration procedure (3.3.2.15). In effect, this equated to a 25pcg/µL on column injection and verged on the limits of detectability of the equipment. Lines of best fit through the origin were calculated by linear regression. The correlation coefficient R, was generally > 0.99 for a 5 point standard curve.

Fig 4.9: **CALIBRATION CURVES FOR S(+) AND R(-) IBUPROFEN**

1: s - IBUPROFEN



2: r - IBUPROFEN



4.3.2.6 Reproducibility and Precision

The precision of the assay for the ibuprofen enantiomers at a high and low concentration is given in Table 4.5:

Table 4.5: **PRECISION OF THE IBUPROFEN ASSAY**

Drug	Theoretical (ng/mL)	Number of Determinations	Experimental Mean ± SD (ng/mL)		CV* (%)
s(+) Ibuprofen	50	5	42.38	4.57	10.8
	150	5	136.2	5.70	4.2
r(-) Ibuprofen	50	5	45.7	3.38	7.4
	150	5	136.3	6.05	4.4

* CV = coefficient of variation

4.3.2.7 Stability

Two sets of sample derivatives were re-chromatographed (3.3.2.5) after 48 hours at 4°C, in order to assess stability, (Table 4.6):

Table 4.6: **SAMPLE STABILITY OVER 48HR PERIOD**

Derivative	Theoretical (ng/mL)	Experimental (ng/mL) 0hr	48hrs	Change (%)
s(+) ibuprofen	20	19.0	19.0	0
	50	45.2	40.5	10.4 (-)
	100	95.2	95.2	0
	150	140.5	128.6	8.5 (-)
	200	247.6	216.7	12.5 (-)
r(-) ibuprofen	20	20.4	20.4	0
	50	46.9	42.9	8.5 (-)
	100	93.9	95.9	2.1 (+)
	150	136.7	126.5	7.5 (-)
	200	234.7	210.2	10.4 (-)

4.4 KETOPROFEN

4.4.1 HPLC Assay

Fig 4.10 depicts a chromatogram of synovial fluid spiked with 200ng/mL racemic ketoprofen, (ie: 100ng/mL of each enantiomer, assuming the racemic drug to be an equal mix of each enantiomer):

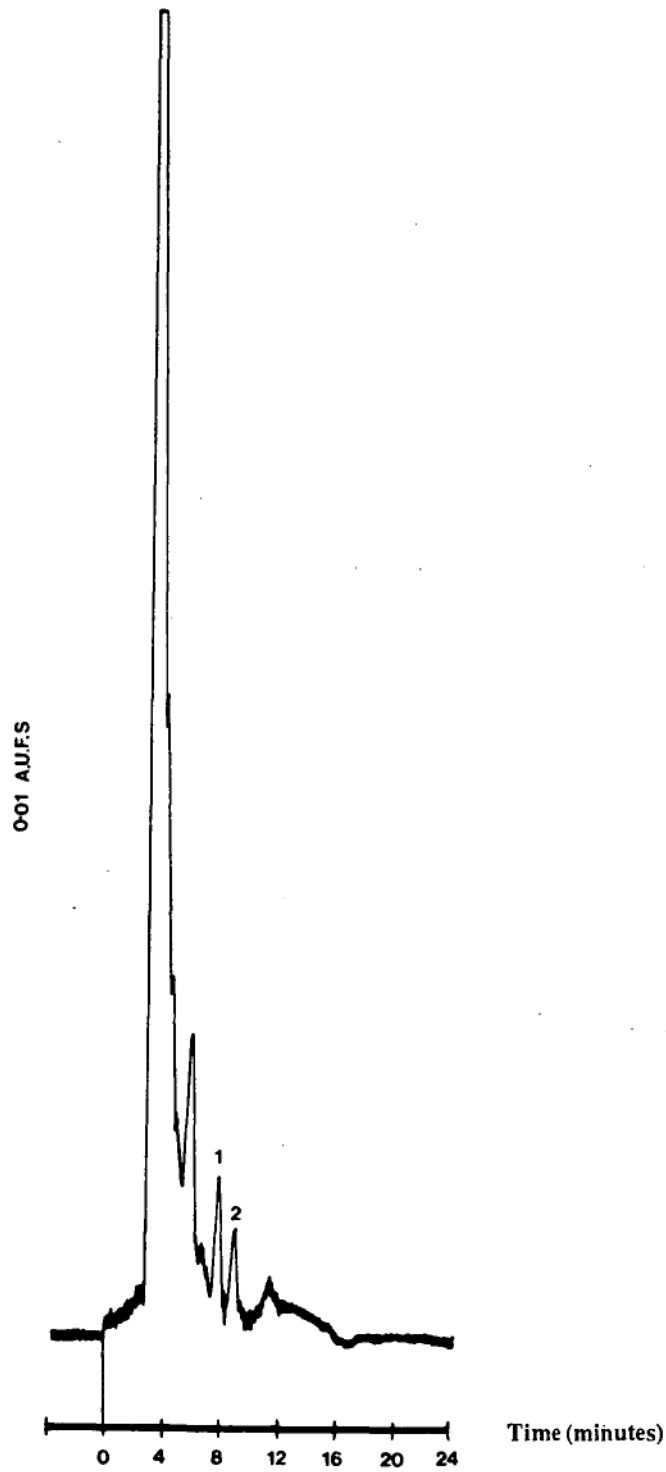


Fig 4.10: **HPLC profile of blank synovial fluid spiked with 100ng/mL of racemic ketoprofen. Peaks: 1 = r(-)- ketoprofen; 2 = s(+)- ketoprofen.**

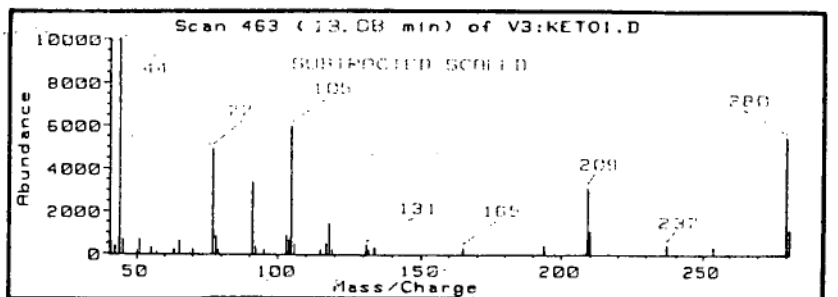
4.4.2 GC/MS Assay Using Dexamphetamine / Ethylchloroformate

4.4.2.1 Identification of Enantiomers

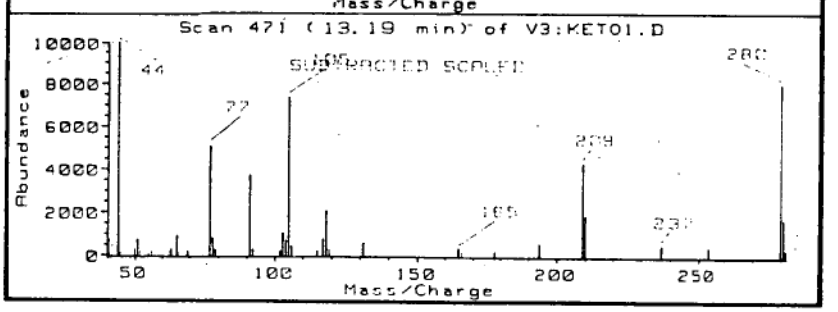
Two peaks at 13.08 and 13.19min for s(+) and r(-) ketoprofen respectively, were eluted. Mass spectra confirmed these to be the required peaks. SIM of m/z 280 provided a clean trace,free from interfering peaks, (Fig 4.11):

Fig 4.11: GC & MASS SPECTRA FOR KETOPROFEN ENANTIOMERS

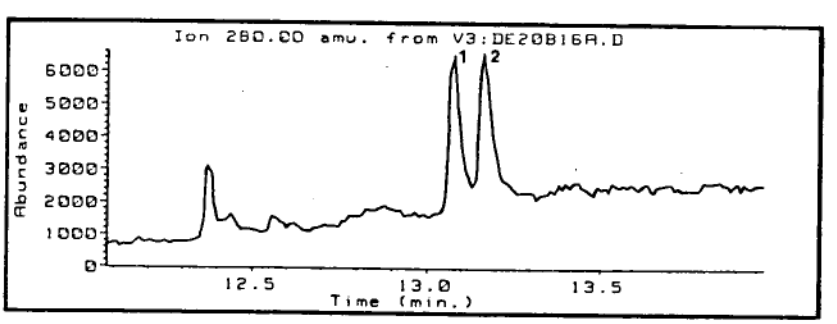
A



B



C



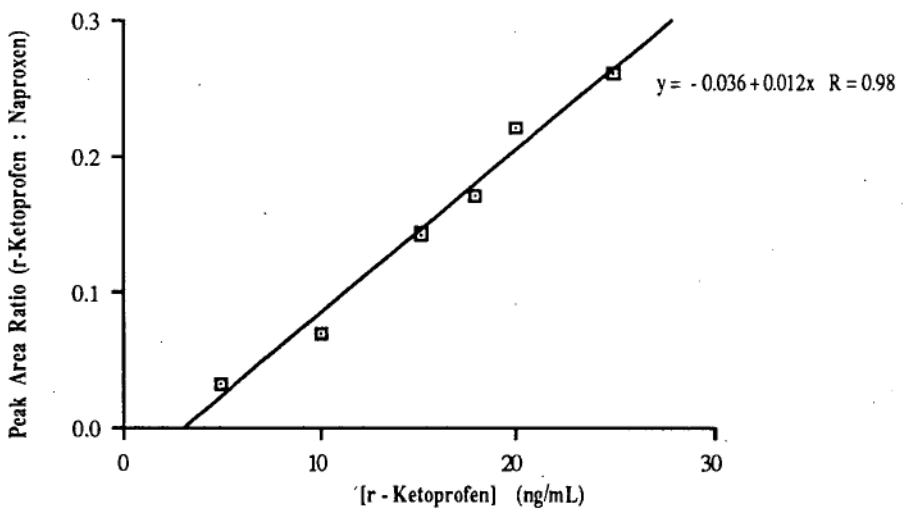
A: mass spectra of s(+)- ketoprofen diastereomer.
B: mass spectra of r(-)- ketoprofen diastereomer.
C: gc/ms(SIM m/z 280)resolution of s(+)- ketoprofen(1); r(-)- ketoprofen(2) diastereomers.

4.4.2.2 Standard Curves

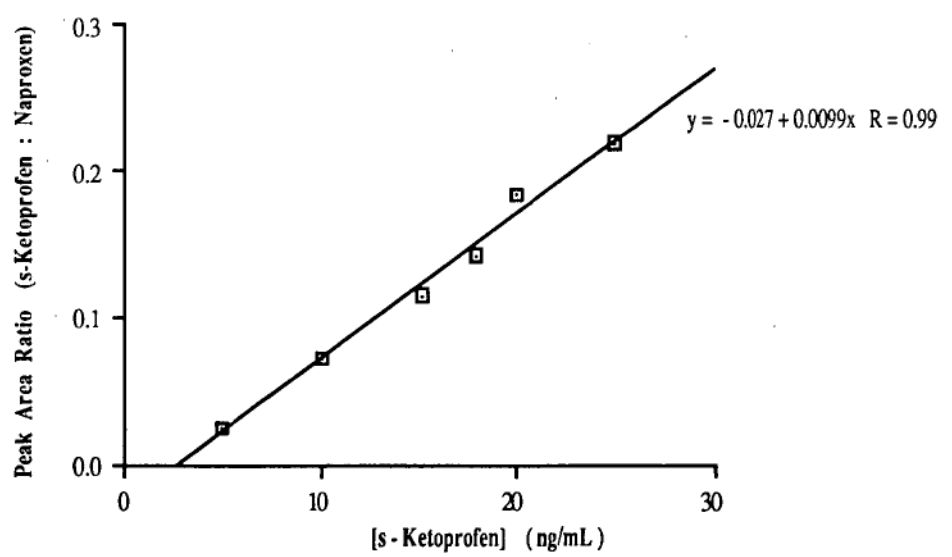
The calibration curves for ketoprofen enantiomers(5-25ng/mL) are illustrated in Fig 4.12:

Fig 4.12: CALIBRATION CURVES FOR S(+) AND R(-) KETOPROFEN

1: r - KETOPROFEN



2: s - KETOPROFEN



CHAPTER 5

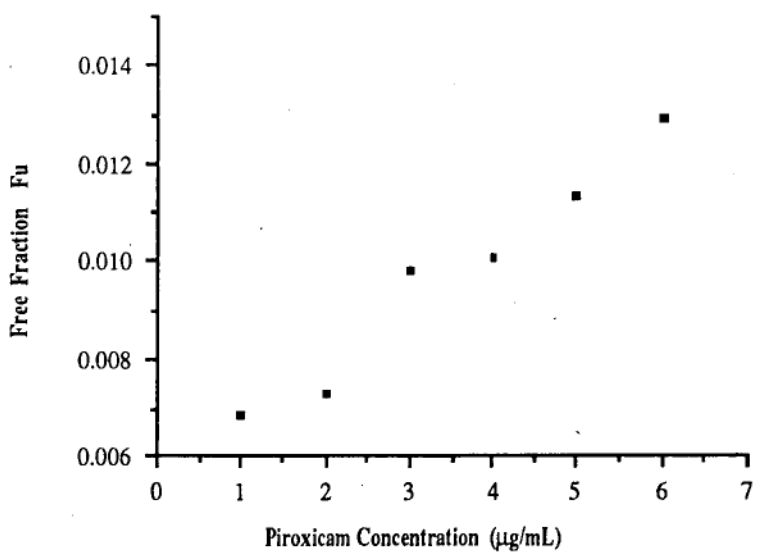
RESULTS : NSAID BINDING STUDIES

5.1 NSAID CONCENTRATION

5.1.1 Piroxicam

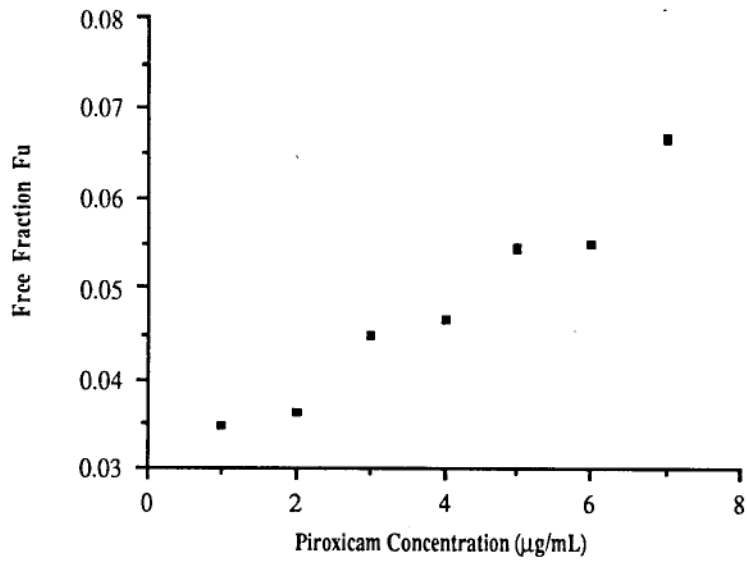
The free fraction(F_u) of unbound piroxicam in HSA solution (18g/L) over a range of piroxicam concentrations (1-6μg/mL), is represented by Fig 5.1:

Fig 5.1: F_u vs [PIROXICAM] - HSA



The experiment was repeated using blank synovial fluid {previously assayed (3.1.3) and found to contain 24g/L albumin}, and the following results obtained (Fig 5.2):

Fig 5.2: F_u vs [Piroxicam] - SYNOVIAL FLUID

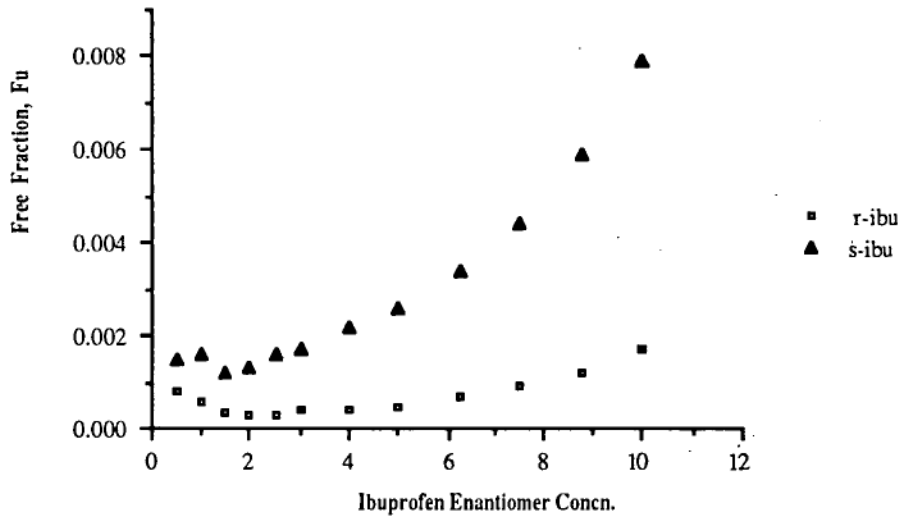


At all total piroxicam concentrations, the free fraction in synovial fluid was greater than in HSA solution.

5.1.2 **Ibuprofen**

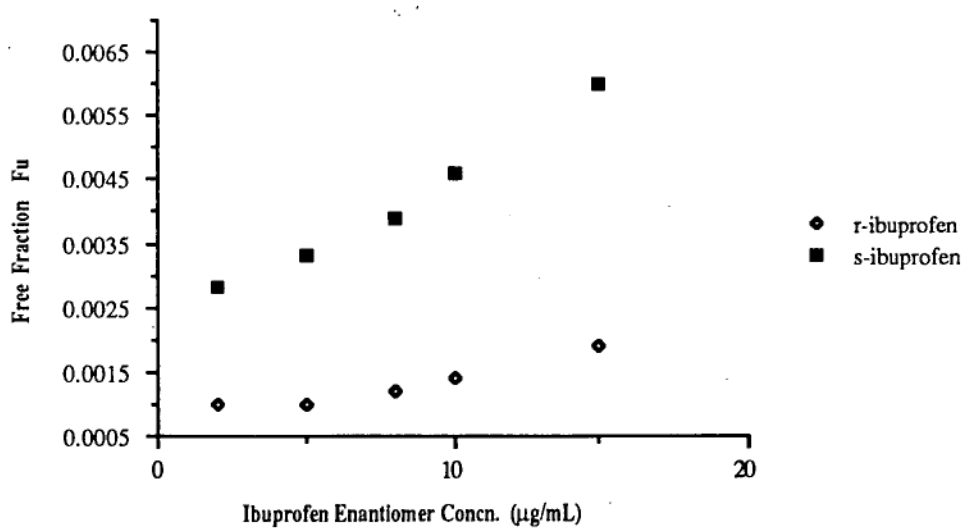
The free fraction of ibuprofen enantiomers in HSA solution (18g/L) was investigated over a range of enantiomer concentrations, (Fig5.3):

Fig 5.3: BINDING OF IBUPROFEN ENANTIOMERS TO HSA (18G/L)



The experiment was repeated in a sample of synovial fluid {previously assayed (3.1.3), and found to contain 18g/L albumin} - Fig 5.4:

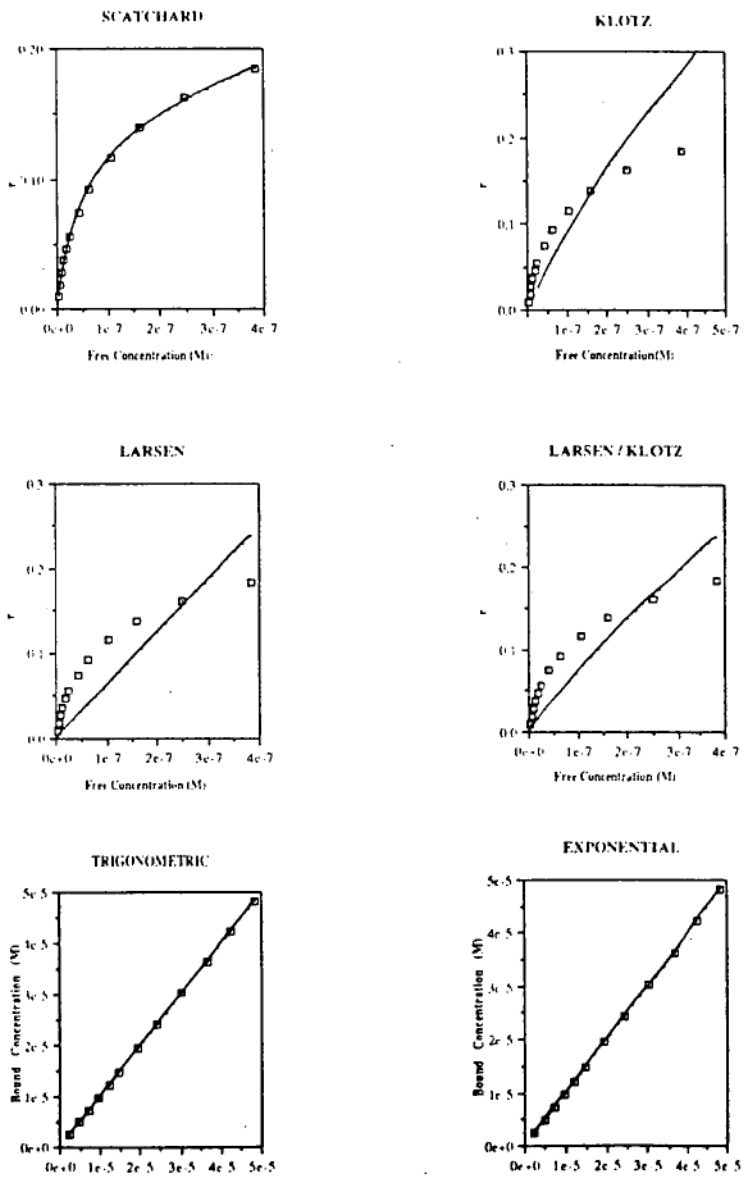
Fig 5.4 BINDING OF IBUPROFEN ENANTIOMERS IN SYNOVIAL FLUID



5.1.3 Binding / Association Constants

The preceeding data (5.1) were fitted to the fore mentioned mathematical models (3.5). Examples of plots and fitted curves are illustrated in Fig 5.5, whilst binding/association constants for the fitted data are shown in Table 5.1:

Fig 5.5: EXAMPLES OF BINDING PLOTS* USED TO CALCULATE BINDING / ASSOCIATION CONSTANTS



* s - ibuprofen in HSA solution

Table 5.1: **BINDING PARAMETER ESTIMATES[#] CHARACTERIZING THE INTERACTION OF IBUPROFEN AND PIROXICAM WITH ALBUMIN IN SYNOVIAL FLUID AND BUFFER SOLUTIONS.**

MODEL	PIROXICAM		r-IBUPROFEN			s-IBUPROFEN		
	HSA	SF[o]	HSA	SF[+]	FAF[Ø]	HSA	SF[+]	FAF[Ø]
Scatchard								
k_1 ($10^{-6}M^{-1}$)	9.81(86)	0.75(41)	70.8(28)	12.5(93)	42.5(58)	20.8(10)	5.9(18)	51.7(48)
n_1	0.05(82)	0.15(47)	0.19(20)	0.34(101)	0.29(40)	0.15(6)	0.22(16)	0.28(30)
k_2 ($10^{-6}M^{-1}$)	10.8(35)	7.62(56)	213(63)	143(270)	187(705)	148(2)	374(114)	99.2(62)
n_2	127(29)	2.0(1000)	12.9(148)	30.7(513)	33.7(745)	8.99(18)	7.46(94)	70.9(6)
Klotz								
k_1 ($10^{-6}M^{-1}$)	0.35(7)	0.07(7)	3.86(14)	3.15(8)	4.26(21)	0.98(9)	0.98(9)	4.55(22)
k_2 ($10^{4}M^{-1}$)	0.04(*)	8.12(*)	50.3(*)	5.67(*)	27.2(*)	0.004(*)	0.004(*)	2.32(*)
Larsen								
b_1 (10^{-3})	2.42(198)	2.19(137)	2.96(983)	5.17(480)	3.23(395)	3.07(2000)	3.33(625)	37.7(1168)
b_2	134(195)	28.4(137)	995(980)	441(479)	763(377)	203(2000)	216(606)	69.4(1157)
Larsen / Klotz								
k_1 ($10^{-6}M^{-1}$)	0.35(7)	0.07(19)	3.77(21)	3.04(16)	3.87(20)	0.79(40)	0.95(19)	4.13(23)
k_2 ($10^{4}M^{-1}$)	0.035(*)	3.07(*)	103(*)	2.30(*)	5.92(*)	99.3(*)	0.003(*)	2.17(*)
Trigonometric								
a_1 (10^{-3})	3.14(0.05)	2.63(0.2)	3.14(0.01)	3.14(0.01)	3.15(0.02)	3.20(0.04)	3.21(0.04)	3.15(0.01)
a_2 (10^{-4})	3.15(0.05)	3.60(0.2)	3.18(0.01)	3.18(0.01)	3.17(0.02)	3.11(0.04)	3.10(0.04)	3.18(0.01)
Exponential								
b_1 (10^{-3})	3.14(0.03)	2.62(0.2)	3.13(0.01)	2.80(0.004)	3.15(0.02)	3.18(0.09)	3.22(0.07)	3.15(0.06)
b_2 (10^{-4})	3.15(0.03)	3.61(0.2)	3.19(0.01)	3.57(0.003)	3.17(0.03)	3.13(0.02)	3.09(0.001)	3.17(0.03)

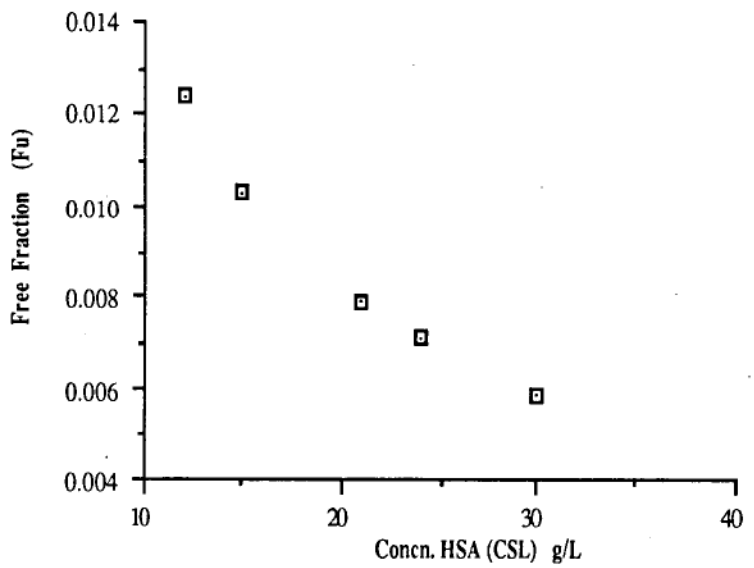
[#] = value ± coefficient of variation; * = (> 10^6); HSA = Human Serum Albumin (18g/L solution); SF = Synovial Fluid : [o] = 24g/L HSA ; [+] = 18g/L HSA ; [Ø] FAF = Fatty Acid Free Albumin Solution(18g/L)

5.2 ALBUMIN CONCENTRATION

5.2.1 Piroxicam

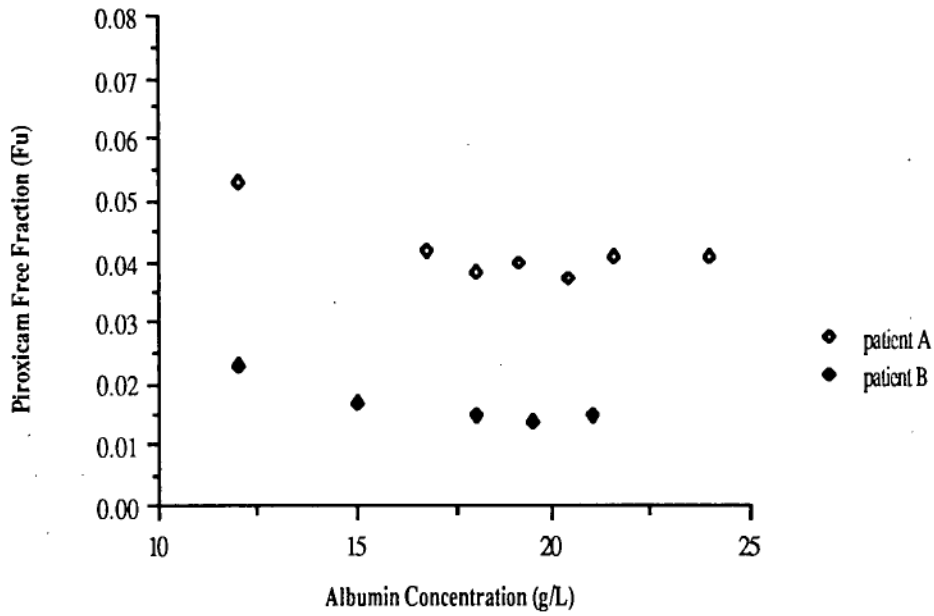
The free fraction of piroxicam (2μg/mL) in HSA solutions of varied concentration are given in Fig 5.6:

Fig 5.6: PIROXICAM - F_U vs [ALBUMIN]



The experiment was repeated using two independent samples of synovial fluid, (Fig 5.7):

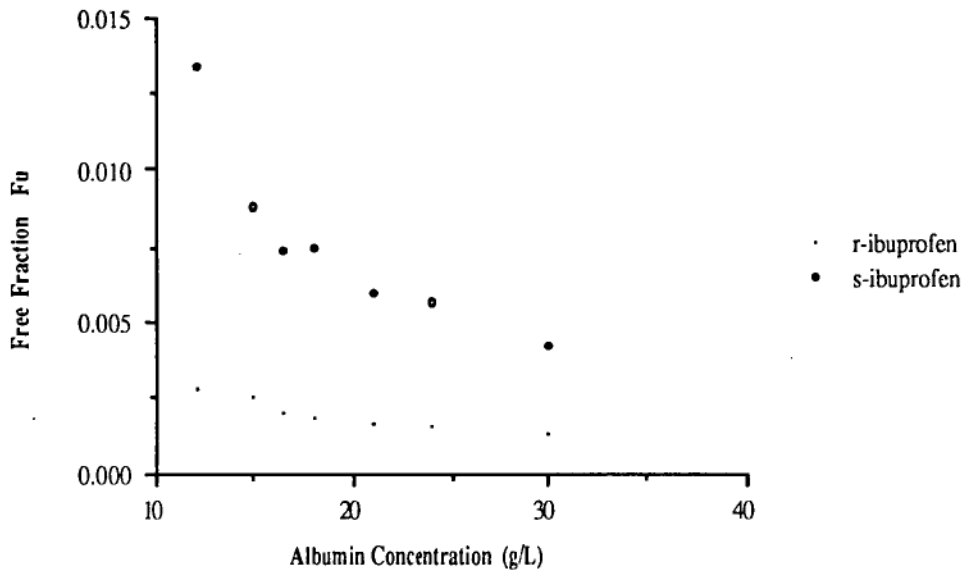
Fig 5.7: PIROXICAM - F_U vs [ALBUMIN] IN SYNOVIAL FLUID



5.2.2 Ibuprofen

The free fraction of ibuprofen enantiomers {10µg/mL r(-) and 10µg/mL s(+)} in HSA solutions of varied concentration are represented in Fig 5.8:

Fig 5.8: F_U vs HSA CONCN. { [R,S-IBUPROFEN] = 20µg/mL }



5.3 CHIRAL NSAID ENANTIOMER RATIO

The effect on free fraction (F_u), of increasing the concentration of the s(+) enantiomer of ibuprofen relative to a fixed concentration of the r(-) enantiomer was examined in HSA solutions (18g/L) - Table 5.2:

Table 5.2: EFFECT OF VARIATION TO IBUPROFEN ENANTIOMER RATIO

Sample No	Enantiomer Concn. (µg/mL)		Fu*	
	R-IBU	S-IBU	R-IBU	S-IBU
1	6	6	0.0012	0.0034
2	6	9	0.0014	0.0040
3	6	12	0.0017	0.0054
4	6	15	0.0053	0.0175
5	6	20	0.0034	0.0204
6	6	30	0.0048	0.0253
7	6	40	0.0084	0.0293

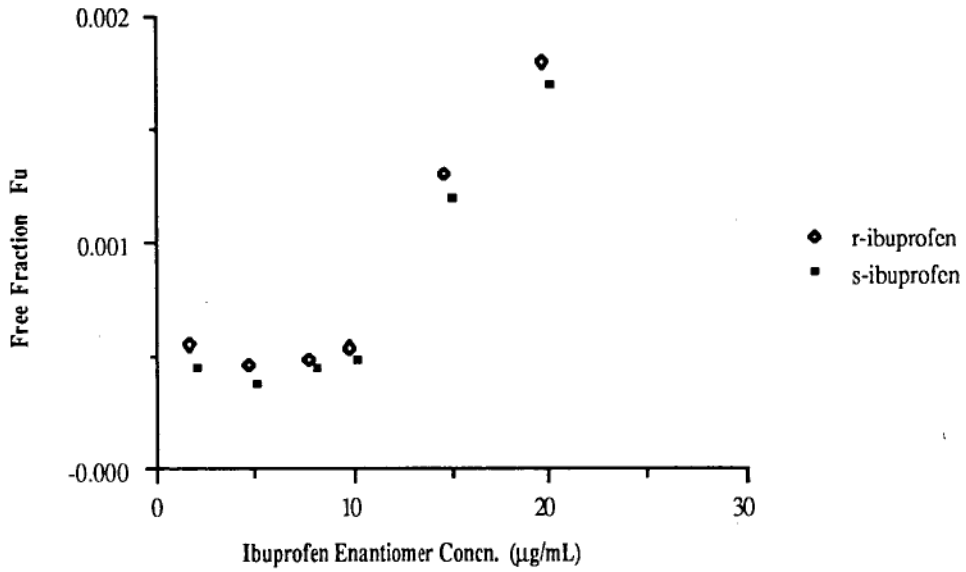
* coefficient of variation: R-IBU = 7.4%; S-IBU = 10.8%

5.4 FATTY ACIDS

5.4.1 Ibuprofen Binding in Stripped Albumin

The binding of ibuprofen enantiomers (2-20µg/mL) in "fatty acid - stripped" HSA solutions (18g/L) was examined, (Fig 5.9):

Fig 5.9: **BINDING OF IBUPROFEN ENANTIOMERS IN FATTY ACID - FREE HSA (18g/L)**



5.4.2 Addition of Fatty Acid

The effect on free drug fraction (F_u), of adding a fatty acid (oleic acid) to "stripped" HSA solutions (18g/L) in the presence of various concentrations of ibuprofen enantiomers and oleic acid concentrations, was examined, (Table 5.3):

Table 5.3: **EFFECT OF FATTY ACID ON IBUPROFEN ENANTIOMER BINDING**

Enantiomer Conc. (µg/mL)	Free Fraction (F_u*) Sodium Oleate Added - (Molar ratio of Oleic Acid to albumin)					
	2:1		3:1		4:1	
	R-IBU	*S-IBU*	*R-IBU*	*S-IBU*	*R-IBU*	*S-IBU*
2	0.00048	0.00072	0.00050	0.00120	0.00140	0.00950
5	0.00033	0.00062	0.00051	0.00150	0.00110	0.00940
10	0.00034	0.00071	0.00062	0.00220	0.00120	0.01300
15	0.00044	0.00120	0.00062	0.00250	0.00190	0.01500
20	0.00073	0.00260	0.00082	0.00340	0.00200	0.02400

* coefficient of variation: R-IBU = 7.4%; S-IBU = 10.8%

5.5 pH

The influence of pH on free fraction (F_u), of piroxicam (2µg/mL) was examined over a pH range typically found in pathological synovial fluid - Table 5.4:

Table 5.4: EFFECT OF pH ON PIROXICAM BINDING IN HSA (18G/L)

pH	F_u Determinations	Mean
7.05	0.0132, 0.0125	0.0129
7.27	0.0121, 0.0125	0.0123
7.37	0.0116, 0.0141	0.0129
7.41	0.0092, 0.0128, 0.0114	0.0111
7.45	0.0090, 0.0100	0.0095
7.48	0.0092, 0.0100	0.0096
7.49	0.0101, 0.0105	0.0103

mean 0.0112 ± 0.0015 S.D.

CHAPTER 6

DISCUSSION

6.1 SYNOVIAL FLUID

6.1.1 Practical Considerations

Normal synovial fluid has a high viscosity and when drawn with a glass pipette tends to form a tenacious string several centimetres in length before finally breaking. This property alone presents difficulties in volumetric analysis and in some instances, this may necessitate densitometric measurement involving weighing. However, pathological fluids generally have reduced viscosity because of dilution and/or enzymatic attack on the hyaluronic acid molecule[25] and therefore tend to pour readily from a pipette. Normal synovial fluid does not clot as fibrinogen and other clotting factors are absent. However, clots do occur in pathologic fluids and their size is roughly proportional to the severity of the inflammation[1]. Mucin clot formation* occurs spontaneously within several days of storage and can vary from a tight ropey mass in a clear solution (good mucin) to small soft masses in a turbid solution (poor mucin). Generally, the more inflamed the joint, the worse the mucin[1].

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* This may be induced in the laboratory immediately following sample collection by mixing with aqueous acetic acid solution. This "mucin test" has been used as a descriptive qualitative test to give an indication as to the physical state of the hyaluronic acid[1].

Whilst protein determinations (3.1.3) are not specifically diagnostic of a specific articular disease, one can be reasonably certain that a fluid is not normal when the total proteins exceed 25g/L and that moderately severe inflammation is present when total proteins exceed 45g/L.

6.1.2 **Electrophoresis Study**

This procedure is commonly used in clinical laboratories for the separation of proteins found in serum. In aqueous medium, proteins form aggregates of charged molecules (micelles), and upon application of an electric field, the individual protein molecules move towards the electrode of opposite charge. For the purposes of this investigation, cellulose acetate electrophoresis was employed as this provided a reliable and convenient medium upon which to separate the principle proteins in synovial fluid. Proteins in synovial fluid dissolve in the buffer on the cellulose acetate strip and the applied current across the strip allows the individual protein molecules to begin migration along the matrix of cellulose fibre "channels" towards the anode. The rates at which the various proteins do this will depend upon their respective mobilities[75].

Schmid and MacNair[29] used electrophoresis to examine the nature of the protein in synovial fluid. They compared synovial fluid and corresponding plasma from rheumatoid patients and noted the similarity between proteins in both fluids. In the current studies, the viscosity and high levels of mucopolysaccharides in synovial fluid made resolution of component proteins difficult[2,75]. However, this latest investigation confirmed the pattern of proteins in synovial fluids used in the current binding studies was comparable to plasma (and in agreement with previous researchers[29]).

6.1.3 **Stability and Storage**

The pH of stored samples increased slightly (≤ 2.1%) at day 10 to ≤ 5.8% after 30 days. This is presumably due to the loss of carbon dioxide. Normal synovial fluid is reported[2] as having a similar pH to serum of 7.31-7.64, though there are reports of higher values of mean 7.77[1].

There was an insignificant amount of variation with respect to the free fraction (F_u) of stored samples. The variation was within the experimental limits of the procedure and considered unlikely to be of practical importance.

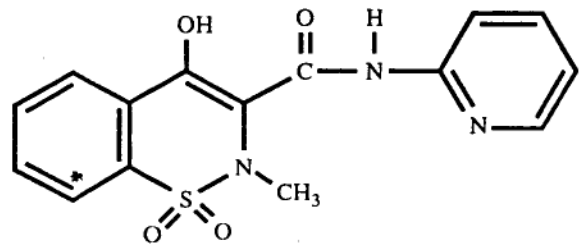
6.2 <u>PIROXICAM</u>

6.2.1 **Radiolabelled Piroxicam**

6.2.1.1 Background

These studies were made possible by the ready availability of radiolabelled ^3H piroxicam (Fig 6.1):

Fig 6.1: **PIROXICAM**



* ^{3}H radiolabel

6.2.1.2 Stability

The stock solution was protected from light at all times, as reports of similar experiments using radiolabelled tenoxicam solutions exposed to sunlight resulted in minor degradation of the drug and subsequent errors in free fraction determination[61].

6.2.2 Precision and Reproducibility of Ultrafiltration Procedure

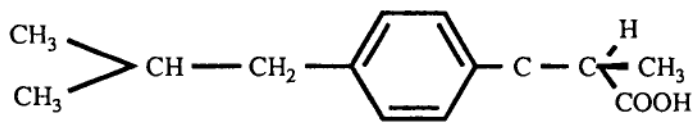
The precision of the assay has been previously assessed[46], and the coefficient of variation for piroxicam at 5µg/mL calculated to be 4.1%. The mean recovery (2.55µg/mL piroxicam) was 92.7% (SD 8.1%). The difference may be attributed to binding associated with the Amicon device and / or YMT membrane. According to previous studies by Whitlam and Brown[24], this is unlikely to be significant.
The radiolabelled drug allowed for rapid and convenient determination of piroxicam free fractions in synovial fluid and albumin solutions (using the Amicon MPS-1 ultrafiltration device), with a coefficient of variation of 9.8%.

6.3 IBUPROFEN

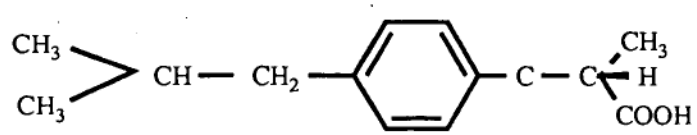
6.3.1 Background

Ibuprofen [2-(4-isobutylphenyl) propionic acid] has a propionic acid side - chain with an asymmetric centre, the alpha carbon atom, (Fig 6.2):

Fig 6.2: IBUPROFEN ENANTIOMERS



R(-) - ibuprofen



S(-) - ibuprofen

Two optical isomers (enantiomers) therefore exist.

6.3.2 Radiolabelled Ibuprofen

Radiolabelled enantiomers of ibuprofen of suitable specific activity would have greatly facilitated the development of a suitable assay procedure. Unfortunately, the difficulty, expense and time involved in producing suitable

radiolabelled material[63,64] made this procedure impractical. It was decided that a suitably sensitive chromatographic assay procedure was the most logical alternative. Cox and co-workers[80] on the other hand, opted to persist with the preparation of radiolabelled enantiomers in their ongoing endevours to examine plasma and tissue protein binding affinities of this drug.

6.3.3 Sensitivity Considerations

The ultrafiltration procedure using the Amicon MPS-1 device provided the ultrafiltrate from which subsequent chromatographic analysis was undertaken. As supplies of drug free synovial fluid from volunteers used in this research (Appendix 1) were infrequent and understandibly small in volume (typically less than 15mL), a maximum of only 1mL of fluid could be allocated to each Amicon MPS-1 device. Of this, only 200μL could be realised as collectable ultrafiltrate. Therefore, the amount of free drug available for chromatographic assay was reduced by a factor of 5 from the outset. Alternative, slower procedures such as equilibrium dialysis, which allow collection of larger amounts of free drug, were considered unsuitable for later binding experiments with competing ligands and other binding modifiers[48,24]. It was envisaged that an assay capable of detecting 2ng/mL and 5ng/mL of ketoprofen and ibuprofen enantiomers respectively was required. This meant that existing assays had to be improved substantially in terms of sensitivity if this technique were to be used.

6.3.4 Previous Assay Attempts

Generally speaking, gas chromatographic methods for separation of NSAID enantiomers are difficult as the isomers have the same physical properties.

There have been numerous attempts at quantification of racaemic ibuprofen in biological fluids[36], yet relatively few attempts at resolving the enantiomers. The limited number of published enantiomeric assays involve either the use of optically active stationary phases[36,57], or more commonly, derivatization to diastereomers by reaction with an optically active reagent[37-40]. The latter methods are usually time consuming procedures, often involving complex derivatization reactions and lengthy incubation periods. This is often necessary, however, as detection and quantification may be improved by selection of chiral resolving agents which confer enhanced detector sensitivity on the resulting diastereomers. Table 6.1 illustrates some examples of earlier enantiomeric NSAID assays.

Table 6.1 **EXAMPLES OF SOME ENANTIOMERIC NSAID ASSAYS**

Author	NSAID(s)	Instrument[n].	Lower Limits	Additional Drawbacks
Augerinos[37]	ibuprofen	HPLC	0.5µg/mL	2hr. reaction time.
Sallustio[56]	ketoprofen	"	0.2µg/mL	-
Geislinger[36]	"	"	0.1µg/mL	-
Foster[41]	ketoprofen	"	50ng/mL	-
Goto[58]	naproxen	"	10ng/mL	complex derivatizing reagent synthesis; expensive; slow.
Lee[39]	ibuprofen	HPLC	0.5µg/mL	1hr. reaction time.
Kaiser[38]	ibuprofen	GC	0.5µg/mL	-
Singh[40]	ibuprofen	"	75ng/mL	2hr reaction time; unstable derivatizing agent.
	ketoprofen	"	n/a	
	naproxen	"	n/a	

Ideally, a relatively simple and straightforward HPLC assay (of sufficient sensitivity to enable detection of free levels of ibuprofen enantiomers in synovial fluid), was required[111]. In addition, it would be convenient if such an assay could be modified to allow measurement of the enantiomers of other chiral NSAID's such as ketoprofen. Royer et al[43] developed an HPLC assay for ketoprofen in plasma using UV detection and applied it to the study of its plasma protein binding, using an equilibrium dialysis system. Whilst the method was rapid and relatively sensitive (2ng/mL), it made no attempt at resolving the individual enantiomers. Meanwhile, Bjorkman[42] and Foster and Jamali[41] reported plasma assays for the enantiomers of indoprofen and ketoprofen respectively. Their methods involved extraction of unchanged drug, conversion to a mixed anhydride with ethylchloroformate, derivatisation with l - leucinamide, extraction of the formed diastereomers with an organic solvent, and subsequent evaporation and injection into the HPLC system. The method developed by these two research groups was appealing, from a speed and simplicity point of view as compared to the fore mentioned GC procedures. The latter work was viewed as an ideal starting point for the development of an assay of sufficient sensitivity for the intended investigation of the unbound enantiomers of ibuprofen and ketoprofen in synovial fluid. As later discussed (6.4.1), the Foster and Jamali HPLC assay[41] did not provide sufficient sensitivity for the intended investigations with ketoprofen. Whilst it was initially intended to further pursue this issue with ketoprofen, the unavailability of ketoprofen enantiomers compared to the ready availability of ibuprofen enantiomers (courtesy of The Boots Company, Nottingham, U.K.), caused the research to orientate towards the development of a suitably sensitive enantiomeric ibuprofen assay (which in turn could be adapted for ketoprofen at a later date). As all practical alternatives for a suitably sensitive HPLC assay had been pursued, attention was focused on the development of a suitably sensitive GC/MS assay.

6.3.5 GC/MS Enantiomeric Assays

6.3.5.1 Background

GC assays are generally more time consuming than HPLC assays, yet often have the inherent advantage of greater sensitivity. Ideally, a GC assay of high sensitivity using mass spectrometry for enhanced sensitivity with a rapid, relatively simple work-up, (typical of many HPLC assays) was required. Singh and co-workers[40] developed an enantiomeric assay for several NSAID's (including ibuprofen and ketoprofen) using optically active amphetamine to form the diastereomers and then separated them using capillary gas chromatography with nitrogen - phosphorous detection. They found dexamphetamine to be a preferred alternative to alpha - methylbenzylamine as used by earlier workers[40]. Furthermore, they used 1,1'-carbonyldiimidazole (CDI) as their coupling reagent instead of thionyl chloride as it has been observed that " the hydroxylated metabolites of ibuprofen are readily dehydrated and hydrohalogenated by this agent[14]". Their work was viewed as the next logical step in the current work, for the development of a suitably sensitive assay. The ready availability of a suitable GC/MS system for this work was seen as a means of not only re-establishing their assay, but also as a possible means of enhancing sensitivity by the use of selected ion monitoring.

6.3.5.2 GC/MS Assay Using Dexamphetamine CDI

The spectra of the CDI reagent (Fig 4.4), obtained in the course of this assay was characteristic of imidazole and included m/z 36; characteristic of HCl (presumably from chloroform in the reagent). When solid CDI was transferred directly to the probe, a mass spectra characteristic of CDI was observed, whilst the m/z 36 ion

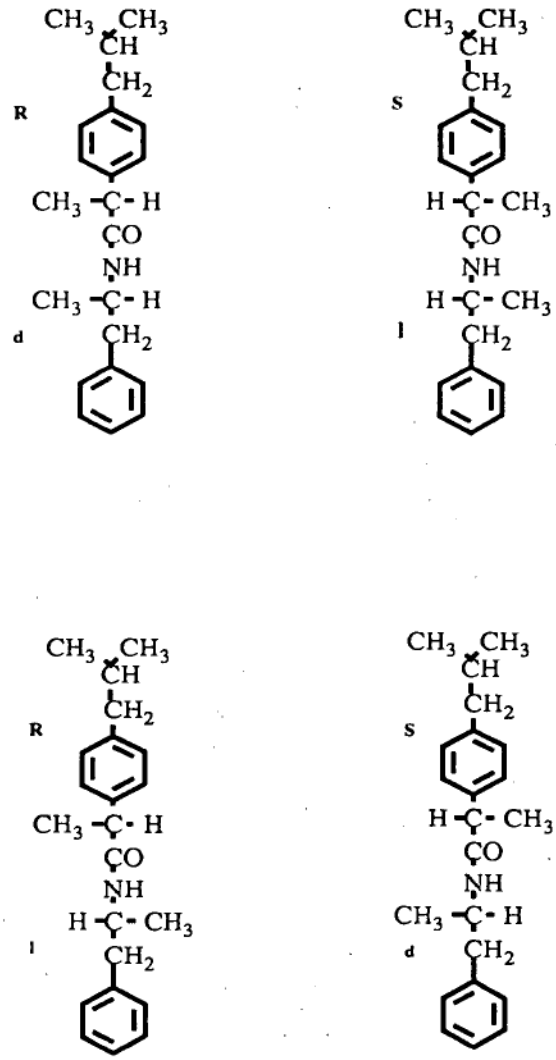
fragment was absent. Data thus far supported the theory that HCl impurity in the chloroform was facilitating the hydrolysis of CDI and rendering it unsuitable for derivatization work.

A 1mg/mL solution of CDI in chloroform was analysed by infra red spectroscopy, and from the IR spectra, there appeared to be an abundance of imidazole and an insignificant amount of CDI present. From this, it appeared that CDI had spontaneously decomposed to its imidazole counterpart. This is consistent with the findings of Kaiser and co-workers[38], who conducted stability studies on CDI reagent and found substantial degredation to imidazole albeit after 48 hours. Several other batches of chloroform available in this laboratory were tested, yet these all failed to provide a reagent of sufficient stability. As further samples of CDI were not readily attainable, it was decided to abandon this reagent in favour of another. Furthermore, the lengthy incubation period for derivatization was viewed as a significant disadvantage. It was therefore decided to explore alternative coupling agents with expectations of a more rapid and less finical assay procedure.

6.3.5.3 GC/MS Assay Using Dexamphetamine / Ethylchloroformate

The ketoprofen HPLC assay (3.3.1) established, provided a rapid derivatization procedure for the enantiomeric leucinamide derivatives by using ethylchloroformate as the coupling reagent. As mass spectra for the amphetamine derivatives of ibuprofen have previously been determined[40], it seemed logical to attempt the derivatization with dexamphetamine as above, but using ethylchloroformate as the coupling reagent. The reaction is a two step procedure; ie: the formation of a mixed anhydride of ibuprofen and ethylchloroformate and its subsequent conversion to an amide by reaction with dexamphetamine to produce the required diastereomers. The expected products are illustrated in Fig 6.3:

Fig 6.3: IBUPROFEN - AMPHETAMINE DIASTEREOMERS



6.3.5.3.1 *Derivatization Reaction Time*

Ethylchloroformate is a commonly used reagent for amide formation in peptide synthesis[42], but not commonly used in this capacity in analytical chemistry. When

used in conjunction with l-leucinamide (as in the current HPLC assay), the coupling reaction is complete in less than 3 min[41,42,62]. In the amphetamine derivatization, there was negligible variation in peak area ratio after 60 seconds, (Fig 4.7). A reaction time of 60 seconds was therefore settled upon.

6.3.5.3.2 *Dexamphetamine Reagent Quantity*

A large excess of dexamphetamine theoretically remains after biological sample derivatization[40]. In the current studies, a volume of 50µL (equivalent to 100µg free base) was settled upon (based on the negligible variation in yield with increasing volume of reagent used - Fig 4.8). This was twice the amount used by Singh[40].

6.3.5.3.3 *Extraction Solvent*

A number of extraction solvents for ibuprofen in synovial fluid were considered. A study by Lee and co-workers[39] compared numerous extraction solvents from earlier NSAID assays and concluded that 0.1% isopropyl alcohol (IPA) in n-hexane was the most suitable for their studies with ibuprofen. It allowed rapid and reasonable recovery (85% or better for both enantiomers in the concentration ranges examined) of drug with minimal interfering peaks.This solvent system was therefore used in these studies.

6.3.5.3.4 *Contamination*

Contamination of both chemicals and reagents are common, ongoing problems with any assay working in the picogram range of detection. The development of this drug assay was severely hampered by such problems in its early stages. Blank (ibuprofen

free) derivatizations in both synovial fluid and water repeatedly produced peaks, consistent with a small amount of ibuprofen contamination. 10mL samples of all solvents involved in the assay were evaporated to near dryness and derivatized and chromatogrammed in the usual manner. However,this failed to locate the offending agent(s).It was not until a new batch of ethylchloroformate and triethylamine were obtained that the problem was resolved. "Blank" samples were assayed during all subsequent runs to monitor this problem.

6.3.5.3.5 *Internal Standard*

P-toluic acid was used in an earlier HPLC assay[46] which had been developed to quantify numerous NSAID's in plasma and subsequently used in the early stages of development of this assay. However, it was considered far from ideal as supplies of p-toluic acid available at these laboratories were contaminated with unknown material, and a clean chromatographic trace could not be readily obtained. A supply of o-toluic acid was readily available which did not cause this problem. Whilst this agent appeared superficially suitable, there were problems, not only in terms of repeatability of results, but also in obtaining a linear correlation with respect to the development of a standard curve. It was hypothesised that problems were occurring in the extraction procedure and/or the evaporation steps. The data (Table 4.4), demonstrate not only the large variation in extraction efficiency of ibuprofen as compared to o-toluic acid (samples 1 & 2), but also the variability in derivatized o-toluic acid of both extracted (1,2& 3) and non extracted (4,5 & 6), samples. Rapid loss of o-toluic acid during the evaporation step is consistent with this data, and also in agreement with guidelines developed by Grob and Muller[47]. They demonstrated rapid and significant losses (10-15%) of solute material (up to C_{21}) when co-evaporated with solvents under a stream of nitrogen gas. In addition, they

demonstrated that losses may be dramatic when a sample is allowed to evaporate to complete dryness. Naproxen, being a larger molecule and more akin to ibuprofen than o-toluic acid was considered a potentially more suitable alternative. The second peak in the trace (Fig 4.6) was attributable to l-amphetamine contamination in the dexamphetamine derivatization reagent and possibly r-naproxen contamination in the naproxen. For the purpose of this assay however, this was to be of no consequence, as total peak areas of both peaks were considered in subsequent calculations.

6.3.5.3.6 *Standard Curves*

The calibration curves were linear over the concentration range 10-200 ng/mL. The minimum quantifiable concentration was 2.5ng/mL, in conjunction with the ultrafiltration procedure (3.3.2.15), and was markedly more sensitive than previous assays (Table 6.1).

6.3.5.3.7 *Assay Reproducibility and Precision*

Using the ultrafiltration procedure (3.3.2.15), the coefficient of variation for determination of ibuprofen enantiomers was ≤ 10.8% (s-ibuprofen) and ≤ 7.4% (r-ibuprofen) - Table 4.5. This compares favourably with the 10% variation of ibuprofen enantiomers obtained in the GC/MS assay procedure of Singh[40].

6.3.5.3.8 *Sample Stability*

Whilst all samples in this current work were analysed within 12 hours of derivatization, the data (Table 4.6), clearly demonstrates that the influence of short term sample storage with regard to assay reproducibility is negligible. This is in

agreement with work by Testa[55] who demonstrated a general degree of increased stability of diastereomeric amide derivatives over that of esters for bioanalytical work.

6.4 KETOPROFEN

6.4.1 HPLC Enantiomeric Assay

6.4.1.1 Background

An HPLC enantiomeric assay for ketoprofen was established (prior to the fore mentioned GC/MS assays), following guidelines previously set by Foster and Jamali[41]. The rationale for this was to:

a) Reproduce the assay described in their publication with a view to modifying physical conditions in an attempt to enhance sensitivity for ketoprofen initially

and b) To convert or modify the assay to allow measurement of ibuprofen enantiomers.

6.4.1.2 Assay Sensitivity

Numerous attempts to enhance sensitivity included variation of composition of the mobile phase and its pH, the substitution of alternative columns and substitution of the variable wavelength UV detector with a Waters fixed wavelength UV detector

Model 441 (used at various wavelengths between 250nm and 300nm). Despite these attempts, it was not possible to increase sensitivity to levels beyond 20ng/mL for each enantiomer. A ten fold increase in sensitivity would be required for this assay to be suitable. A possible alternate means of enhancing sensitivity considered was to incorporate continuous - flow, post column derivatization (as used by Anhalt and Brown[44] in their HPLC assay of aminoglycoside antibiotics in serum). In their assay, o-phthalaldehyde was used to form fluorescent products for detection. It was subsequently determined that leucinamide derivatives of ketoprofen would be unlikely to readily react with such reagents[54], and so an alternative procedure was sought.

6.4.2 **Ketoprofen GC/MS Enantiomeric Assay**

A major incentive for the development of the fore mentioned ibuprofen GC/MS assay was to adapt it for the measurement of the free levels of ketoprofen enantiomers in synovial fluid, at much lower levels than achieved with the HPLC assay (6.4.1). Time constraints prevented refinement of the ketoprofen assay, however it was confirmed that standard curves for the procedure (within the required concentration range of 2-25ng/mL for each enantiomer) were possible.

Whilst the lowest concentration of enantiomers assayed was 5ng/mL, the data (Fig 4.12) suggest that with refinement of procedure and optimisation of GC/MS conditions, measurement of ketoprofen enantiomers at 2ng/mL is feasible.

6.5 FACTORS AFFECTING NSAID BINDING

6.5.1 NSAID Concentration

6.5.1.1 Piroxicam

The normal therapeutic range of piroxicam in plasma is 3 - 8μg/mL[26], and the corresponding synovial fluid levels will be approximately 40% of the plasma levels[27]. There was a marked trend of piroxicam concentration-dependent HSA binding, (Fig 5.1). Within the piroxicam concentration range 1 to 6μg/mL (and at an albumin concentration of 18g/L), the free drug fraction, F_u increased from 0.69% to 1.29% respectively.

The experiment was repeated with a sample of synovial fluid (Fig 5.2). The free fraction ranged from 3.5% - 5.5%, and was consistently higher than for the 18g/L albumin solutions over the same piroxicam concentration range. Whilst there are no published data comparing piroxicam binding in albumin solutions to synovial fluid, the current findings are consistent with previous studies, where the binding in synovial fluid and plasma (at comparable drug concentrations), were approximately 98% and 99% respectively[7]. Chan et al[90] found a similar trend in their studies with diclofenac, whilst contrary to these findings, Trnavska´ and co-workers[34] failed to detect any difference with piroxicam binding in synovial fluid and corresponding patient plasma. It is worth noting however, that they used much higher piroxicam concentrations (14.9 - 298μg/mL) than in the present study, which used concentrations more likely to be found in the clinical situation.

6.5.1.2 Ibuprofen

The fraction of ibuprofen enantiomers not bound to albumin (F_u) increased significantly from a value of 0.00083 (r) and 0.0015(s) at 1μg/mL to 0.0017(r) and 0.0079(s) at 20μg/mL of r,s-ibuprofen. At all times, the free fraction of s-ibuprofen exceeded that of r-ibuprofen, (mean ratio s:r = 4.4 ± 1.15 S.D.) - Fig 5.3. The experiment was repeated using blank synovial fluid {previously assayed and found to contain 18g/L albumin}. F_u increased from 0.0010 (r) and 0.0028 (s) at 4μg/mL r,s-ibuprofen to 0.0019 (r) and 0.0060 (s) at 30μg/mL. Free fractions of s-ibuprofen exceeded those of r-ibuprofen at all times (mean ratio s:r = 3.14 ± 0.19 S.D.) - Fig 5.4. In the above studies, similar free fractional ratios of s - ibuprofen : r - ibuprofen were observed in albumin solution and synovial fluid (at comparable drug concentrations). Hansen and co-workers[101] studied the plasma protein binding of ibuprofen enantiomers using equilibrium dialysis and observed a mean free fractional s:r ratio of 1.7:1 ± 0.29S.D. However, care must be exercised with extrapolation of their findings to the current work with HSA solutions as allied studies by Vowles and Marchant[95] displayed a higher degree of ibuprofen binding to serum (or plasma) proteins compared to albumin solutions. Their work suggested that HSA was not a suitable model for human serum.

6.5.1.3 Data Interpretation

There are numerous difficulties involved with regard the analysis of results of binding experiments with relatively small NSAID molecules to proteins. In most instances, a theoretical model is used which plays a crucial role in terms of interpretation of the

experimental results. Many authors have used the Scatchard equation (Equation 6.1), which was deduced from the law of mass action and from the probability of binding to a carrier protein:

$$r = \sum_{i=1}^{n} \frac{k_i C}{1 + k_i C} \qquad \text{Equation 6.1}$$

where r is the average number of bound drug molecules per molecule of albumin, C is the free drug concentration, n is the number of binding sites and k_i is the Scatchard site binding constant. In the simplest possible situation where it has been established unequivocally by means other than binding measurements that there is only one receptor site, then it is reasonable to prepare a Scatchard plot (r / C vs r) of binding data and calculate the binding constant, k_i. In practice however, many authors have attempted to decompose their complex, upward curved Scatchard plots into two or more independent components[88]. Furthermore, the number of classes of binding sites is often arbitarily chosen, based upon the minimum number of classes consistent with the observed experimental data[83] - a procedure in itself which casts doubt upon the validity and significance of such values. Semi-logarithmic plots (log free drug vs moles of bound drug) of their Scatchard data should theoretically produce characteristic "S - shaped" curves. This rarely occurs, as the data for the upper part of the curves are usually absent[84]. In practice, this happens because a point is reached where drug concentrations cannot be increased any further due to limited solubility or because the experiment must be terminated at a certain point for practical

reasons[82]. An advantageous alternative[85,89] can be the use of a stoichiometric binding description, such as the Klotz equation(Equation 6.2):

$$r = \frac{k_1C + 2k_1k_2C^2 + 3k_1k_2k_3C^3 + ...nk_1k_2..k_nC^n}{1 + k_1C + k_1k_2C^2 + k_1k_2k_3C^3 + ... k_1k_2..k_nC^n} \qquad \textbf{Equation 6.2}$$

where $k_1....k_n$ are the set of stoichiometric binding constants[82].

Even so, within the confines and limitations of laboratory procedures for binding measurements, it becomes increasingly difficult to assign meaningful values to binding constants > k_2 when dealing with multiple binding sites. The rationale for binding data analysis is seemingly reduced to the condensation of large quantities of experimental data to a small number of parameters suitable for reconstruction of a binding curve. A more appropriate equation[82] for the non-saturated binding studies in the current work is Equation 6.3:

$$r = b_1 \ln (b_2 C + 1) \qquad \textbf{Equation 6.3}$$

where b_1 and b_2 are positive constants.

An examination of the relation of these two constants to the Klotz equation (Equation 6.2), where a plot of r vs C from Equation 6.3 has the same slope and curvature as a similar plot from Equation 6.2 allows calculation of the first and second

stoichiometric binding constants (k_1 and k_2) - Equation 6.4 :

$$r = \frac{k_1 \ln [\, 2 (k_1 - 2k_2) C + 1 \,]}{2 (k_1 - 2k_2)} \qquad \textbf{Equation 6.4}$$

where $k_1 > 2k_2$

However, even these later equations (Equations 6.3 and 6.4) are essentially descriptive models, which are merely mathematical tools with little physiological meaning[83]. Monot and co-workers[83] described a global association function to characterize the apparent overall equilibrium of drug protein binding, and a binding transfer function to describe the relative rates of change in bound and free concentrations from the total concentration. There are two asymptotic approximation functions which have been proposed and used[86]:

(i) The Trigonometric Model: $B = a_1 \arctan (a_2 T)$ **Equation 6.5**

and (ii) The Exponential Model: $B = b_1 (1 - \exp (- b_2 T))$ **Equation 6.6**

where B = concentration of bound drug, T = concentration of total drug and a_1 and a_2, b_1 and b_2 are the two parameters of the models as determined from the experimental data.

These two models are considered useful when the saturation limit of the bound concentration is unknown[83].

Preceeding data were compared to the fore mentioned models for goodness of fit, using FUNFIT®, (Fig 5.5 and Table 5.1). The data were well described by the Scatchard Model (assuming two classes of binding sites). Values for k_1 and k_2 were about ten times higher than previously quoted in the literature[107], whilst values for n_1 and n_2 were of similar order of magnitude, as previously estimated in plasma. The Larsen Model gave a poor fit to all experimental data, which was contrary to that predicted by the authors[82]. Better fits were obtained with the Klotz model. This was expected, as there were a larger number of parameters that varied in the curve fitting procedure for the latter model. The k_2 values using the Klotz Model however, were too small to be meaningful. Similar values for k_1 to above were calculated using the Larsen / Klotz Model, whilst again, k_2 values were extremely small. The two asymptotic functions (Trigonometric and Exponential Models) gave the best fits to the experimental data. Monot and co-workers[83] described these functions as being particularly useful when the saturation limit of the bound concentration is unknown. Values for k_1, a_1 and b_1 were consistently greater in the piroxicam / HSA solutions compared to piroxicam in synovial fluid. This is consistent with previous studies by Monot et al[83] using sodium salicylate, in which they found " the association function to be slightly less for synovial fluid than for plasma". Scatchard (k_1,k_2), Klotz (k_1) and Larsen / Klotz (k_1) constants calculated for the current studies were slightly larger for r - ibuprofen in HSA solution compared to synovial fluid. The potential for r - ibuprofen to be displaced by endogenous substances present in synovial fluid may be a plausible explanation for these findings. This theory is also supportive of the generally higher association constant values obtained in fatty acid "stripped" albumin as compared to those in corresponding HSA solutions and synovial fluid.

6.5.2 **Protein Concentration**

6.5.2.1 Background

As outlined in chapter 1, albumin (the principle protein to which NSAID's bind at therapeutic levels) generally appears at a lower concentration in synovial fluid to plasma. From Table 1.2 it may be inferred that whilst synovial fluid albumin concentration will vary according to disease state, it generally would be expected to lie within the range 11g/L (normal) to 30g/L (gout).

6.5.2.2 Piroxicam

When binding experiments were performed with piroxicam at an albumin concentration of 1g/L there was no evidence of binding. Piroxicam, at a concentration of 2μg/mL was highly bound to HSA (≥ 98.8% - Fig 5.6), at all other albumin concentrations studied, with only a small fraction present in the free form. Only at albumin concentrations less than 12g/L was there a more pronounced trend of albumin concentration - dependent binding. When the above procedure was repeated with diluted synovial fluid samples, higher fractions of unbound drug were present at comparable concentrations of albumin solutions, (Fig 5.7). Piroxicam binding remained independent of albumin concentrations over 12g/L. Also noteworthy was the two fold variation in free drug fractions of the synovial fluid samples examined. Interpatient variations of similar magnitude (with respect to binding in synovial fluid), were also noted by Trnavska´ and co-workers[34]. In their studies, binding of piroxicam (albeit at much higher concentrations; ≥ 15μg/mL), to protein in synovial fluid was the same as in corresponding patients' plasma. These researchers did not examine lower concentrations of piroxicam, presumably due to the limited sensitivity

of their assay technique.

6.5.2.3 Ibuprofen

Experiments were conducted with r,s-ibuprofen (20μg/mL) mixed and incubated in the presence of blank HSA(Fraction V - CSL, Melbourne, Australia) in phosphate buffer, over a range of albumin concentrations typically found in synovial fluid (12-30g/L). At the r,s-ibuprofen concentration examined,the free fraction of both enantiomers were shown to be markedly dependent upon the albumin concentration. Fig 5.8 illustrates that enantiomers of ibuprofen (at the concentration studied), were highly bound to HSA (r-ibuprofen ≥ 99.7% ; s-ibuprofen ≥ 98.7%) within the albumin concentration range examined. At all times, the free fraction of s-ibuprofen was higher than the corresponding r-ibuprofen free fraction (mean s:r ratio = 3.40 ± 1.31 S.D.), which is consistent with the fore mentioned (6.5.1.3) findings of Hansen[101].

6.5.3 **Chiral NSAID Enantiomer Ratio**

The well documented metabolic inversion of r - ibuprofen to s - ibuprofen[31] provided the basis for an in-vitro investigation of the effect on binding with increasing ratio of s : r - ibuprofen. The initial concentrations of r(-) - and s(+) - ibuprofen enantiomers were both 6μg/mL. As the concentration of s-ibuprofen was gradually increased, the free fractions of both enantiomers increased significantly from 0.0012(r),0.0034(s) at 6μg/mL r + 6μg/mL s - ibuprofen, to 0.0084(r), 0.0293(s) at 6μg/mL r + 40μg/mL s - ibuprofen (Table 5.2). Furthermore, at s-ibuprofen : r-ibuprofen ratios greater than 2:1, there was a dramatic increase in the free fractions

of both enantiomers. The data is suggestive of incremental displacement of r-ibuprofen from low affinity binding sites with increasing concentrations of s-ibuprofen. This theory is consistent with the findings of Jones and co-workers[48], whose studies with r,s-2PPA* in rabbit albumin suggested the possibility of a single saturable site of relatively low affinity (greater for r-2PPA than for s-2PPA) together with a capacity for non-specific binding that was also greater for r-2PPA. In the current work, r-ibuprofen was not increased in the presence of s-ibuprofen as this has never been shown to occur in vivo, however, Jones et al[48] illustrated that the reverse situation does in fact occur with 2PPA.

As the chiral NSAID's have relatively low hepatic extraction ratios, displacement of r - enantiomer from plasma and synovial fluid protein binding sites will lead to an increase in its clearance and volume of distribution[104,105]. Lee et al[33] noted the clearance of r - ibuprofen was greater when administered as part of the racemate, than when administered alone. Their data also suggested that s - ibuprofen was similarly affected by co-administration of the enantiomers. A plausible explanation for these observations may be competitive binding of the r(-) and s(+) enantiomers. Patients with renal dysfunction for example, will have a reduced net clearance of the r - and s - enantiomers to their glucuronides. This would result in an increase in the pharmacologically active s - enantiomer, which in turn may be further increased due to metabolic inversion of the r - enantiomer to its s - counterpart. It is possible therefore, that in these patients, the effects could combine to produce elevated levels of the pharmacologically active species, with the likelihood of increases in both beneficial and unwanted effects[102].

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* enantiomers of r(-) - and s(+) - 2-phenylpropionic acid

6.5.4 **Fatty Acids**

6.5.4.1 Background

Fatty acids interact with different sites on the albumin molecule and have been demonstrated to have a variable influence on drug binding[28,96]. However, these interactions appear particularly prevalent on sites 1 and 2 where most NSAID's locate (Table 1.4). Whilst fatty acid binding has been demonstrated to be remote from these two sites, it has been postulated that long chain fatty acids bind at a location which is very close to site 2 and it is from there that they exert their allosteric effects, thus causing significant variability in terms of the nature and extent of associated drug binding[28,110].

There are numerous pathological, physiological and pharmacological conditions under which free fatty acid concentrations vary as illustrated in Table 6.3:

Table 6.3: FACTORS AFFECTING FATTY ACID CONCENTRATIONS[#]

Factor	**[Fatty Acids]**
Meals	increase
Exercise	increase
Pregnancy	increase
Ethnicity	variable
Myocardial infarct (acute)	increase
Heparin	increase
Caffeine	increase
Rheumatic Disease	increase

adapted from Naranjo and Sellers[28] and Geigy Tables[2]

6.5.4.2 Ibuprofen

The unbound fraction of ibuprofen enantiomers in "stripped" albumin solution (18g/L) was examined. The free fraction of ibuprofen enantiomers increased significantly at concentrations above 20μg/mL of total r,s-ibuprofen, (Fig 5.9). There were insignificant differences in the s:r enantiomer ratios within the ibuprofen concentration range examined. In comparison to an earlier experiment using "unstripped" albumin (Fig 5.3), it was observed that whilst the extent of binding of r-ibuprofen is similar, s-ibuprofen is more highly bound in "stripped" albumin at comparable concentrations of racemic drug. The addition of oleic acid to "stripped" albumin was examined (Table 5.3). At molar ratios of oleic acid : albumin between 3:1 and 4:1, noteworthy increases in free fractions of both enantiomers were observed at all concentrations of ibuprofen examined.

No attempt at fitting parameters of best fit to a model of the interaction between oleic acid binding and combined specific and non-specific ibuprofen enantiomer to albumin was made as the number of parameters involved would be too large to be useful[48]. However, the current observations are in agreement with those of Birkett et al[97] who also noted significant inhibition of binding of similar drugs to albumin at oleic acid : albumin molar ratios of 3:1. Trnavska´ et al[34], (whilst undertaking their binding studies with piroxicam), measured the content of free fatty acids in synovial fluid and plasma of patients with rheumatoid arthritis and noted molar ratios of free fatty acid to albumin were around 0.4 : 1 for synovial fluid and 0.5 : 1 for plasma. They suggested that " in these conditions, inhibition of piroxicam binding by free fatty acids could not occur". These findings do not explain the current studies in which ibuprofen was more highly bound in "stripped" than "non-stripped" albumin, at a given drug concentration. Zona et al[112] examined the influence of several environmental parameters on the binding of indomethacin and indoprofen to HSA.

Their data suggested that "protein - drug binding is dependent upon the conformational status of the protein, and that it is favoured or permitted by relatively loose structure of the HSA". The above discrepancy may be a reflection of minor but significant conformational changes in the albumin "stripping" technique[49,103].

6.5.5 **pH**

6.5.5.1 Background

Whilst normal synovial fluid pH lies within the range 7.31-7.64, it is generally reduced with onset of inflammatory joint disease, presumably in connection with increased lactate concentration[2]. It was therefore considered of interest to examine the effect of piroxicam binding in-vitro, (at drug / albumin / pH conditions typically found in normal and pathological synovial fluid).

6.5.5.2 Study Using Piroxicam

Piroxicam in HSA {(Fraction V - CSL) 18g/L in 0.067M Sorensen's phosphate buffer} was examined over the pH range 7.05-7.49, (typical of what occurs in pathological synovial fluid).

In the current study, the protein binding of piroxicam (2μg/mL), was independent of pH at an albumin concentration of 18g/L, (Table 5.4). This experiment was not repeated using synovial fluid over the same pH range due to practical difficulties in obtaining sufficient drug free samples from patients. Furthermore, there was concern that adjustment of synovial fluid pH to comparable values with exogenous agents may in some way interfere with the fluids' binding properties. However, earlier stability studies in this current work with piroxicam (2μg/mL) in synovial fluid (Table 4.2)

demonstrated insignificant free fractional fluctuation over the pH range : 7.45 - 7.95. Further experiments using synovial fluid pose problems, as alteration of pH in isolation of other physical or environmental factors (such as $ppCO_2$, ppO_2 etc), have the potential to introduce undesirable variation to subsequent free fraction calculations.

CHAPTER 7

OVERVIEW AND FUTURE WORK

7.1 PROTEIN BINDING DETERMINATIONS

In this current work, the Amicon MPS-1 ultrafiltration device provided rapid and consistent separation of unbound NSAID in synovial fluids and HSA solutions. Radiolabelled piroxicam provided a convenient and relatively simple means of investigating the protein binding characteristics of this drug in synovial fluid.A major drawback with using a radiolabelled NSAID as a marker for subsequent binding studies may occur when the specific activity of the radiolabelled material is too low to enable a synovial fluid sample (containing the NSAID) to be "spiked" with radiolabelled drug, without causing a significant alteration to the initial drug concentration in the fluid, prior to determination of the unbound drug fraction. From a practical viewpoint, the above radiolabelling procedure was inappropriate for examination of the binding characteristics of ibuprofen and ketoprofen enantiomers in these laboratories. An assay of sufficient sensitivity and reproducibility was therefore developed to measure the extremely low levels of unbound drug enantiomers expected in these fluids.

The TLC/HPLC assay system established to monitor the radiochemical purity of the radiolabelled ^{3}H piroxicam was relatively simple and rapid, and considered necessary to eliminate potential sources of experimental error in the calculation of free drug fractions. The specific activity of the radiolabelled material was sufficiently high to enable ultrafiltration binding experiments to be performed with small

volumes of prefiltrate (≤ 1mL) within a range of piroxicam concentrations typically found in synovial fluid, (1-7μg/mL). The coefficient of variation of the procedure (using the Amicon MPS-1 ultrafiltration device), was ≤ 9.8%.

The GC/MS assay procedure developed, allowed resolution of ibuprofen enantiomers at concentrations of 2ng/mL with a coefficient of variation of ≤ 10.8% and ≤ 7.4% for s-ibuprofen and r-ibuprofen respectively.

The entire procedure (complete in a matter of minutes), is in marked contrast to the more lengthy methods found in the literature. The assay was readily adaptable to allow resolution of ketoprofen enantiomers. Whilst this ketoprofen assay was left at a relatively early stage of development, it became abundantly clear from the data that with refinement of procedure and GC/MS conditions, measurement of ketoprofen enantiomers at 2ng/mL (or possibly lower) is feasible.

7.2 SUMMARY OF FINDINGS

The clinical pharmacokinetic properties of NSAID's are markedly influenced by the affinity and extent of their protein binding properties in plasma and synovial fluid. Whilst the total level of NSAID is dependent upon concentration and affinity of local protein, it is the unbound / free fraction that is the species which readily diffuses between plasma and synovial fluid. It is therefore not suprising that the free fraction of these drugs at or near the presumed site of action continue to attract much interest. From the outset, a major strategy with the current work was to ensure all parameters of the in-vitro studies were as representative of the situation in-vivo as practically possible (with respect to protein concentration, NSAID concentration and associated environmental conditions of temperature, pH etc). The current studies concentrated on the influence of several physical factors thought to influence the in-vitro protein

binding of piroxicam and ibuprofen in synovial fluid and/or HSA solutions(at albumin concentrations typically found in normal or pathological synovial fluid). The free fractions of piroxicam and ibuprofen enantiomers were found to be markedly dependent upon their respective drug and associated albumin concentrations in synovial fluid according to pre-determined physical conditions. One should exercise caution with interpretation of binding constants determined from experimental data as most of the commonly calculated values are based on saturation functions such as those described by Scatchard and Klotz[83].Consideration should also be given to alternative mathematical functions when interpreting the results of non-saturated binding studies[83].

Binding of piroxicam was found to be independent of pH at concentrations of drug and albumin typically found in synovial fluid, whilst similar studies (albeit a small population sample over a somewhat higher range of pH) in samples of pathological synovial fluids also supported this finding.

7.3 FUTURE ISSUES

7.3.1 Determinants of NSAID Free Fraction

Clearly current and previous studies leave many questions unanswered. Physical and environmental factors play primary roles with regard to the binding affinities of NSAID's in synovial fluid and plasma. Other factors such as ppO_2, $ppCO_2$, temperature and other biochemical/serological functions should be examined.These factors should be examined not only in isolation, but also as a composite mix, (as expected in-vivo). However, there are numerous practical problems to be overcome in designing such studies. For example, partial pressures of synovial fluid gases

would be expected to change rapidly following collection from a patient, and these shifts may cause difficulties with protein binding experiments monitoring one variable at a time.

7.3.2 Can NSAID Levels be Correlated with Clinical Response?

Despite isolated reports[106] of a positive relationship between plasma NSAID concentration and therapeutic effect, the majority of studies have been disappointing. As synovial fluid is close to the target tissue where a NSAID's effect is exerted, it is reasonable to expect its concentration in this fluid to be a better indicator of drug activity in rheumatic disease than in plasma. However, before an attempt to correlate synovial fluid NSAID concentration with clinical response can be made, further characterisation of the protein binding of the whole class of these drugs in synovial fluid may be beneficial[70].

Furthermore, it is essential to examine the free levels of active enantiomer of chiral NSAID's in synovial fluid, because interindividual differences in metabolic inversion could lead to erroneous results if enantiospecific differentiation were ignored in the assay procedures. Despite the fact that the enantiomers of the 2-arylpropionic acid NSAID's differ in pharmacological properties, we are still witnessing articles in the literature that generate data of a non stereospecific nature[98,113]. In addition, such studies would require consideration of individual differences in NSAID disposition of both drug and its active metabolites.

APPENDIX

APPENDIX

1. SELECTION OF PATIENTS

1.1 Inclusion Criteria

Male or female adult patients with effusions into the knee of either mechanical or inflammatory origin.

1.2 Exclusion Criteria

- Patients with severe renal or hepatic disease.
- Manifest cardiac insufficiency or severe hypertension.
- Patients under treatment with oral anticoagulants.
- Patients under treatment with corticosteroids.
- Malabsorption syndrome or any other g.i. condition likely to affect drug absorption.
- Pregnant women or women actively seeking pregnancy.
- Concomittant NSAID therapy.
- Concomittant therapy with Salazopyrin.
- Treatment with aspirin or any other NSAID within 48 hours (or 6 drug half-lives : whichever is the greater), prior to recruitment.
- Patients with hepatitis or HIV antibodies.

2. PROCEDURE

2.1 The collection of such samples will be via retrieval of excess synovial fluid remaining after routine non-trial related patient care.This procedure will eliminate unnecessary and invasive sampling of body fluids.

2.2 The volume of synovial fluid collected will vary with the extent of the effusion, and shall be an amount as deemed appropriate to normal patient care.

3. METHOD

Synovial fluid samples will be collected according to the method of Day et al* and stored at 4°C until assayed.

4. CONCOMITANT MEDICATIONS / THERAPY

- Any other non-steroidal anti-inflammatory agent is strictly prohibited for 48 hours or six drug half-lives, (whichever is the greater) prior to sample collection.
- Paracetamol will be permitted as an analgesic, if required.
- Patients on chronic therapy with specified drugs considered not to interfere with NSAID protein binding may be included.

5. ANALYSIS OF DATA

The data will be analysed by Pharmacy School, University of Tasmania (D. Jack / R. Rumble), and a report generated, suitable for publication.

6. FUNDING

This study is totally funded by the School of Pharmacy / University of Tasmania and external grants. No costs to the hospital or institution will be incurred.

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* Day RO, Williams RM, Lee EJD, Knihinicki RD and Graham GG. The Stereoselective Disposition of the Enantiomers of Ibuprofen in Synovial Fluid. Agents Actions[Suppl] 1985;17.

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