Pharmacokinetics and pharmacodynamics of pentoxifylline and metabolites

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Pharmacokinetics and pharmacodynamics of pentoxifylline and metabolites in humans

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The thesis is based on the following papers, referred to by their Roman numerals:

I. Stereoselective metabolism of pentoxifylline in vitro and in vivo in humans. 
   **Nicklasson, M.**, Björkman, S., Roth, B., Jönsson, M., Höglund, P. *Chirality*. 
   2002;14:643-652

II. A placebo-controlled study of retinal blood flow changes by pentoxifylline and 
    metabolites in humans. **Magnusson, M.**, Bergstrand, I.C., Björkman, S., Heijl, A., 

III. Effects of pentoxifyllin and its metabolites on platelet aggregation in whole blood 
     from healthy humans. **Magnusson, M.**, Gunnarsson M., Berntorp E., Björkman S., 

IV. Pentoxifylline and vitamin E treatment for prevention of radiation-induced side 
    effects in women with breast cancer: a phase two, double-blind, placebo-controlled, 
    randomised clinical trial (Ptx-5). **Magnusson, M.**, Höglund P., Johansson K., Jönsson 
    C., Killander F., Malmström P., Weddig A., Kjellén E. *Manuscript*
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>b.i.d</td>
<td>Twice a day</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CL</td>
<td>Clearance</td>
</tr>
<tr>
<td>CL\textsubscript{app}</td>
<td>Apparent clearance</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>Maximum concentration</td>
</tr>
<tr>
<td>$C_{\text{min}}$</td>
<td>Minimum concentration</td>
</tr>
<tr>
<td>$C_{\text{ss}}$</td>
<td>Mean concentration at steady state</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LENT-SOMA</td>
<td>Late Effects Normal Tissue; Subjective, Objective, Management Analytic</td>
</tr>
<tr>
<td>ptx</td>
<td>Pentoxifylline</td>
</tr>
<tr>
<td>RIF</td>
<td>Radiation induced fibrosis</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>Elimination half-life</td>
</tr>
<tr>
<td>TGF$\beta$1</td>
<td>Transforming growth factor beta 1</td>
</tr>
<tr>
<td>t.i.d</td>
<td>Three times a day</td>
</tr>
<tr>
<td>VAS</td>
<td>Visual Analogue Scale</td>
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INTRODUCTION

In this thesis the pharmacokinetics and pharmacodynamics of pentoxifylline have been studied. Pharmacokinetics describes how a drug is absorbed, distributed, metabolised in and eliminated from the body. Pharmacodynamics describes the relationship between the drug concentration and effect. Pentoxifylline is an interesting drug to study since it exhibits complex pharmacokinetics with both reversible metabolism, and active metabolites. Difficulties in finding consistent clinical effects of pentoxifylline may be due to the drug acting at least in part through formation of active metabolites, the rate and extent of which may vary between individuals.

1. Pharmacokinetics of pentoxifylline

When the biotransformation of pentoxifylline was studied in man, seven phase 1 metabolites (denoted M1-M7) were identified in human urine (Hinze, 1972b). The structures of the metabolites were determined: the biotransformation yields three hydroxy metabolites (M1, M2, M3a, and M3b), two carboxy metabolites (M4, M5) and two demethylated metabolites (M6, M7), figure 1. The major metabolite excreted in urine is M5 followed by M4. The excretion of unchanged pentoxifylline and M1 each accounts for less than 1% of the dose (Hinze, 1972a). When pentoxifylline is administered to healthy humans the areas under the plasma concentration curves (AUC) for M5 and M1, but not M4 are larger than pentoxifyllines (Beermann et al., 1985; Bryce et al., 1989, Smith et al., 1986).
**Figure 1:** The structural formulae of pentoxifylline and its metabolites, R-M1, S-M1, M2, M3, M4, M5, M6 and M7.
Studies have shown that clearance (CL) for pentoxifylline was much higher than hepatic blood flow and higher than, or in the same level as, cardiac output (Ings et al., 1982; Rames et al., 1990). Since the metabolism of pentoxifylline to M1 is reversible the clearance is further underestimated, as the AUC is increased due to continuous addition of pentoxifylline formed from M1 (therefore the term CL<sub>apparent</sub> is used in this these studies). The high clearance indicates that pentoxifylline is metabolised also at other sites than the liver, such as blood, since CL is not limited by hepatic blood flow. Patients with impairment of liver function due to cirrhosis had significantly increased AUCs for pentoxifylline and M1 compared with healthy volunteers but the AUC ratio for Pentoxifylline/M1 was the same in the two groups (Rames et al., 1990). Studies have shown that pentoxifylline is metabolised to M1 when incubated in whole blood (Bryce et al., 1980; Ings et al., 1982). Taken together this suggests that erythrocytes are the major site for pentoxifylline - M1 interconversion.

Metabolites M1 and M5 of pentoxifylline have significant haemorheologic effects but M2, M3, M4, M6 and M7 had little haemorrheologic effect (Ambrus et al., 1995). Consequently, the present thesis focuses on the active metabolites M1 and M5 and the M4 metabolite due to the pharmacokinetic data.

The hydroxy metabolite M1 is formed by reduction of pentoxifylline, the reaction is rapid and reversible (Lee et al., 1997). It takes place both in the liver (Lillibridge et al., 1996) and the erythrocytes (Bryce et al., 1989; Ings et al., 1982; Poirier et al., 1989). M1 has a carbon atom (marked *) that carries four different substituents and is therefore a chiral compound. A chiral molecule has two non-superimposable mirror forms called enantiomers that are as one's left and right hands to each other: "the same" but opposite. The R (rectus=right) and S (sinister=left) nomenclature refer to the absolute configuration based on atomic number of the substituents. Different enantiomers of chiral compounds can have different effects as drugs. The enantiomers of M1 are shown in figure 1.

The R – enantiomer of M1 was manufactured as a drug in its own right, lisofylline. Lisofylline has been tested in several clinical trials with varying results. A study in healthy volunteers showed that lisofylline causes a prolonged and marked decrease in the levels of circulating free fatty acids but these effects were not seen when lisofylline was administered to patients with acute lung injury or acute respiratory distress syndrome (ARDS-Network, 2002; Bursten et al., 1998) Lisofylline 3mg/kg t.i.d. (but not 2mg/kg) reduced the incidence of infections and
improved 100-day survival in patients receiving related-donor allogeneic bone marrow transplantation; but no effects were seen on time to neutrophil recovery and platelet recovery (List et al., 2000). Co-administration with lisofylline did not decrease the toxicities of high dose i.v. IL-2 (Margolin et al., 1997). Recently, a new therapeutic use for lisofylline was proposed, suggesting that lisofylline can prevent autoimmune disorders including type 1 diabetes (Yang et al., 2005). However, despite many clinical trials lisofylline is still in the experimental and development stages and has not been approved for sale due to limitations in its therapeutic efficacy.

It is unclear how much R-M1 is formed after administration of pentoxifylline. Limited in vitro data indicates that R-M1 is formed to a much lesser extent than S-M1 in humans (Lillibridge et al., 1996). The stereoselective formation of M1 has only been studied in vitro in human liver microsomes and in a few cancer patients who received pentoxifylline together with ciprofloxacin and interleukin-2 (Thompson et al., 1994). There is a pharmacokinetic interaction between ciprofloxacin and methylxanthines that may influence the formation of M1 (Fuhr et al., 1992). A recent study showed that the mechanism behind the interaction is inhibition of CYP1A2 and that it seems likely that CYP1A2 catalyses xanthine 7-demethylation of pentoxifylline to M6 and M1 to M7 (Peterson et al., 2004; Raoul et al., 2007).

Since the pharmacokinetics for the formation of the stereoisomers of M1 has only been partly investigated this prompted us to perform paper I. The aims in paper I were to study the kinetics of the reversible transformation of pentoxifylline to R-M1 and S-M1 in erythrocyte suspension and also to quantify the formation of the enantiomers of M1, as well as M4 and M5 after intravenous and oral administration to healthy volunteers.

2. Therapeutics and pharmacodynamics

A) Blood flow

Pentoxifylline was developed by Hoechst Aktiengesellschaft in the seventies. Pentoxifylline obtained marketing authorisation in Germany 1972 and in the USA 1984 for the treatment of intermittent claudication on the basis of chronic occlusive arterial disease of the limbs. Pentoxifylline has never been granted authorisation in Sweden but it is possible to prescribe and obtain permission for its use on a named patient bases from the Medical Product Agency.
Pentoxifylline has been classified as a haemorheological drug and a phosphodiesterase inhibitor. Studies have shown that pentoxifylline improves peripheral blood flow. The mechanisms for this are several. Blood flow in the capillaries and blood viscosity are influenced by red cell deformability and aggregation, haematocrit, and plasma viscosity. Pentoxifylline significantly reduced whole blood viscosity in patients with peripheral arterial disorders, significantly increased red cell deformability in healthy subjects and patients with peripheral vascular disease (Samlaska et al., 1994; Ward et al., 1987). Pentoxifylline decreased platelet adhesion and aggregation to vessel walls in patients with peripheral vascular disorder and decreased fibrinogen levels. In blood from healthy volunteers pentoxifylline increased the filterability of monocytes and polymorphonuclear leucocytes. (Ward et al., 1987).

After incubation with pentoxifylline both mononuclear and polynuclear cells increase intracellular cyclic adenosine monophosphate (cAMP) levels through inhibition of phosphodiesterase (Bessler et al., 1986). Phosphodiesterase catalyzes the hydrolysis of cAMP to adenosine monophosphate (5’-AMP); inhibition of this enzyme leads to elevated levels of cAMP. Increased cAMP contents of platelets interfere with platelet aggregation and leads to inhibition of cyclooxygenase (Sha et al., 2003). Further, elevated intracellular cAMP levels inhibit cytokine production through inhibition of activation of monocytes and lymphocytes.

The exact mechanism of how pentoxifylline alters red blood cell physiology is not entirely understood but might be a reduction of intracellular calcium due to inhibition of calcium influx. It is known that older erythrocytes accumulate more Ca\(^{2+}\) and are less deformable. Transglutaminase is a Ca\(^{2+}\) dependent enzyme that irreversibly crosslinks membrane proteins, and thereby rigidifies the erythrocytes. Pentoxifylline can reduce intracellular Ca\(^{2+}\) and inhibited activation of Ca\(^{2+}\) dependent transglutaminases (Swislocki et al., 1989; Swislocki et al., 1991). This enables the erythrocytes to remain deformable and makes it easier for them to pass through the capillaries. However, exactly how pentoxifylline alters red blood cells is not completely understood. Another study showed that pentoxifylline improved whole blood filterability in healthy volunteers, but the investigators did not find any increased red cell deformability (Cummings et al., 1992).

At least 630 clinical trials with pentoxifylline have been performed in order to investigate the effects on different indications e.g. – intermittent claudication, vascular dementia, venous leg
ulcers, bone marrow transplant, cerebrovascular disease, diabetes mellitus, endometriosis, male infertility and many more (Cochrane, 2009; Martindale, 2009). Most of the studies are small and the magnitude of effects varies. The only approved indication for pentoxifylline is intermittent claudication. Below a short summary will follow for the most common therapeutic usages of pentoxifylline.

Intermittent claudication develops during exercise, such as walking, when the flow to the lower limb is insufficient to meet the needs of the exercising muscle. The patient experience muscle pain that disappears when resting. The reduced blood flow is often caused by atherosclerosis. Intermittent claudication is often associated with coronary heart disease and morbidity and mortality is raised in patients with intermittent claudication. The risk factors for developing intermittent claudication are the same as coronary heart disease: hypertension, diabetes, smoking, and high cholesterol levels. Intermittent claudication reduces the patients’ mobility due to pain when walking, but at the same time the walking exercise together with smoking cessation are the primary treatment (LB, 2007-2008; Watson et al., 2008). Pentoxifylline has been used in the treatment of intermittent claudication for a long time due to its haemorheologic properties (Ernst et al., 1992; Moher et al., 2000; Porter et al., 1982; Reich et al., 1987).

In a meta-analysis all randomised, placebo-controlled, double blind clinical trials on the indication intermittent claudication were reviewed: 52 studies met the inclusion criteria of the meta-analysis and pentoxifylline were studied in 17 of these, including 1041 patients (Moher et al., 2000). The meta-analysis showed that, after 24 weeks treatment (but not 8 weeks), pentoxifylline was more effective than placebo and the other drugs for the primary effect parameter, maximum walking distance. The other primary effect parameter was pain free walking distance: pentoxifylline seems better than the other medications here as well, but this was not significant. Mortality was studied in seven of the studies including 479 patients, conclusions regarding mortality could not be made from this, only one patient in the treatment group and two in the placebo group died. The authors conclude that pentoxifylline therapy may be efficacious in improving the walking capacity, although its treatment effect is modest (Moher et al., 2000).

Patients with vascular dementia have reduced cerebrovascular perfusion. Pentoxifylline has been tested in clinical trials and used for treatment of vascular dementia (Black et al., 1992; Hartmann, 1985), its mechanism of action in vascular dementia was said to be due to reduced blood
viscosity and inhibition of the production of pro-inflammatory cytokines (Sha et al., 2003). A systematic review was performed in order to evaluate the use of pentoxifylline in patients with vascular dementia (Sha et al., 2003). Only four studies including 469 patients met their inclusion criteria i.e. randomised, double blinded, and placebo controlled. None of the four studies showed a significant improvement in the pentoxifylline group compared with the placebo group, but a trend towards improvement could be noticed. A subgroup analysis was done in three of the studies, using a stricter definition for vascular dementia. The subgroup analysis showed significant improvement or decreased impairment in the pentoxifylline group compared with the placebo group (Sha et al., 2003). The authors conclude that pentoxifylline has a potential role in treatment of vascular dementia, but that few studies met the quality criteria for inclusion in the review and that new larger well contained clinical trials are needed to confirm the results.

When a patient has chronic venous hypertension in the tissues of the lower leg, this leads to a chronic inflammatory condition and a leukocyte activation and may eventually result in venous leg ulcer (Pascarella et al., 2005). Pentoxifylline has been tested in clinical trials for treatment of venous leg ulcer (Barbarino, 1992; Dale et al., 1999; Falanga et al., 1999). A Cochrane review was performed in order to investigate the effects of pentoxifylline for treating venous leg ulcer: 11 randomised, placebo controlled clinical trials including 841 patients met the inclusion criteria (Jull et al., 2007). A random effect model was used to combine the results from the studies and it was found that treatment with pentoxifylline leads to significant improvement or complete healing more often compared with placebo (RR 1.70, 95% CI 1.30-2.24) (Jull et al., 2007).

Pentoxifylline has been tested and used in the treatment of microalbuminuria (Navarro et al., 2005; Rodriguez-Moran et al., 2005). A systematic review on all studies with patients with kidney disease and pentoxifylline treatment where done: 10 randomised controlled clinical trials were found including 476 patients (McCormick et al., 2008). The effect parameter was change in proteinuria. Proteinuria increases the risk for cardiovascular mortality and morbidity and end stage renal disease. By decreasing proteinuria the cardiovascular mortality and morbidity decreases and the progression of chronic kidney diseases is delayed. The meta-analysis showed that pentoxifylline significantly decreased proteinuria compared with placebo or standard care (McCormick et al., 2008). The most likely explanation for the antiproteinuric effect of pentoxifylline was said to be inhibition of the production of proinflammatory cytokines. The authors conclude that pentoxifylline seems to decrease proteinuria in patients with chronic
kidney diseases, but that the studies were small and few and that larger high quality studies with mortality and morbidity as endpoint parameters are needed to verify this.

It has been suggested that pentoxifylline may be used in diseases affecting retinal blood flow, such as diabetic retinopathy (Schmetterer et al., 1996; Sebag et al., 1994; Sonkin et al., 1993a; Sonkin et al., 1993b) or macular degeneration (Kruger et al., 1998), but a recent Cochrane review on diabetic retinopathy (Lopes de Jesus et al., 2008) stated that no conclusions could be drawn due to lack of large randomised controlled clinical trials.

Pentoxifylline was generally well tolerated in all studies with few undesirable effects, nausea being the most common one. Few patients discontinue treatment with pentoxifylline due to side effects.

Measurement of blood flow is difficult since the capillaries are “hidden” in the body. Retinal blood flow is an exception since it can be measured through the eye using a validated method quantifying all the flows in a selected temporal area of the retina (Michelson et al., 1995; Michelson et al., 1998). The aim of paper II was to investigate the possible contribution of the metabolites (R-M1, S-M1, M4, and M5) of pentoxifylline to its effect, assessed as retinal blood flow, in healthy humans.

Since pentoxifylline inhibits platelet aggregation we used platelet aggregation in whole blood as effect parameter in paper III (Ambrus et al., 1995; de la Cruz et al., 1993). The aim in paper III was to investigate the relative potencies of pentoxifylline and metabolite R-M1, S-M1, M4 and M5 to inhibit platelet aggregation in whole blood, and in particular to clarify contributions of the two enantiomers of M1, to this effect.

B) Fibrosis

More recent studies have shown that pentoxifylline in combination with vitamin E can reduce radiation induced fibrosis (RIF) (Chiao et al., 2005; Delanian, 1998; Delanian et al., 1999; Delanian et al., 2003; Lefaix et al., 1999; Okunieff et al., 2004). Previously, RIF was considered irreversible. But in a double-blind, placebo-controlled study in women previously treated with radiotherapy for breast cancer, co-administration of pentoxifylline and vitamin E reduced the mean area of RIF up to 60% compared with placebo or single treatment with pentoxifylline or
vitamin E (Delanian *et al.*, 2003). RIF develops months to years after radiotherapy and constitutes of local and unavoidable damage to normal tissue. The development is spontaneous and is characterised by a gradual stepwise aggravation over several years resulting in irreversible sequelae.

According to Delanian and colleges (Delanian *et al.*, 2004) the “fibrotic process can be divided in three steps: a) an initial pre-fibrotic phase that last for the first few months after radiotherapy and is often asymptomatic but may be marked by signs of a specific chronic local inflammation; b) a constitutive phase of organised fibrotic sequelae, during the first few years after radiotherapy, in which the local inflammation signs have disappeared and the tissues have thickened and hardened c) a phase of late fibroatrophy that lasts for 5-30 years after radiotherapy, with retractile atrophy and concomitant gradual destruction of the normal tissues included in the irradiated volume”.

After radiation a co-ordinated cellular response occurs which involves interaction of cytokines with their receptors and the extracellular matrix (ECM). When fibrosis develops, imbalance in cytokine response occurs and leads to over production and accumulation of ECM in tissues (O'Sullivan *et al.*, 2003). The first signs of radiation injury usually are loss of tissue elasticity followed by mild induration. Worse degree of injury involves significant induration with rigidity of the surface layers and of surface contours generally related to fibrosis of the dermis and subcutaneous tissue. Anatomic regions usually affected are the breast, the head and neck and the connective tissue.

Transforming growth factor beta-1 (TGF\(\beta\)1) is considered to be the main cytokine involved in the RIF’s development in vivo, it seems to be responsible for the initiation, development and persistence of fibrosis. Circulating TGF\(\beta\)1 during the constitutive and chronic fibrous phases may contribute to the self-perpetuation of the fibrotic process (Delanian *et al.*, 2004). Breast cancer patients with high pre-radiation plasma levels of TGF\(\beta\)1 had a higher risk of developing fibrosis after radiotherapy (Li *et al.*, 1999). CD105 is a specific vascular membrane glycoprotein with high affinity binding TGF\(\beta\)1, CD-105 diminish TGF\(\beta\) signal transduction. Li *et al* also showed that soluble CD-105 binds TGF\(\beta\)1 and forms circulating CD105- TGF\(\beta\)1 complexes. Patients that developed fibrosis had significantly lower CD105-TGF \(\beta\)1 levels. By forming CD-105 - TGF\(\beta\)1 complexes, CD-105 seems to inactivate the over produced TGF\(\beta\)1 and therby diminish the potential of developing tissue fibrosis after radiotherapy (Li *et al.*, 1999). Variability
in incidence and severity of fibrosis after radiotherapy can partly be explained by TGFβ1 gene polymorphism. Patients with -509 polymorphic allele can partly be predisposed to severe fibrosis due to over expression of TGFβ1. (Giotopoulos et al., 2007; Quarmby et al., 2003).

Pentoxifylline appears to stimulate prostacyclin release from normal endothelial cells to inhibit some of the cytokine cascade resulting from tissue injury, and it indirectly inhibits the production of thromboxane, a potent vasoconstrictor and a strong stimulator of platelet aggregation. In vitro studies have indicated that pentoxifylline inhibits human dermal fibroblast proliferation and extracellular matrix production and increases collagenase activity (Berman et al., 1989; Berman et al., 1990).

During inflammatory reactions and RIF development reactive oxygen species, such as singlet oxygen, superoxide anion hydrogen peroxide, and hydroxyl radicals are generated (Delanian et al., 2004). If they are not scavenged efficiently, oxidative stress may lead to cell necrosis or apoptosis. Vitamin E is used due to its antioxidant properties; it protects membrane phospholipids from oxidative damage. Vitamin E deficiency has been associated with abnormal connective tissue repair, resulting in the formation of scar like tissue.

The first report that the combination of pentoxifylline and vitamin E can reduce RIF was published in 1999. It was shown that pentoxifylline/vitamin E treated pigs had a histopathologic normalisation of the subcutaneous tissues surrounding a small residual scar, as well as a large reduction in the immunohistochemical expression for the TGFβ1 (Lefaix et al., 1999). Since then more studies have shown that therapy with pentoxifylline and vitamin E can decrease or even reverse RIF in humans (Chiao et al., 2005; Delanian et al., 1999; Delanian et al., 2003). Still it would be even better if the fibrosis could be prevented. We hypothesised that treatment with pentoxifylline and vitamin E could prevent or reduce the development of fibrotic tissue. To determine the preventive effects of pentoxifylline and vitamin E against a range of radiation-induced side effects, a randomised, double-blind, placebo-controlled clinical trial was planned (paper IV). The primary aim in paper IV was to investigate if pentoxifylline + vitamin E prevent radiation induced side effects measured as impaired shoulder mobility in women treated for breast cancer with radiotherapy to the axilla and breast.
AIMS

- To investigate the reversible transformation of pentoxifylline to R-M1 and S-M1 in erythrocyte suspension (Paper I)

- To investigate the pharmacokinetics of pentoxifylline and some important metabolites after administration of pentoxifylline to healthy volunteers (Paper I, II) and breast cancer patients (Paper IV)

- To investigate the effects of pentoxifylline and metabolites R-M1, S-M1, M4 and M5 on retinal blood flow in healthy humans after intravenous infusions of pentoxifylline (Paper II)

- To investigate the relative potencies of pentoxifylline and metabolites R-M1, S-M1, M4 and M5 to inhibit platelet aggregation in whole blood, and in particular to clarify contributions of the two enantiomers of M1, to this effect (Paper III)

- To investigate if the combination of pentoxifylline and vitamin E can prevent radiation induced side effects in women with breast cancer (Paper IV)
MATERIAL AND METHODS

The aim of this section is to highlight some of the most important methods used in this thesis. Detailed descriptions of the material and methods used are given in the papers.

Methods for analysis of pentoxifylline and metabolites

HPLC method for determination of pentoxifylline, R-M1 and S- M1 (Paper I, II, IV)
The concentrations of pentoxifylline and the enantiomers of M1 were determined by reversed-phase HPLC after chiral derivatisation of the metabolite.\textsuperscript{12} Thalidomide was used as internal standard. The sample was extracted with dichloromethane and diacetyl-L-tartaric acid anhydride solution was used for derivatisation. A LiChrosorb RP-18, 250×4 mm, 7 μm particle size column was used with 17% acetonitrile in 0.05 M NaH\textsubscript{2}PO\textsubscript{4} buffer, pH 4.0. as mobile phase. The detection wavelength was 274 nm.

HPLC method for determination of M4 and M5 (Paper I-II)
For the determination of metabolites 4 and 5, enprofylline was used as internal standard. The sample was extracted with ethyl acetate. A RP-18 column was used with 13% acetonitrile in 0.005 M NaH\textsubscript{2}PO\textsubscript{4} buffer, pH 3.6 as mobile phase and the detection wavelength was 274 nm.

Paper I

Interconversion of pentoxifylline, R-M1 and S-M1 by erythrocytes
The reductive metabolism of pentoxifylline to R-M1 and S-M1 and the oxidative metabolism of R-M1 and S-M1 to pentoxifylline were investigated in haemolysed erythrocyte suspension from healthy humans.

For the reductive metabolism of pentoxifylline to R-M1 and S-M1, NADPH and Mg\textsuperscript{2+} were added to the aliquots of haemolysed erythrocytes. The incubations were started by the addition of pentoxifylline in the respective samples. The mixtures were incubated for 20 min at 37°C.
For the oxidative metabolism of R-M1 and S-M1 to pentoxifylline, NADP and Mg\(^{2+}\) were added to the aliquots of haemolysed erythrocytes. The incubations were started by the addition of R-M1 or S-M1 respectively. The samples were incubated for 20 min at 37°C.

NONMEM version V (The NONMEM project group, San Francisco, CA, USA) was used to determine the Michaelis – Menten parameters, \(V_{\text{max}}\) and \(K_m\) of the enzymes involved in the metabolism of pentoxifylline to R-M1 and S-M1. Two models were used, representing the action of either one or two enzymes:

\[
V = \frac{V_{\text{max}} \times [C]}{K_m + [C]} \quad V = \frac{V_{\text{max}(1)} \times [C]}{K_m(1) + [C]} + \frac{V_{\text{max}(2)} \times [C]}{K_m(2) + [C]}
\]

In these equations, \(V\) is the measured rate of metabolite formation in nmoles/min and \([C]\) is the concentration of substrate (mM). From the obtained values of \(V_{\text{max}}\) and \(K_m\) for the various enzymes, the rates of conversion of pentoxifylline to R-M1 and S-M1 at a therapeutic blood concentration were calculated and compared.

**Pharmacokinetics in humans**

The study was performed according to the declaration of Helsinki and approved by the Ethics Committee of Lund University and by the Swedish Medical Products Agency. After giving written informed consent six healthy, non-smoking volunteers were included in the study. They received, in a randomised cross-over design, three doses of pentoxifylline with washout periods of at least one week in between. The three doses were: 300 mg and 600 mg as intravenous infusion and a 600 mg controlled release tablet (Trental®). Blood was sampled from an indwelling venous catheter before start of infusion, during infusion and until 6 h after termination; for oral administration before and until 25 h after intake of the tablet.

**Paper II**

**Study design**

The study was performed according to the declaration of Helsinki and approved by the Ethics Committee of Lund University and by the Swedish Medical Products Agency. After giving written informed consent eight healthy, non-smoking volunteers were included in the study. Each
subject passed a pre-study ophthalmic examination. The study was randomized, placebo controlled, observer-blinded, and partly blinded for the subject in a four period cross-over design.

The subjects were given the four treatments in random order. During one session they were given placebo (0.9% saline solution) as intravenous infusion. During the three other sessions they were given pentoxifylline by intravenous infusion. In one session the subjects were pre-treated with ciprofloxacin and in another with rifampicin. The pre-treatments with ciprofloxacin and rifampicin could not be blinded but the pentoxifylline and placebo administrations were.

**Measurement and analysis of retinal blood flow**

Retinal blood flow was assessed using the Scanning Laser Doppler Flowmetry (SLDF) (Heidelberg Retina Flowmeter, Heidelberg Engineering) (Michelson et al., 1995). The method of SLDF provides a high definition tomographic image of perfused retinal vessels with simultaneous evaluation of blood flow using an optical Doppler effect. The measurements were performed in a selected area (2.7x0.7mm) of the central temporal retina, at baseline, during the infusion period and up to 5 hours after termination.

Quantification of capillary retinal blood flow was stated in arbitrary units (AUs) describing the product of mean flow velocity and mean amount of blood cells in a standardised volume. The mean values from each time point have been used in the calculations.

**Data presentation and statistical considerations**

Two main statistical models were tested after an initial check for lack of period effects. The first one comprised only the treatments given as fixed effects, thus disregarding the obtained plasma concentration data. In the second model treatments were not included; instead the AUCs of pentoxifylline and its metabolites were used as regressor variables. Such a model can only be successfully used if the correlations between the regressors are low. All models used subjects as random effects, and a repeated measures spatial exponential covariance structure design.
Paper III

Platelet aggregation

The study was performed according to the declaration of Helsinki and approved by the Ethics committee of Lund University. The subjects were informed about the study and gave oral informed consent prior to blood sampling. Measurement of platelet aggregation were done in whole blood from 8 healthy volunteers by impedance technology (electrical resistance between two electrodes immersed in whole blood), using a whole blood lumi-aggregometer, (Chrono log modell 560 Ca, Chrono Log Corp, Havertown, PA, USA) (Podczasy, 1997; Vucenik, 1998).

Blood was sampled at two occasions from each subject by venepuncture. At one occasion pentoxifylline, rac-M1, M4 and M5 were investigated and on another occasion rac-M1 and R-M1.

Platelet aggregation was studied in whole blood diluted in a 1:1 ratio with 0.9 % saline, the samples were incubated in 37°C prior to testing. The test procedure was started by adding luciferin luciferase to the sample; the samples were incubated in 37 °C for 1 minute prior to addition of the test substances. Pentoxifylline, rac-M1, R-M1, M4, M5 or saline (positive control) was added to the sample and incubated in 37°C for 1 minute prior to addition of Adenosindiphosphate (ADP), aggregation was monitored for 6 minutes. The effects of S-M1 were calculated from a comparison between R-M1 and rac-M1 since we did not have access to pure S-M1.

Paper IV

Study design

The study was a randomised, placebo controlled for pentoxifylline, double blinded clinical trial with a parallel study design. Randomisation was stratified for previous use of chemotherapy. The study was conducted at Lund University Hospital, Department of Oncology in accordance with the principles of Good Clinical Practice (GCP) and the ethical principles stated in the current revision of the declaration of Helsinki. The study was approved by the Ethics Committee of Lund University and by the Swedish Medical Products Agency. The trial is registered on the ISRCTN.org website, number ISRCTN39143623.
Patients
Patients fulfilling the following criteria were included in the study: primary breast cancer, axillary dissection, mastectomy or segmental resection of the breast, radiotherapy to the breast/thoracic wall, and axilla and fossa supra/infra calvicularis. The inclusion period was one to three months after termination of radiotherapy. Exclusion criteria were; known sensitivity to pentoxifylline or vitamin E, disorders related to muscles or joints, treatment with corticosteroids during the radiotherapy treatment.

Treatments
After written informed consent and baseline assessments were obtained, patients were randomly assigned on a 1:1 basis, with stratification for chemotherapy, to pentoxifylline 400 mg (Pentoxifylline Ratiopharm®, extended-release tablet) 3 times daily or a matching placebo. All patients were treated with vitamin E 100 mg 3 times daily. The duration of treatment was 12 months.

Safety assessment
Patients were assessed for breast cancer recurrence and survival as a measurement of drug safety. At each visit patients were asked if they had experienced any change in health status. Adverse events and serious adverse events were classified and reported according to the ICH GCP guidelines.

Efficacy assessment
The endpoints consisted of change in the passive abduction of the shoulder, and changes in arm volume, Late Effects on Normal Tissue; Subjective, Objective, Management and Analytic, breast score (LENT-SOMA) as assessed by the patients’ physicians (Rubin et al., 1995); and patients’ subjective assessment of somatic sensations and discomfort during the last week as measured by Visual Analogue Scale (VAS) (Scott et al., 1976). Abduction of the shoulder was measured using a goniometer. Arm volume was measured by the water displacement method; the volumes were presented as percentage difference between arms (Bednarczyk et al., 1992; Kettle et al., 1958). Blood samples were collected by venipuncture into vacuum collection tubes containing sodium heparin. Plasma concentrations of pentoxifylline, R-M1 and S-M1, were determined by HPLC.
Statistical Methods
Continuous variables are presented as medians and quartiles. Categorical are presented as frequencies. Abduction, percentage difference in arm volumes, LENT-SOMA score and VAS observations were regarded as ordinal data. A two-way method developed for repeated measures of ordinal scale data (Shah et al., 2004) using SAS statistical software version 8.2 (SAS, Cary, NC, USA) was used to elucidate differences between treatment over time and the time by treatment interaction in the effect data. Statistical significance was defined as two tail p < 0.05.
RESULTS & DISCUSSION

Papers I and IV

Pharmacokinetics in humans

In paper I pentoxifylline was administered intravenously and orally to healthy volunteers. Plasma concentration curves of pentoxifylline and its metabolites after oral administration of pentoxifylline are shown in figures 2 and 3 and pharmacokinetic parameters of pentoxifylline and its metabolites after oral and intravenous administration of pentoxifylline are shown in table 1. The AUCs for S-M1 and M5 were larger than for pentoxifylline and the AUCs for M4 and R-M1 were lower. The AUCs for M4 and M5 are in agreement with previous observations but the AUCs for R-M1 and S-M1 have not been studied previously (Beermann et al., 1985; Bryce et al., 1989; Smith et al., 1986). The plasma concentrations of all metabolites declined in parallel with pentoxifylline, indicating formation rate-limited disposition after intravenous administration of pentoxifylline.

The AUC ratios of each metabolite to pentoxifylline were compared after oral and i.v. administration. The AUC ratios of M4 and M5, but not S-M1 (or total M1), are higher after oral administration. Enhanced formation of M4 and M5 but not M1 during the first pass metabolism of orally administered pentoxifylline indicates difference in sites of the two different metabolic pathways. The slight difference in AUC ratio for R-M1 after oral and i.v. administration however show some influence of first-pass metabolism, possibly suggesting a contribution of the liver to the formation of R-M1 (Lillibridge et al., 1996). In healthy humans we found that the R/S plasma concentration ratio of M1 showed no change over time, during and after the administration of pentoxifylline. The overall median R/S ratio in each subject ranged from 0.019 to 0.037 after 600 mg orally. The R/S plasma concentration ratio for M1 in breast cancer patient (0.01-0.05) was similar to that in healthy volunteers.

The CL_{app} of pentoxifylline was 2-6 L/min and is similar to data from two other studies (Ings et al., 1982; Rames et al., 1990). In comparison hepatic blood flow can be estimated at around 1.5 L/min in young healthy volunteers. A third study reported a plasma CL of 1.3 L/min, the discrepancy of the results between the studies are not clear (Beermann et al., 1985). However the true CL of pentoxifylline is even higher since CL is underestimated due to reversible metabolism. This large CL emphasizes the mainly extrahepatic metabolism of the drug.
Figure 2: The plasma concentrations of pentoxifylline and its metabolites during and after the intravenous infusion of 600 mg of the drug over 3 hours in one subject.

Figure 3: The plasma concentrations of pentoxifylline and its metabolites after administration of a 600 mg controlled release pentoxifylline tablet.
Table 1. Pharmacokinetic parameters of pentoxifylline (Ptx) and its metabolites (R-M1, S-M1, M4, M5) after intravenous or oral administration of pentoxifylline to healthy humans, median (range).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parameter</th>
<th>Intravenous, 300mg (n = 6)</th>
<th>Intravenous, 600mg (n = 5)</th>
<th>Oral 600 mg (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentoxifylline</td>
<td>AUC/dosea</td>
<td>4.0 (2.7 - 6.0)</td>
<td>4.1 (3.8 - 7.3)</td>
<td>1.4 (0.9 - 1.8)</td>
</tr>
<tr>
<td></td>
<td>CLapp (L/min)</td>
<td>4.2 (2.8 - 6.3)</td>
<td>4.1 (2.3 - 4.6)</td>
<td>not applicable</td>
</tr>
<tr>
<td></td>
<td>Terminal t½ (h)</td>
<td>0.90 (0.75 - 1.0)</td>
<td>0.81 (0.75 - 1.1)</td>
<td>2.7 (1.2 - 3.7)</td>
</tr>
<tr>
<td>(R)-M1</td>
<td>AUC/dosea</td>
<td>0.14 (0.06 - 0.26)</td>
<td>0.23 (0.19 - 0.40)</td>
<td>0.13 (0.06 - 0.22)</td>
</tr>
<tr>
<td></td>
<td>Terminal t½ (h)</td>
<td>0.94 (0.76 - 1.0)</td>
<td>0.99 (0.81 - 1.2)</td>
<td>1.7 (n=1)</td>
</tr>
<tr>
<td></td>
<td>Molar AUCratio (R)-M1/Ptx</td>
<td>0.02 (0.02 - 0.05)</td>
<td>0.01 (0.01 - 0.02)</td>
<td>0.06 (0.03 - 0.16)</td>
</tr>
<tr>
<td>(S)-M1</td>
<td>AUC/dosea</td>
<td>12 (9.2 - 18)</td>
<td>13 (11 - 22)</td>
<td>4.9 (3.2 - 6.9)</td>
</tr>
<tr>
<td></td>
<td>Terminal t½ (h)</td>
<td>0.81 (0.61 - 0.93)</td>
<td>0.86 (0.67 - 1.2)</td>
<td>2.6 (2.5 - 3.0)</td>
</tr>
<tr>
<td></td>
<td>Molar AUCratio (S)-M1/Ptx</td>
<td>3.0 (2.4 - 3.7)</td>
<td>2.8 (2.1 - 3.5)</td>
<td>3.2 (2.1 - 4.5)</td>
</tr>
<tr>
<td>M4</td>
<td>AUC/dosea</td>
<td>1.7 (0.77 - 3.3)</td>
<td>1.2 (0.85 - 2.3)</td>
<td>0.89 (0.70 - 2.1)</td>
</tr>
<tr>
<td></td>
<td>Terminal t½ (h)</td>
<td>1.0 (0.42 - 2.1)</td>
<td>0.81 (0.71 - 1.8)</td>
<td>2.8 (1.8 - 5.3)</td>
</tr>
<tr>
<td></td>
<td>Molar AUCratio M4/Ptx</td>
<td>0.35 (0.19 - 0.72)</td>
<td>0.24 (0.16 - 0.32)</td>
<td>0.69 (0.51 - 1.0)</td>
</tr>
<tr>
<td>M5</td>
<td>AUC/dosea</td>
<td>11 (9.6 - 16)</td>
<td>11 (8.9 - 21)</td>
<td>10 (9.5 - 23)</td>
</tr>
<tr>
<td></td>
<td>Terminal t½ (h)</td>
<td>0.94 (0.63 - 1.0)</td>
<td>0.85 (0.68 - 1.1)</td>
<td>2.9 (1.7 - 3.6)</td>
</tr>
<tr>
<td></td>
<td>Molar AUCratio M5/Ptx</td>
<td>2.6 (1.9 - 3.5)</td>
<td>2.1 (1.5 - 3.3)</td>
<td>8.6 (5.7 - 10)</td>
</tr>
</tbody>
</table>

a Area under the curve (ng*h/ml) divided by dose of pentoxifylline in mg.

In paper IV oral pentoxifylline 400 mg three times daily was administered to breast cancer patients. Plasma concentrations after 3, 6, 9 and 12 months treatment are shown in table 2.
Table 2: Plasma concentrations of pentoxifylline, after administration of 400 mg three times daily in breast cancer patients and predicted steady state, median, (range).

<table>
<thead>
<tr>
<th>Compound</th>
<th>3 months</th>
<th>6 months</th>
<th>9 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ptx</td>
<td>Measured point Conc (ng/ml) 174 (72-234)</td>
<td>116 (78-214)</td>
<td>152 (80-230)</td>
<td>97 (60-134)</td>
</tr>
<tr>
<td></td>
<td>Expected mean Css (ng/ml) 70 (45-90)</td>
<td>70 (45-90)</td>
<td>70 (45-90)</td>
<td>70 (45-90)</td>
</tr>
<tr>
<td>S-M1</td>
<td>Measured point Conc (ng/ml) 417 (250-645)</td>
<td>382 (239-525)</td>
<td>403 (249-586)</td>
<td>313 (208-596)</td>
</tr>
<tr>
<td></td>
<td>Expected mean Css (ng/ml) 245 (160-345)</td>
<td>245 (160-345)</td>
<td>245 (160-345)</td>
<td>245 (160-345)</td>
</tr>
<tr>
<td></td>
<td>Molar ratio S-M1/Ptx 2.54 (1.85-3.56)</td>
<td>2.87 (1.88-3.81)</td>
<td>2.85 (1.71-4.73)</td>
<td>3.71 (2.52-4.24)</td>
</tr>
<tr>
<td>R-M1</td>
<td>Measured point Conc (ng/ml) 15 (1-27)</td>
<td>15 (1-26)</td>
<td>15 (1-21)</td>
<td>3.5 (1-13)</td>
</tr>
<tr>
<td></td>
<td>Expected mean Css (ng/ml) 6.5 (3-11)</td>
<td>6.5 (3-11)</td>
<td>6.5 (3-11)</td>
<td>6.5 (3-11)</td>
</tr>
<tr>
<td></td>
<td>Molar ratio R-M1/Ptx 0.08 (0.04-0.12)</td>
<td>0.07 (0.03-0.12)</td>
<td>0.06 (0.02-0.14)</td>
<td>0.05 (0.02-0.11)</td>
</tr>
</tbody>
</table>

For drugs with linear kinetics single dose data can be used to predict concentration at steady state, but sometimes the kinetics is altered due to saturable first pass metabolism. This seems not to be a problem for pentoxifylline since a study in healthy volunteers showed that AUCs for pentoxifylline and rac-M1 were not altered when a single dose of pentoxifylline were compared with the corresponding AUCs after multiple dosing (Beermann et al., 1985). Hinze et al showed that plasma concentration and renal excretion increased in proportion to dosage and time when three different dosing intervals were compared: 400 mg once daily, 400 mg b.i.d. and 400 mg t.i.d. (Hinze, 1976).

The AUC/dose data after oral administration in healthy volunteers was used to calculate expected mean steady state concentration (C_{ss}) after administration of pentoxifylline 400 mg three times daily (table 2). The calculated expected concentration is a mean concentration over the dosing
interval, therefore the actual concentration should be higher than this concentration after intake of pentoxifylline and lower in the end of the dosing interval, i.e. before the next tablet is taken. The data from one healthy volunteer, the same as shown in figure 3, was used to simulate oral administration of 400 mg three times daily, figure 4. The fluctuation between $C_{\text{max}}$ and $C_{\text{min}}$ was approximately a factor four and this shows that the measured point concentrations are in the interval of fluctuations. We do not know the time between intake of study drug and blood sampling in the breast cancer study, but all patients visited the clinic at lunch time or in the afternoon and it seem likely that they had taken the second dose before visiting the study team. This could explain the measured point concentrations being in the upper range of predicted concentrations. The measured point concentrations are in agreement with what should be expected in patients with good compliance. Measured molar ratio for S-M1/pentoxifylline and R-M1/pentoxifylline are as expected from the calculations.

![Graph](image)

**Figure 4.** Simulated plasma concentrations of 400 mg pentoxifylline three times daily

**Conclusion**

When pentoxifylline was administered to healthy humans AUCs for S-M1 and M5 were higher, and the AUCs for M4 and R-M1 were lower, than the AUC for pentoxifylline (Paper I). The same pattern was seen for mean concentration in breast cancer patients treated with pentoxifylline (Paper IV).
Paper I

Interconversion of pentoxifylline and M1 by erythrocytes

The metabolism of pentoxifylline to R-M1 by haemolysed erythrocytes was best described by a one-enzyme reaction model. In contrast, the metabolism of pentoxifylline to S-M1 was best described by a two-enzyme model, with inter-individual variance only in the $V_{\text{max}}$ of one of the enzymes. At a therapeutic blood concentration of pentoxifylline (0.5 $\mu$g/ml, 1.8 $\mu$M) the calculated rates of formation of R-M1 and S-M1 were $6.8 \times 10^{-6}$ and $1.0 \times 10^{-4}$ nmoles/min per 5 $\times 10^9$ erythrocytes, and the low $K_m$ enzyme would account for 89% of the metabolism to S-M1. The rate of formation of S-M1 would thus be 15 times higher than that of the R-enantiomer.

The formation of pentoxifylline from S-M1 was approximately 3 times faster than the formation from R-M1 at the investigated concentrations. If the two enantiomers were to be present at an equal concentration of 0.5 $\mu$g/ml (1.8 $\mu$M), the calculated rates of formation of pentoxifylline would be $1.8 \times 10^{-5}$ and $7.4 \times 10^{-5}$ nmoles/min per 5 $\times 10^9$ erythrocytes from R-M1 and S-M1, respectively, i.e. a 4-fold higher rate of formation from the S-enantiomer, figure 5.

Figure 5: Formation of R-M1 and S-M1 from pentoxifylline and the re-formation of pentoxifylline in erythrocyte suspension (nmoles/min per 5 $\times 10^9$ erythrocytes).
Conclusion

In erythrocyte suspension the transformation of pentoxifylline to M1 was highly stereospecific in favor of the S-enantiomer and was reversible from both enantiomers. A 15 fold faster formation of S-M1 than of R-M1 was estimated in erythrocytes. This seems to be the main reason for the marked difference in plasma concentration and AUC of the two enantiomers.

Paper II

This study was planned in order to investigate the possible contributions of the metabolites of pentoxifylline to the haemorheologic effect in humans. Measurement of retinal blood flow was chosen as a convenient experimental model. By pre-treatment of the subjects with ciprofloxacin a known inhibitor of CYP1A2 (Fuhr et al., 1992; Peterson et al., 2004) and rifampicin, an inducer of several of the cytochrome P450 enzymes (Niemi et al., 2003) we succeeded to diminish the usual high correlation between the concentrations of pentoxifylline and metabolites. Thereby enabling us to investigate whether pentoxifylline’s metabolites have any effect on retinal blood flow. We found that AUCs for pentoxifylline and S-M1 were significantly higher after pre-treatment with ciprofloxacin compared with pentoxifylline administration alone. The effect was seen on R-M1 as well but was not statistically significant. However the R/S plasma concentration ratio remained unchanged. The AUC for M5 was significantly lower after pre-treatment with rifampicin compared with after pentoxifylline alone. The estimated intercept and coefficients from the mixed model applying a simple linear AUC-effect model are shown in table 3. We found that pentoxifylline; R-M1 and M5 had coefficients with positive signs indicating that they all enhanced retinal blood flow. In contrast M4 and S-M1 had negative sign and therefore appeared to either decrease retinal blood flow or to counteract the pentoxifylline, R-M1 and M5.

R-M1 has a high potency, this is reflected in the values of the coefficients (Table 3). It is particularly noticeable that R-M1 exerts a significant positive effect in spite of being present in concentrations that are approximately two orders of magnitude lower than those of pentoxifylline and M5.
The expected mean flow should be calculated as:

\[ 245 + 17.1 \times \text{AUC}_{\text{ptx}} + 303 \times \text{AUC}_{\text{R-M1}} - 9.58 \times \text{AUC}_{\text{S-M1}} - 54.5 \times \text{AUC}_{\text{M4}} + 6.66 \times \text{AUC}_{\text{M5}} \]

**Table 3.** Estimates (95% CI) of the intercept and gradients from the regression analysis of the influence from pentoxifylline (Ptx) and its metabolites on retinal blood flow measured as mean flow.

<table>
<thead>
<tr>
<th>Mean flow</th>
<th>Intercept AU† 245 (186 - 304)</th>
<th>AUC Ptx AU†/(μg<em>h/ml) 17.1</em> (4.31 - 29.9)</th>
<th>AUC R-M1 AU†/(μg<em>h/ml) 303</em> (147 - 459)</th>
<th>AUC S-M1 AU†/(μg<em>h/ml) -9.58</em> (-13.6 - -5.52)</th>
<th>AUC M4 AU†/(μg<em>h/ml) -54.5</em> (-93.4 - -15.5)</th>
<th>AUC M5 AU†/(μg<em>h/ml) 6.66</em> (0.675 - 12.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conclusion</strong></td>
<td></td>
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</tbody>
</table>

The R-M1 and M5 metabolites of pentoxifylline contribute significantly to pentoxifylline enhancement on retinal blood flow in humans.

**Paper III**

The aim of this study was to investigate the relative potencies of pentoxifylline and metabolite R-M1, S-M1, M4 and M5 to inhibit platelet aggregation in whole blood, and in particular to clarify contributions of the two enantiomers of M1, that are formed to very different extents in vivo, to this effect.
Figure 6 A-B: The estimated least square mean impedance values (ohm) and their 95% confidence limits from the mixed model after incubation with pentoxifylline, rac-M1, M4 and M5, figure 6A and rac-M1, R-M1, figure 6B. pentoxifylline △, rac-M1 □, M4 ◊, and M5 ▼, R-M1 ×, and S-M1 dotted line.

Pentoxifylline, rac-M1, R-M1, S-M1 and M4 significantly inhibits platelet aggregation in a concentration-dependent manner, R-M1 being the most potent followed by rac-M1, S-M1, pentoxifylline, M4 and M5, figure 6 A,B.

A previous study showed that rac-M1, pentoxifylline and M5 but not M4 inhibited aggregation in platelet rich plasma, and that rac-M1 was more potent than pentoxifylline (Ambrus et al., 1995). Our results are in agreement with the findings of Ambrus and co-workers regarding the most potent substances, but not regarding M4 and M5. A direct comparison between studies is always difficult and between these two studies the methodologies differ. The method used here should be more relevant for in vivo situations since we studied aggregation in whole blood instead of aggregation in platelet rich plasma. Aggregation in whole blood is preferable, both since it evaluates the platelets in a physiologic milieu in the presence of red and white blood cells, which are known to modulate platelet function and also since aggregation in platelet rich plasma require centrifugation that causes injury to the platelets and loss of giant thrombocytes (Dyszkiewicz-Korpanty et al., 2005). Another study showed that pentoxifylline inhibits platelet
aggregation in whole blood more than in platelet rich plasma (de la Cruz et al., 1993). In addition, Ambrus et al. could not distinguish between the enantiomers of M1, which, as shown here, differ significantly in their potencies.

In vivo there will always be a mixture of the parent compound and its metabolites after administration of pentoxifylline, which allows pharmacological interactions between the different species (an aspect that has not been investigated in vitro, where only one specimen is added at the time, with the exception of the racemate). In addition, the relative plasma concentrations of pentoxifylline and the metabolites will be very different from those in the in vitro experiments, so that activities are compared for different parts of the underlying concentration – effect curves.

We therefore wanted to estimate the relative contribution of each substance to the total effect of pentoxifylline on platelet aggregation in vivo. The potencies found in this study was used together with the concentration data of pentoxifylline and its metabolites in humans after administration of pentoxifylline (paper I). When combing these results we conclude that the main effect on platelet aggregation in vivo should actually be brought about by S-M1 and pentoxifylline, and that the remaining metabolites would contribute by less than 10% each. Thus, even if R-M1 is twice as potent as S-M1 in vitro the low concentration achieved after administration of pentoxifylline results in only a small contribution to the total effect on platelet aggregation in vivo. However, further studies are needed in order to confirm this.

In this thesis the effects of the R and S enantiomers of M1 have been described for the first time. Both enantiomers were active in inhibiting platelet aggregation in whole blood whereas only R-M1 was effective in increasing retinal blood flow. The activity of M4 and M5 also differs between the two effect models, M5 increases retinal blood flow but does not inhibit platelet aggregation in whole blood, whereas M4 inhibits platelet aggregation but does not increase retinal blood flow. The effects of both S-M1, M4 and M5 are limited whereas R-M1 was more effective than pentoxifylline in both effect models. On retinal blood flow the R-enantiomer was up to 17 times as effective as pentoxifylline but on aggregation it was only approximately 1.25 as effective. The differences in activities for the metabolites may be explained by different mechanism of action in the two effect models.
Conclusion
In the following potency order R-M1, rac-M1, pentoxifylline, S-M1 and M4 all significantly inhibit platelet aggregation in whole blood in vitro.

Paper IV

Between May 2004 and May 2007, a total of 83 patients were included in the study, 42 patients were randomised to pentoxifylline treatment and 41 to the placebo group. Most patients (67) were treated with previous chemotherapy, 33 in the pentoxifylline group and 34 in the placebo group.

Both treatments were generally well tolerated. Only four patients discontinued due to adverse events: two due to nausea and one due to bruising in the pentoxifylline group and one due to neuropathic pain in the placebo group. A safety analysis was done in September 2008 when all patients had been included in the study for a median period of 31 months (range 16-52 months). Although the study was not dimensioned for safety, we observed no significant differences between the study groups in terms of safety, including disease recurrence, death, and adverse events. This shows that pentoxifylline in combination with vitamin E is safe to use prophylactically.

Abduction
No treatment effect was found on the primary effect parameter, abduction of the shoulder. The median value for passive abduction at inclusion was 121° in the pentoxifylline group and 117° in the placebo group (figure 7). Both treatment groups improved in passive abduction during the 12 months treatment period. At the end of this the median improvement in the pentoxifylline group was 3.7° (p=0.0035) and in the placebo group 9.4° (p=0.0041). These changes were not significantly different between treatments (p=0.20). A reduction in passive abduction of the shoulder was expected since abduction in a previous study in breast cancer women treated with radiotherapy to the breast and axilla continuously decreased during the two years they were followed (Johansson et al., 2001).
Instead, the fortunate result was found that shoulder abduction improved in both treatment groups. Changes that can explain this has been made in both in the physiotherapy and the radiation technique in order to diminish radiation-induced side effects. The changes in physiotherapy involve changes in the exercise program that patients receive after radiotherapy, before the exercise program only included shoulder motion. Now, all patients receive a training program mostly focused on stretching the shoulder and breast area. Additionally, the radiation technique has been refined to diminish radiation-induced side effects. The radiation dose to the muscle tissue around the caput humerus and tissue around the axilla has been diminished. The discrepancies between the studies could be explained by one or a combination of both factors. A third but less likely explanation could be that vitamin E causes the improvement since all patients received vitamin E.

![Box-plot of passive abduction of the shoulder by treatment group (blue placebo, red pentoxifylline) and visit.](image)

However, the VAS score for pain described as stiffness in the skin significantly decreased in the pentoxifylline group during the treatment time (p=0.0003), but not in the placebo group (p=0.97), figure 8. When comparing scores for VAS stiffness to abduction of the shoulder, it appears that
high VAS stiffness score is connected to lower degrees of abduction, i.e. less ability to move the arm. The sense of stiffness may be a harbinger of reduced abduction of the shoulder. Radiation induced side effect develops over time and all patients will therefore be followed for 5 years.

Figure 8: Box-plot of VAS for stiffness of the skin by treatment group (blue placebo, red pentoxifylline) and visit in the 38 patients reporting this phenomenon at least once during the study.

Volume
At study start there was no significant median difference in arm volume between the patient’s affected and unaffected arms (figure 9). Arm volume increased over time in the placebo group but not in the pentoxifylline group. At the end of the treatment period the median increase was 1.04% in the placebo group and 0.50% in the pentoxifylline group, and was significant between groups (p=0.0172).
Previously RIF had been considered irreversible, but a few recent publications have shown that therapy with pentoxifylline and vitamin E may decrease or even reverse RIF (Chiao et al., 2005; Delanian, 1998; Delanian et al., 2003). Most of the studies are case reports or small uncontrolled studies; however, Delanian et al. performed a double-blind, placebo-controlled study in 24 women previously treated for breast cancer (Delanian et al., 2003). After 6 months of treatment, mean RIF surface regression was significant with combined pentoxifylline (800mg/day) and vitamin E (1000U/day) treatment versus double placebo treatment (60% ± 11% v 43% ± 17%). The treatment of pentoxifylline or vitamin E alone was ineffective (39% ± 37 v 40% ± 32%).

Prior to initiation of this study, no other prophylactic studies with pentoxifylline and vitamin E were published. Since any interaction of pentoxifylline and vitamin E with the efficacy of radiation was unknown, for safety reasons it was decided that the radiotherapy should be completed before the patients were included in the study. Since then, there have been two other studies published using pentoxifylline and vitamin E to prevent radiation-induced side effects. Ozturk et al. administered pentoxifylline 400 mg t.i.d. during the entire radiotherapy in order to prevent radiation-induced lung toxicity in patients with lung or breast cancer (Ozturk et al., 2003).
A significant protective effect of pentoxifylline compared with placebo for both early and late lung radiotoxicity was reported. In another study, pentoxifylline 400 mg t.i.d. was given during radiotherapy to patients with squamous cell carcinoma of the head and neck (Aygen et al., 2004). Late skin changes, fibrosis, and soft tissue necrosis were more severe in the control group than in the pentoxifylline group. These studies indicate that it is safe to give pentoxifylline and vitamin E during the radiotherapy. When planning further studies, we intend to administer these treatments concomitant with the radiotherapy; earlier treatment may lead to greater protective effects.

**Conclusion**

The combination of pentoxifylline and vitamin E is safe and can be used to prevent some radiation-induced side effects. It was found that pentoxifylline in combination with vitamin E diminished the development of increased arm volume. No significant effects on abduction of the shoulder were seen but VAS for stiffness in the skin was significantly decreased in the pentoxifylline group.
CONCLUSIONS

- In erythrocyte suspension the transformation of pentoxifylline to M1 was highly stereospecific in favor of the S-enantiomer and was reversible from both enantiomers (Paper I).

- When pentoxifylline was administered to healthy humans AUCs for S-M1 and M5 were higher, and the AUCs for M4 and R-M1 were lower, than the AUC for pentoxifylline (Paper I, II). The same pattern was seen for mean concentration in breast cancer patients treated with pentoxifylline (Paper IV).

- Pentoxifylline, R-M1 and M5 all significantly increased retinal blood flow. In contrast S-M1 and M4 appeared to decrease the blood flow after intravenous infusions of pentoxifylline to healthy humans (Paper II).

- In the following potency order R-M1, rac-M1, pentoxifylline, S-M1 and M4 all significantly inhibit platelet aggregation in whole blood in vitro (Paper III).

- Pentoxifylline in combination with vitamin E is safe and can be used to prevent some radiation induced side effects such as increased arm volume in women with breast cancer (Paper IV).
SUMMARY

Pentoxifylline is a haemorheologic drug that has been used for a long time for the treatment of intermittent claudication and other diseases with impaired microcirculation. Pentoxifylline is an interesting drug to study since it exhibit complex pharmacokinetics with both reversible metabolism, and active metabolites. Difficulties in finding consistent clinical effects of pentoxifylline may be due to the drug acting at least in part through formation of active metabolites, the rate and extent of which may vary between individuals. In humans, pentoxifylline is metabolised into at least seven phase 1 metabolites (M1-M7). The reversible metabolism of pentoxifylline to the enantiomers of M1 has only been partly studied.

This thesis investigates the pharmacokinetics of pentoxifylline and metabolites and their contributions to the haemorheological effects.

When pentoxifylline is administered either orally or intravenously to healthy humans the plasma concentrations of M5 and S-M1 are higher than the pentoxifylline concentrations, whereas the ones for M4 are lower and R-M1 much lower. In-vitro studies showed that this can be mainly explained by a 15 times faster formation of S-M1 than R-M1 from pentoxifylline. Had the enantiomers been present at equal concentrations the reversible metabolism would have been 4 times faster from S-M1 than from R-M1.

Pentoxifylline was administered to healthy volunteers and retinal blood flow was measured. By pre-treatment of the subjects with ciprofloxacin and rifampicin, the usual high correlation between the concentrations of pentoxifylline and the metabolites was diminished and the effects of pentoxifylline and its metabolites could be studied. Pentoxifylline, R-M1 and M5 seem to increase retinal blood. When present at equal concentration R-M1 is approximately 15 times as effective as pentoxifylline. M4 and S-M1 appears to either decrease retinal blood flow or to counteract the pentoxifylline, R-M1 and M5.

Further, the effects of pentoxifylline and its metabolites on platelet aggregation in whole blood was investigated. The study showed that pentoxifylline, rac-M1, R-M1, S-M1 and M4 significantly inhibits platelet aggregation in a concentration-dependent manner. R-M1 being the most potent followed by rac-M1, S-M1, pentoxifylline, M4 and M5.
More recent studies have shown that pentoxifylline in combination with vitamin E can reduce radiation induced fibrosis (RIF). A study was planned to investigate whether the same drug can prevent radiation induced fibrosis in women with breast cancer, treated with radiotherapy to the breast and axilla. The clinical trial was randomised, double-blind, placebo-controlled, and the 83 patients included in the study were treated with pentoxifylline or placebo in combination with vitamin E for 12 months. Abduction of the shoulder was the primary effect parameter and arm volume the secondary. The study showed that pentoxifylline in combination with vitamin E diminishes the development of increased arm volume. Significant effects on abduction of the shoulder were not seen, but the VAS for stiffness in the skin decreased: this may be a harbinger of reduced abduction of the shoulder. Radiation induced side effect develops over time and all patients will be followed for 5 years. The combination of pentoxifylline and vitamin E appeared safe and may be used for prevention of some radiation-induced side effects.

Huvudmålet med denna avhandling var att titta noggrannare på pentoxifyllins farmakokinetik och farmakodynamik, samt att objektivt försöka mäta effekten av pentoxifyllin och dess metaboliter. Detta för att se om någon eller några av metaboliterna bidrar till pentoxifyllins effekter och därmed förklarar skillnaderna i effekt.


I de två följande delarbeteerna studeras effekterna av pentoxifyllin och det undersöks om dess metaboliter bidrar till dess effekter vilket skulle kunna förklara variabiliteten i effekt hos patienterna.
En klinisk prövning på friska frivilliga genomfördes för att undersöka pentoxifyllin och dess metaboliters effekt på ögonblodflöde i näthinnan mätt med objektiv metodik. Pentoxifyllin visade sig öka retinalt ögonblodflöde jämfört med placebo. När area under kurva (AUC) av plasmakoncentrationerna användes som förklaringsvariabler till retinalt ögonblodflöde fann vi att R-M1 och M5 ökar retinalt blodflöde medan S-M1 och M4 minskar retinalt blodflöde eller minskar effekten av pentoxifyllin och R-M1.

I delarbete III undersöktes om pentoxifyllin och dess metaboliter hämmar trombocyt aggregation i helblod. Det visade sig att pentoxifyllin racemiskt M1 (50% R-M1, 50% S-M1), R-M1, S-M1 och M4 hämmar trombocyt aggregation i helblod på ett koncentrationsberoende sätt. Vi fann att R-M1 var den potentaste substansen följt av rac-M1, S-M1, pentoxifyllin och M4.

På senare tid har studier visat att pentoxifyllin kan minska eller till och med tillbakabilda fibrområden i huden vilka uppkommit efter strålbehandling. Dessa fibrosytor utvecklas ofta några år efter avslutat strålning och medför besvår med nedsatt rörlighet, smärta och ödemutveckling. I delarbete IV undersöks om pentoxifyllin kan förhindra dessa biverkningar efter strålbehandling.

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REFERENCES


Hinze, HJ (1972a) [Pharmacokinetics of 3,7-dimethyl-1-(5-oxo-hexyl)-xanthine (BL 191) in man]. *Arzneimittelforschung* 22(9): 1492-1495.


Stereoselective Metabolism of Pentoxifylline In Vitro and In Vivo in Humans
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ABSTRACT  Pentoxifylline increases erythrocyte flexibility, reduces blood viscosity, and inhibits platelet aggregation and is thus used in the treatment of peripheral vascular disease. It is transformed into at least seven phase I metabolites, of which two, M1 and M5, are active. The reduction of the keto group of pentoxifylline to a secondary alcohol in M1 takes place chiefly in erythrocytes, is rapidly reversible, and creates a chiral center. The aims of this study were: to develop HPLC methods to separate the enantiomers of M1, to investigate the kinetics of the reversible biotransformation of pentoxifylline to (R)- and (S)-M1 in hemolysed erythrocyte suspension, and to quantify the formation of the enantiomers of M1 (as well as M4 and M5) after intravenous and oral administration of pentoxifylline to human volunteers. (R)- and (S)-M1 could be separated preparatively on a cellobiohydrolase column, while determination in blood or plasma was by HPLC after chiral derivatization with diacetyl-L-tartaric acid anhydride. The metabolism of pentoxifylline to (R)-M1 in suspensions of hemolysed erythrocytes followed simple Michaelis-Menten kinetics ($K_m = 11\text{ mM}$), while that to (S)-M1 was best described by a two-enzyme model ($K_m = 1.1$ and $132\text{ mM}$). Studies with inhibitors indicated that the enzymes were of the carbonyl reductase type. At a therapeutic blood concentration of pentoxifylline, the calculated rate of formation of (S)-M1 is 15 times higher than that of the (R)-enantiomer. Back-conversion of M1 to pentoxifylline was 3–4 times faster for the (S)- than for the (R)-enantiomer. In vivo, the R:S plasma concentration ratio of M1 ranged from 0.010–0.025 after intravenous infusion of 300 or 600 mg of pentoxifylline, and from 0.019–0.037 after oral administration of 600 mg. The biotransformation of pentoxifylline to M1 was thus highly stereoselective in favor of the (S)-enantiomer both in vitro and in vivo. Chirality 14:643–652, 2002. © 2002 Wiley-Liss, Inc.

KEY WORDS: stereospecific analysis; HPLC; ketone reductase; blood; erythrocytes; pharmacokinetics; humans

Pentoxifylline (oxpentifylline) is a methylxantine derivative used in the treatment of peripheral vascular disease and other conditions involving a defective regional microcirculation. Since pentoxifylline acts primarily by increasing erythrocyte flexibility, by reducing blood viscosity, and by decreasing the potential for platelet aggregation and thrombus formation it is characterized as a hemorheological agent.

Pentoxifylline is transformed in humans into at least seven phase I (i.e., nonconjugated) metabolites, denoted M1–7 (Fig. 1). In four of these the metabolism has created at least one chiral center. After oral administration of pentoxifylline to healthy volunteers, the areas under the plasma concentration curves (AUCs) of M5 and M1 exceeded that of pentoxifylline, while that of M4 was lower. The major species excreted in the urine is M5, followed by a diol metabolite (M3) and M4, while excretion of unchanged pentoxifylline and M1 each account for less than 1% of the dose. However, small amounts of M1 may be excreted in conjugated form.

The transformation of pentoxifylline to M1 is rapidly reversible. It takes place both in erythrocytes and liver. The apparent clearance of pentoxifylline after intravenous administration has been shown to considerably exceed hepatic blood flow. In addition, impairment of liver function due to cirrhosis lowered the total clearance of pentoxifylline but did not affect the plasma AUC ratio of M1 to pentoxifylline. This suggests that erythrocytes are the major site for the pentoxifylline–M1 interconversion.

Metabolites 1 and 5 have shown biological activity similar to pentoxifylline on erythrocyte flexibility and inhibition of thrombocyte aggregation. In addition, the (R)-enantiomer of M1 (lisofylline) inhibits the effects of tumor
necrosis factor-α and suppresses serum levels of free fatty acids and is being developed as a drug in its own right. Difficulty in finding consistent clinical effects of pentoxifylline may be due to the drug acting at least in part through formation of its active metabolites, the rate and extent of which may vary between individuals. Limited data indicate that (R)-M1 is formed to a much lesser extent than (S)-M1 in humans. The stereoselective formation of M1 has, however, only been studied in vitro in human liver microsomes and cytosol and in cancer patients (peak and trough plasma concentrations in four patients) who received pentoxifylline together with ciprofloxacin and interleukin-2. Ciprofloxacin has been reported to raise the plasma concentration of pentoxifylline and M1, presumably by the same mechanism as it interacts with other methylxantines, i.e., inhibition of cytochrome P450 1A2. Formation of the enantiomers of M1 from pentoxifylline in the absence of drug interactions has not previously been studied.

The aims of this study were: 1) to develop high-performance liquid chromatographic (HPLC) methods to separate the enantiomers of M1 and to determine them in blood or plasma; 2) to investigate the kinetics of the reversible transformation of pentoxifylline to (R)− and (S)−M1 in erythrocyte suspension, and also the distribution of pentoxifylline and its metabolites in blood; and 3) to quantify the formation of the enantiomers of M1 (as well as M4 and M5) after intravenous and oral administration of pentoxifylline to human volunteers.

**MATERIALS AND METHODS**

**HPLC**

Pentoxifylline and metabolites 1 (racemic), 4, and 5 were kindly supplied by Hoechst Marion Roussel AS (Oslo, Norway) and Aventis Pharma AB (Stockholm, Sweden). The (R)-enantiomer of M1 (lisofylline) was a gift from Cell Therapeutics (Seattle, WA, USA), thalidomide from Grünenthal GmbH (Stolberg, Germany), and enprofylline from AstraZeneca (Lund, Sweden). Diacetyl-L-tartaric acid anhydride was purchased from Sigma-Aldrich Sweden (Stockholm, Sweden). Analytical-grade solvents for chromatography were from Merck (Darmstadt, Germany). The liquid chromatographic systems consisted of SpectraSystem P1000 (Thermo Separation Products, San Jose, CA, USA) pumps, Rheodyne 7725 loop injectors with 20 µl loops and SpectraSystem UV1000 variable-wavelength UV detectors.

Semipreparative separation of the enantiomers of M1 was performed on a 100 × 4 mm HPLC column packed with cellulobiohydrolase immobilized on 5 µm spherical silica particles (Chiral-CBH, ChromTech, Hägersten, Sweden). The mobile phase was 10 mM sodium phosphate buffer, pH 6.15, containing 50 µM EDTA, flow rate 0.9 ml/min. The detection wavelength was 274 nm.

The concentrations of pentoxifylline and the enantiomers of M1 in blood, hemolysed erythrocyte suspension, plasma, or plasma water were determined by reversed-phase HPLC after chiral derivatization of the metabolite. To (typically) 0.50 ml of sample were added 0.1 ml of 1.0 M HCl and (typically) 30 µl of thalidomide solution, 50 µg/ml in methanol. The sample was then extracted with 5 ml of dichloromethane and the phases were separated by centrifugation at 700g for 10 min. The organic phase was evaporated to dryness under a stream of dry air. Then 100 µl of diacetyl-L-tartaric acid anhydride solution, 100 mg/ml in dichloromethane:glacial acetic acid (4:1) solution (prepared maximum 1 h before use), was added to the residue. The mixture was heated at 75 °C for 2 h. After cooling, 1.5 ml of 0.02 M NaH2PO4 solution, pH 4.4, and 5.0 ml of dichloromethane were added. The samples were mixed for 30 sec and the phases were separated by centrifugation. The organic phase was then evaporated to dryness. The residue was dissolved in 50 µl of mobile phase, of which 20 µl were injected into the chromatographic system. A LiChrosorb RP-18, 250 × 4 mm, 7 µm particle size column was eluted at 1.0 ml/min with 17% acetonitrile in 0.05 M NaH2PO4 buffer, pH 4.0. The detection wavelength was 274 nm.

For the determination of metabolites 4 and 5, (typically) 0.5 ml of plasma was acidified with 0.1 ml of 1.0 M HCl and 50 µl of enprofylline solution, 1 µg/ml in water, was added as internal standard. The sample was extracted with 5 ml of ethyl acetate. The phases were separated by centrifugation and the organic phase evaporated to dryness. The residue was dissolved in 50 µl of mobile phase, of which 20 µl were injected. The RP-18 column was eluted with 13% acetonitrile in 0.005 M NaH2PO4 buffer, pH 3.6, and the detection wavelength was 274 nm.

Validation of the methods comprised investigation of ex-
traction yields from plasma and blood, confirming the absence of racemization during derivatization of the enantiomers of M1, establishing the accuracy and precision of the assays at several concentration levels of the analytes and investigating the stability of the compounds during storage and sample handling.

The extraction yields of pentoxifylline and rac-M1, 0.50 µg/ml, from plasma and blood were determined using four aliquots of each. After the extraction, 8.0 µg of thalidomide was added as external standard and the samples were injected into the chromatograph without derivatization. The extraction yields of metabolites 4 and 5 and enprofylline were checked analogously at 0.10 µg/ml of the metabolites and 0.050 µg/ml of enprofylline.

The derivatization of rac-M1 (1 µg) was initially investigated using 50 mg/ml diacetyl-L-tartaric acid anhydride in dichloromethane:glacial acetic acid (4:1) and heating either for 16 h at 55°C or for 2, 4, or 6 h at 75°C. A 100-ng/ml solution heated to 75°C for 2 h was then tested. The extraction yields of the diacetyl-L-tartaric acid monoesters of the enantiomers of M1 from the reaction mixture were determined with various extraction solvents. In order to investigate the possibility of racemization during derivatization, a sample of the (R)-enantiomer of M1 obtained from Cell Therapeutics was further purified by chromatography on the Chiral-CBH column. A 1.0-µg sample was then derivatized by the normal procedure with heating for 2 h and another with heating for 6 h.

Standard curves were prepared over the concentration intervals 2.5–600 or 200–800 ng/ml for pentoxifylline, 2.5–160 or 200–800 ng/ml for the enantiomers of M1, 10–700 ng/ml for M4, and 20–700 ng/ml for M5. Within-day accuracy and precision were checked by the assay of eight plasma samples, 1.0 ml each, to which analytes had been added to the following concentrations: 3, 15, or 150 ng/ml of pentoxifylline, (R)-M1 and (S)-M1, or the same concentrations of M4, or 15 or 150 ng/ml of M5. Between-day variance was determined by the assay of quality-control samples (0.50 ml) containing 200 ng/ml each of pentoxifylline and racemic M1 or 200 ng/ml of M4 and 600 ng/ml of M5. In addition, the accuracy and precision of the stereospecific determination of the enantiomers of M1 was checked by assay of eight plasma samples to each of which the purified enantiomers had been added in the ratios R:S 0.03 or 0.05:1 to a total concentration of 100 ng/ml.

The stability of pentoxifylline and metabolites 1, 4, and 5 in stock solutions, 100 µg/ml in methanol, was investigated at room temperature (23°C), in the refrigerator (5°C), and in the freezer (~25°C) over 308 days. The possibility of ex vivo metabolism of pentoxifylline to M1 and vice versa in blood samples was investigated. Either compound was added to freshly drawn blood to a concentration of 2 µg/ml. The samples were left on the bench for 10 min and then assayed (in duplicate) for formed M1 or pentoxifylline, respectively.

**Distribution of Pentoxifylline and Its Metabolites in Blood**

The putative concentration-dependence of the distribution of pentoxifylline and its metabolites in blood was investigated in samples from four healthy donors (three female, one male, 26–42 years). The blood:plasma concentration ratio ($\lambda$), erythrocyte:buffer concentration ratio ($r_e$), and unbound fraction in plasma ($f_u$) were determined at concentrations of 0.030, 0.10, 0.30, 1.0, and 3.0 µg/ml of pentoxifylline and at double these concentrations of rac-M1. Blood was collected from the donors in Vacutainer (Becton-Dickinson, San Jose, CA) EDTA-K$_3$ tubes and the erythrocyte volume fraction (EVF; hematocrit) was determined. Blood drawn on a single occasion from each donor was used for the entire experiment and all assays were performed in duplicate.

For the preparation of an erythrocyte suspension, 9 ml of blood was centrifuged for 10 min at 1,000g and 4°C. The erythrocytes were collected and suspended in 4 ml of physiological saline solution containing 2 mM EDTA-Na$_2$. The suspension was centrifuged for 10 min at 100g and 4°C. The supernatant was discarded. This washing was repeated three times. The erythrocytes were then centrifuged for 10 min at 1,000g. They were then suspended in phosphate-buffered saline solution (0.02 M K$_2$HPO$_4$, 0.08 M NaH$_2$PO$_4$, and 0.036 M NaCl, pH 7.4) in a proportion corresponding to the EVF of the originally used blood.

The $\lambda$ of the compounds were determined after incubation in whole blood for 30 min at 37°C and assay of plasma and frozen and thawed blood as previously described.$^{20}$ Their $r_e$ were determined by incubation in erythrocyte-buffer suspension for 30 min at 37°C. Their $f_u$ were determined in 1.0-ml aliquots of plasma using Centrifree® centrifugal filter devices (Amicon/Millipore, Bedford, MA, USA). The devices were centrifuged for 30 min at 1,000g. Duplicate samples for assay were taken from the unfiltered plasma and from the plasma water obtained.

The $f_u$ of pentoxifylline and of the enantiomers of M1 were also determined in blank plasma from each subject in the human pharmacokinetic study. Pentoxifylline was added to these samples at a concentration of 1 µg/ml and rac-M1 to a concentration of 2 µg/ml.

**Interconversion of Pentoxifylline and M1 by Erythrocytes**

The reductive metabolism of pentoxifylline to M1 and the oxidative metabolism of M1 to pentoxifylline were investigated in hemolyzed erythrocyte suspension. Blood was collected from the four healthy donors and the erythrocyte count was determined. Erythrocyte suspensions were prepared as described above and then hemolyzed by freezing and thawing. The suspension was divided into 0.25-ml aliquots and incubations at all the concentrations given below were performed in samples from all four donors.

For the reductive metabolism of pentoxifylline to M1, 1.2 mM NADPH and 10 mM Mg$^{2+}$ (as MgCl$_2$) were added to the 0.25-ml aliquots of hemolysed erythrocytes. The incubations were started by the addition of pentoxifylline to concentrations of 0.022, 0.072, 0.22, 0.72, 2.2, and 7.2 mM (corresponding to 6.2–2,000 µg/ml) in the respective samples. The mixtures were incubated for 20 min at 37°C and the incubations were stopped by the addition of 100 µl of 1 M HCl.
For the oxidative metabolism of M1 to pentoxifylline, 1.3 mM NADP and 10 mM Mg	extsuperscript{2+} were added to the 0.25-ml aliquots of hemolysed erythrocytes. The incubations were started by the addition of (R)- or (S)-M1, to final concentrations of 0.022 and 0.072 mM, respectively. The samples were incubated for 20 min at 37°C.

NONMEM version V (The NONMEM project group, San Francisco, CA, USA) was used to determine the Michaelis-Menten parameters, V\textsubscript{max} and K\textsubscript{m}, of the enzymes involved in the metabolism of pentoxifylline to (R)- and (S)-M1. Two models were used, representing the action of either one or two enzymes:

\[
V = \frac{V_{\text{max}(1)} \times [C]}{K_{m(1)} + [C]} + \frac{V_{\text{max}(2)} \times [C]}{K_{m(2)} + [C]}
\]

In this equation, V is the measured rate of metabolite formation in nmoles/min and [C] is the concentration of substrate (mM). The models were fitted in a population mode, i.e., to all data from each reaction (n = 6 substrate concentrations × 4 subjects). Interindividual variance was described by a multiplicative model, e.g.:

\[
V_{\text{max}(1)} = V_{\text{max}(1),TV} \times (1 + \eta)
\]

where V\text{max}(1),\text{TV} is the typical value and \eta is the interindividual variance. If the \eta of a parameter turned out to be very low, the model was rerun after deletion of this \eta. The criteria for selecting a final model from the investigated ones (one or two enzymes, with or without \eta on the V\text{max} and K\text{m} values) were the values of the NONMEM objective function and the distributions of residuals. The V\text{max} is given as nmoles/min per 5 × 10\textsuperscript{8} erythrocytes, which corresponds to the normal erythrocyte count in 1 ml of blood.

From the obtained values of V\text{max} and K\text{m} for the various enzymes, the rates of conversion of pentoxifylline to (R)- and (S)-M1 at a therapeutic blood concentration of 0.5 μg/ml (1.8 μM) were calculated and compared.

**Inhibition of the Metabolism of Pentoxifylline to M1**

Blood from one healthy donor was used in all these incubations. Inhibition of the reductive metabolism of pentoxifylline to M1 was investigated\textsuperscript{21,22} by the addition of 5 mM of SKF525-A, ethacrynic acid, mendonaine, dainorubicin, ketamine, or phenobarbital to the incubation mixtures. The incubations were started by the addition of pentoxifylline to concentrations of 0.22 and 2.2 mM and proceeded as described above.

**Pharmacokinetics in Humans**

The study was approved by the Ethics Committee of Lund University and by the Swedish Medical Products Agency. Six healthy, nonsmoking volunteers (three female, ages 39–46 years, weight 63–69 kg, and three male, ages 27–44 years, weight 71–92 kg), who were free of medication and had no history of allergy to drugs, gave written informed consent to the study. They received, in a randomized cross-over design, three doses of pentoxifylline (Trental®, Hoechst Marion Roussel) with a washout period of at least 1 week in between. The subjects had fasted since 10 PM the evening before and were given a light meal 5 h after the start of the study session. The three doses were: intravenous infusion over 180 min at a rate of 1.66 mg/min, the same infusion at a rate of 3.33 mg/min, and a 600 mg controlled release tablet. Blood was sampled from an indwelling venous catheter (in the opposite arm to the infusion) into sodium heparin Venoptic® tubes. The sampling times were: for intravenous infusion before and at 5, 15, 30, 45, 60, 120, and 180 min during the infusion and at 15, 30, 45, 60 min and 2, 3, 4, 5, and 6 h after termination; for oral administration before and at 0.33, 0.66, 1, 1.3, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 14, 23, and 25 h after intake of the tablet. In order to minimize ex vivo metabolism of pentoxifylline and M1 the samples were immediately centrifuged at 4°C and the plasma was collected. Adverse events were recorded ad hoc during the study session and by interviewing the subject at the end of it.

From the plasma concentration data, the terminal half-lives of pentoxifylline and its metabolites were estimated by nonlinear regression using the RSTRIP software (MicroMath, Salt Lake City, UT, USA). Areas under the curves (AUC) were calculated by the logarithmic trapezoidal method from 0 to infinity using the MKMODEL software (N. Holford, Auckland, New Zealand). The AUC of (R)-M1 was, however, calculated as the AUC of (S)-M1 multiplied by the median ratio of plasma concentration ratio. Dose linearity of pentoxifylline was tested by paired Student's t-test on AUC/dose after the two intravenous administrations. Apparent clearance (CL\textsubscript{app}) of pentoxifylline was calculated as dose/AUC after the intravenous administrations and apparent bioavailability of pentoxifylline as AUC/dose after oral administration divided by AUC/dose after intravenous administration. The AUC of unbound drug or metabolite (AUC\textsubscript{u}) was calculated from the AUC based on total plasma concentrations by means of the f\textsubscript{u} values determined for the compounds in vitro. Finally, for comparisons of metabolite concentrations the AUC\textsubscript{u} in weight units (ng·h/ml) was recalculated to molar AUC\textsubscript{u}.

**RESULTS**

**HPLC**

The semipreparative separation of the enantiomers of M1 is illustrated in Figure 2. The capacity factors (k') for the separation were 2.7 and 3.7 for (S)- and (R)-M1, respectively. The amount separated at each injection was 2 µg of the compounds in vetro. Finally, for comparisons of metabolite concentrations the AUC\textsubscript{u} in weight units (ng·h/ml) was recalculated to molar AUC\textsubscript{u}.
When derivatization of M1 was performed as originally described, using 50 mg/ml diacetyl-L-tartaric acid anhydride in dichloromethane:glacial acetic acid (4:1) and heating to 55°C for 16 h, only 91% of M1 was derivatized. With heating to 75°C for 2, 4, or 6 h the yields were 88, 83, and 88%, respectively. When the concentration of the diacetyl-L-tartaric acid anhydride was increased to 100 mg/ml complete derivatization was achieved with heating at 75°C. Derivatization of pure (R)-M1 for either 2 or 6 h gave apparent S:R ratios of less than 1:1,500, indicating less than 0.07% formation of the wrong stereoisomer.

Final extraction of the M1 derivative with dichloromethane was found to be quantitative. After evaporation of the volatile components the samples could be left overnight at room temperature without degradation of either pentoxifylline or the derivatives of (R)-M1 and (S)-M1.

The standard curves of pentoxifylline and of (R)- and (S)-M1 were linear over the ranges 2.5–800 ng/ml. The limits of detection of pentoxifylline, (R)-M1 and (S)-M1 were 0.4, 0.7, and 0.6 ng/ml, respectively. The between-day coefficient of variation (CV) for determination of concentrations in plasma were 6.6% at 200 ng/ml of pentoxifylline (n = 35), 7.7% at 100 ng/ml of (R)-M1 (n = 35) and 7.6% at 100 ng/ml of (S)-M1 (n = 35). The standard curves of M4 and M5 were linear over 10–700 and 20–700 ng/ml and the limits of detection were 0.9 and 0.5 ng/ml, respectively. The between-day CVs were 12% at 200 ng/ml of M4 (n = 29) and 9.5% at 600 ng/ml of M5 (n = 29). Within-day accuracy and precision data for the assays are given in Table 1. The samples to which M1 had been added at R:S ratios of 0.03 or 0.05 showed measured ratios of 0.031 ± 0.0037 and 0.048 ± 0.0025, respectively, or CV = 12% and 5.3%.

After 308 days storage of pentoxifylline and its metabolites in stock solution at 23°C, 5°C, or −25°C comparison with freshly prepared solutions did not indicate any significant decrease in concentration (P > 0.11).

After 10 min of incubation of 2 µg/ml of pentoxifylline in blood (without NADPH) during cooling to room temperature, i.e., under similar conditions as during centrifugation of a blood sample to obtain plasma, 0.07% of the drug had been metabolized to M1. The same experiment with M1 showed a 0.3% conversion to pentoxifylline.

**Distribution of Pentoxifylline and Its Metabolites in Blood**

The λ, r, and f, of pentoxifylline and its metabolites are concentration-dependent, as illustrated in Figure 3, while fu was not. The difference in λ between the enantiomers of M1 was entirely due to the difference in f. The median and range f of pentoxifylline in the plasma from the subjects in the pharmacokinetic study was 0.70 (0.60–0.73) and the fu of (R)- and (S)-M1 were 0.58 (0.51–0.60) and 0.67 (0.58–0.72), respectively.

The λ’s of M4 and M5 could not be directly determined due to interfering peaks in chromatograms from whole blood. Their r values, however, imply very limited distribution to erythrocytes. Consequently, the λ values should not much exceed (1 − EVF), i.e., approximately 0.6, which is the λ of a compound that does not distribute to erythrocytes at all.

### TABLE 1. Accuracy and precision of the HPLC assays for pentoxifylline and its metabolites (M1, M4 and M5) in plasma

<table>
<thead>
<tr>
<th>Compound</th>
<th>Added concentration (ng/ml)</th>
<th>Found concentration (ng/ml, mean ± S.D.)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentoxifylline</td>
<td>3.0</td>
<td>3.0 ± 0.23</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>14.7 ± 0.43</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>149 ± 4.31</td>
<td>2.9</td>
</tr>
<tr>
<td>(R)-M1</td>
<td>3.0*</td>
<td>3.0 ± 0.18</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>15*</td>
<td>15.4 ± 0.84</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>150*</td>
<td>158 ± 4.46</td>
<td>2.8</td>
</tr>
<tr>
<td>(S)-M1</td>
<td>3.0*</td>
<td>3.0 ± 0.19</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>15*</td>
<td>15.8 ± 1.22</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>150*</td>
<td>152 ± 4.58</td>
<td>3.0</td>
</tr>
<tr>
<td>M4</td>
<td>3.0</td>
<td>3.0 ± 0.16</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>14.8 ± 0.80</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>151 ± 6.28</td>
<td>4.2</td>
</tr>
<tr>
<td>M5</td>
<td>15</td>
<td>14.5 ± 0.45</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>156 ± 4.32</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*As 6.0, 30, or 300 ng/ml of racemate.
The metabolism of pentoxifylline to (R)-M1 by heme-
ysed erythrocytes was best described by a one-
enzyme reaction model. The Michaelis-Menten parameter values
(population mean value and interindividual range) were
\( V_{\text{max}} = 0.041 (0.035 – 0.056) \) nmoles/min per 5 \( \times \) 10^9 erythro-
cytes and \( K_m = 11 \text{ mM} \). There was clear interindividual
variance only in \( V_{\text{max}} \). In contrast, the metabolism of pen-
toxifylline to (S)-M1 was best described by a two-
enzyme model, with interindividual variance only in the \( V_{\text{max}} \) of one
of the enzymes. The parameter values of the two enzymes
were \( V_{\text{max}} = 0.054 (0.031–0.096) \) nmoles/min per 5 \( \times \) 10^9
erythrocytes and \( K_m = 1.1 \text{ mM} \), and \( V_{\text{max}} = 0.86 \) nmoles/
min per 5 \( \times \) 10^9 erythrocytes and \( K_m = 132 \text{ mM} \), respec-
tively. At a therapeutic blood concentration of pentoxifyl-
line (0.5 \( \mu \text{g/ml} \), 1.8 \( \mu \text{M} \)) the calculated rates of formation of
(R)- and (S)-M1 were \( 6.8 \times 10^{-6} \) and \( 1.0 \times 10^{-4} \) nmoles/min
per 5 \( \times \) 10^9 erythrocytes, and the low \( K_m \) enzyme would
account for 89% of the metabolism to (S)-M1. The rate of
formation of (S)-M1 would thus be 15 times higher than
that of the (R)-enantiomer.

At the concentrations investigated (0.022 and 0.072 mM)
of (R)- and (S)-M1, the median rates of formation of pen-
toxifylline from (R)-M1 were \( 6.5 \times 10^{-4} \) and \( 1.9 \times 10^{-3} \)
nmoles/min per 5 \( \times \) 10^9 erythrocytes, respectively, and
from (S)-M1 2.2 \( \times \) 10^{-3} and \( 5.0 \times 10^{-3} \) nmoles/min per 5 \( \times \)
10^9 erythrocytes. The formation from (S)-M1 was thus ap-
proximately 3 times faster at these concentrations. By ex-
trapolation, the rate of formation of pentoxifylline from (R)-
M1 would be \( 7.2 \times 10^{-7} \) nmoles/min in 1 ml of blood at an
in vivo concentration of 0.02 \( \mu \text{g/ml} \) (0.07 \( \mu \text{M} \)) while the
formation from (S)-M1 at its 50-fold higher in vivo concen-
tration (see below) would be \( 1.5 \times 10^{-4} \) nmoles/min. If the
two enantiomers were present at an equal concentration of
0.5 \( \mu \text{g/ml} \), the calculated rates of formation of pentoxifylline would be \( 1.8 \times 10^{-5} \) and \( 7.4 \times 10^{-5} \) nmoles/
min per 5 \( \times \) 10^9 erythrocytes from (R)- and (S)-M1, respec-
tively, i.e., a 4-fold higher rate of formation from the (S)-
enantiomer.

### Inhibition of the Metabolism of Pentoxifylline to M1

The effects of the various enzyme inhibitors are shown
in Figure 4. The metabolism of pentoxifylline both to (R)-
and (S)-M1 was inhibited by ethacrynic acid, daunorubicin,
and menadione but not by SKF525-A or ketamine. Inhibi-
tion by phenobarbital could not be evaluated due to inter-
ference with the HPLC assay of M1.

### Pharmacokinetics in Humans

Plasma concentration curves of pentoxifylline and its me-
tabolites after administration of oral and intravenous pen-
toxifylline are shown in Figures 5, 6 and corresponding
pharmacokinetic data given in Table 3. In one subject the
high-dose infusion (600 mg/3 h) had to be stopped after 1
h due to nausea. The extrapolated parts of the AUCs of
pentoxifylline accounted at most for 0.2, 0.2, and 1.6% of the
total AUC after intravenous (300 and 600 mg) and oral
administration, respectively. The bioavailability of oral pen-
toxifylline (median and range) was 35 (24–47)% when cal-
culated in comparison to the 300-mg intravenous dose and
28 (20–46)% in comparison to the 600-mg intravenous dose.
The difference in plasma AUC/dose of pentoxifylline after
administration of 300 or 600 mg intravenously was statisti-

---

**TABLE 2. Blood:plasma concentration ratio (\( \lambda \)), erythrocyte:buffer concentration ratio (\( r_e \)), and unbound fraction in plasma (\( f_u \)) of pentoxifylline and its metabolites (M1, M4, and M5) in blood from four healthy volunteers with normal
erythrocyte volume fractions (EVF). Figures are five determinations in duplicate in each, mean ± SD.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>EVF</th>
<th>( \lambda )</th>
<th>( r_e )</th>
<th>( f_u )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentoxifylline</td>
<td>0.41 ± 0.045</td>
<td>—</td>
<td>—</td>
<td>0.65 ± 0.040</td>
</tr>
<tr>
<td>(R)-M1</td>
<td>0.41 ± 0.045</td>
<td>0.77 ± 0.059</td>
<td>—</td>
<td>0.54 ± 0.057</td>
</tr>
<tr>
<td>(S)-M1</td>
<td>0.41 ± 0.045</td>
<td>0.84 ± 0.063</td>
<td>1.09 ± 0.104</td>
<td>0.83 ± 0.017</td>
</tr>
<tr>
<td>Ratio R:S</td>
<td>0.41 ± 0.027</td>
<td>0.92 ± 0.004</td>
<td>0.96 ± 0.051</td>
<td>0.83 ± 0.017</td>
</tr>
<tr>
<td>M4</td>
<td>0.41 ± 0.027</td>
<td>—</td>
<td>0.19 ± 0.17</td>
<td>0.62 ± 0.046</td>
</tr>
<tr>
<td>M5</td>
<td>0.41 ± 0.027</td>
<td>—</td>
<td>0.13 ± 0.14</td>
<td>0.58 ± 0.058</td>
</tr>
</tbody>
</table>

*Concentration-dependent, see Figure 3.
Not measured because of technical problems.
cally significant \((P < 0.05)\), indicating a lower CL app at the higher dose. However, conversion of the plasma concentrations to blood concentrations by means of the concentration-dependent \(\lambda\) determined in vitro (Fig. 3) abolished this significance. Calculated whole-blood CL app was 4.4 (3.0–6.3) L/min after the 300-mg and 4.4 (2.7–4.9) L/min after the 600-mg infusion.

The R:S plasma concentration ratio of M1 showed no change over time, during or after the administration of pentoxifylline. The overall median R:S ratio in each subject ranged from 0.010–0.018 during and after the 300-mg infusion, from 0.013–0.025 with the 600 mg oral dose. The higher ratio after oral administration was statistically significant, \(P < 0.05\) in two and \(P < 0.01\) in three subjects (Wilcoxon rank sum test of 600 mg orally vs. 600 mg infusion). When pentoxifylline was administered orally M4 and M5 were also formed to a greater extent, in relation to the AUCu of pentoxifylline and (S)-M1, than after intravenous administration (\(P < 0.05\) and 0.001, compared to 300 and 600 mg, respectively). The plasma concentrations of all metabolites declined in parallel with pentoxifylline, indicating formation rate-limited disposition.

**DISCUSSION**

The semipreparative chromatography of the enantiomers of pentoxifylline metabolite 1 on a cellobiohydrolase column is to our knowledge the first reported direct separation of these molecules. For the quantitative analysis of pentoxifylline and the enantiomers of M1 we adopted and modified a published method\(^{12}\) entailing derivatization with diacetyl-L-tartaric acid anhydride to form diastereoisomeric esters of rac-M1. Since the R:S concentration ratios of M1 in the biological samples were very low, it was imperative to show that the small amounts of (R)-enantiomer found were not formed by racemization during the derivatization. In addition, it had to be ascertained that peaks ascribed to the (R)-enantiomer were not due to the reaction of the (S)-enantiomer with an optically impure reagent. We found that formation of the "wrong" diastereoisomer during derivatization gave rise to apparent enantiomeric ratios of less than 1:1,500, as compared to at least 1:100 in the biological samples. Adequate precision of the assay could also be documented at (R)-M1 concentrations of 3.0 and 5.0 ng/ml in the presence of 33- and 20-fold higher concentrations of (S)-M1 (there was no need to investigate opposite R:S concentration ratios since these were never found in biological samples). The assay of M4 and M5 was an adaptation of a published method,\(^{23}\) and accuracy and precision were documented as needed for our study.

Pentoxifylline is reduced to M1 in human liver. In the cytosol, which is the predominant site of metabolism, only (S)-M1 is formed, while the reduction in microsomes is 85% stereoselective in favor of (S)-M1.\(^{12}\) Microsomes from human liver oxidize (S)-M1 back to pentoxifylline, while (R)-M1 is metabolized both to pentoxifylline and to the diol metabolite, M3.\(^{12,24,25}\) These findings are in qualitative agreement with the observed stereoselectivity of pentoxifylline disposition in vivo. However, liver is apparently not the main site of the pentoxifylline-M1 interconversion. The CL app of pentoxifylline exceeds liver blood flow\(^{10,13}\) by 2–4-fold. In the absence of significant renal excretion, this in-
pentoxylline to M1. This metabolism in blood has been
erythrocytes appear to be the main site of the reduction of
was partially purified from human erythrocytes.29 The Km
reaction.27 Erythrocytes from rats and humans reduced
daunorubicin to daunorubicinol in an NADPH-dependent
man blood components reduce the antineoplastic agent
of the reaction nor the conversion of M1 back
to pentoxifylline have been investigated.

AUC/dose* 12 (9.2 – 16)
AUC/dose 12 (9.2 – 16)
Terminal t1/2 (h) 0.81 (0.61 – 0.93)
Tmax (h) 3.0 (2.0 – 3.1)
Cmax (ng/ml) 1222 (897 – 1558)

Intravenous, 300 mg
(n = 5)

Intravenous, 600 mg
(n = 6)

Oral, 600 mg
(n = 6)

Molar AUC ratio (R)-M1/Ptx 0.02 (0.00 – 0.05)
Molar AUC ratio (S)-M1/Ptx 3.0 (2.4 – 3.7)
Molar AUC ratio M4/Ptx 0.25 (0.19 – 0.72)
Molar AUC ratio M5/Ptx 2.6 (1.9 – 3.5)

Pharmacokinetic parameters of pentoxylline (Ptx) and its metabolites (M1, M4, M5) after intravenous or oral administration of pentoxylline, median (range)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parameter</th>
<th>Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intravenous, 300 mg</td>
</tr>
<tr>
<td>Pentoxifylline</td>
<td>AUC/dose*</td>
<td>4.0 (2.7 – 6.0)</td>
</tr>
<tr>
<td></td>
<td>AUC/dose</td>
<td>2.7 (2.0 – 3.6)</td>
</tr>
<tr>
<td></td>
<td>Cl_{app} (L/min)</td>
<td>4.2 (2.8 – 6.3)</td>
</tr>
<tr>
<td></td>
<td>Terminal t1/2 (h)</td>
<td>0.90 (0.75 – 1.0)</td>
</tr>
<tr>
<td></td>
<td>Tmax (h)</td>
<td>2.5 (1.0 – 3.0)</td>
</tr>
<tr>
<td></td>
<td>Cmax (ng/ml)</td>
<td>428 (332 – 625)</td>
</tr>
<tr>
<td>(R)-M1</td>
<td>AUC/dose*</td>
<td>0.14 (0.06 – 0.26)</td>
</tr>
<tr>
<td></td>
<td>AUC/dose</td>
<td>0.07 (0.03 – 0.16)</td>
</tr>
<tr>
<td></td>
<td>Terminal t1/2 (h)</td>
<td>0.94 (0.76 – 1.0)</td>
</tr>
<tr>
<td></td>
<td>Tmax (h)</td>
<td>3.3 (3.0 – 3.3)</td>
</tr>
<tr>
<td></td>
<td>Cmax (ng/ml)</td>
<td>14 (7.9 – 27)</td>
</tr>
</tbody>
</table>

*Area under the curve (ng × h/ml) divided by dose of pentoxylline in mg.

dicates that most of the metabolism takes place outside the
liver. AS regards transformation specifically to M1, the
AUC ratios of M1 to pentoxylline were not different be-
tween patients with cirrhosis and healthy volunteers, even
though the total clearance of pentoxylline was reduced by
60% in the former group.15 Renal insufficiency did not in-
fluence the AUC ratios of M1 to pentoxylline.26 Thus, the
erthrocytes appear to be the main site of the reduction of
pentoxifylline to M1. This metabolism in blood has been
described previously,14 but neither the stereochemistry
and kinetics of the reaction nor the conversion of M1 back
to pentoxifylline have been investigated.

The NADPH-dependent reduction of a ketone group in a
drug by erythrocytes has been described previously. Hu-
mans components reduce the antineoplastic agent
daunorubicin to daunorubicinol in an NADPH-dependent
reaction.27 Erythrocytes from rats and humans reduced
donol, a beta-adrenergic blocking agent, to dihydrobuno-
lor in the presence of NADPH and also catalyzed the re-
verse reaction with NADP as cofactor.28 Later, an NADPH-
dependent ketone reductase that catalyzed the transforma-
tion of 4-nitroacetophenone to 4-nitrophenylmethylcarbinol
was partially purified from human erythrocytes.28 The K_m
value was 0.39 mM. Haloperidol was transformed to re-
duced haloperidol in human liver cytosol, with K_m values of
0.61 and 0.50 mM in two liver samples.21 The same reduc-
tion takes place in human erythrocytes,22,30 and K_m values
ranging from 0.16 – 2.6 mM in six individuals have been
reported.31 Also, the oral antidiabetic agent acetohexamide
was reduced to hydroxyhexamide in human liver and
erthrocytes. The V_max and K_m values in human erythro-
cyttes were 9.2 ± 0.88 nmoles/min/g hemoglobin and 0.70
blood,22 while ethacrynic acid, menadione, and daunorubi-
cin, but not SKF525-A, inhibited haloperidol reduction in
the liver.22,30 The same pattern of inhibitor activities was found
also for the reduction of pentoxylline. We also investigat-
gated putative inhibition by ketamine, another ketone
STEREOSELECTIVE METABOLISM OF PENTOXIFYLLINE

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drug, but did not find any. From the similarities in inhibitor pattern and in \(K_m\) values of the enzymes, it seems clear that pentoxifylline is also reduced by enzymes of the ketone reductase (or carbonyl reductase\(^{32,35}\)) family. A contribution from aldehyde reductase cannot, however, be completely ruled out.

Hydrogen transfer from NADPH is stereospecific for a given enzyme and substrate.\(^ {33,34}\) The reduction of haloperidol by ketone reductase was shown to be highly stereoselective. More than 99% of the reduced haloperidol produced in the human putamen, liver, and blood had the (S)-configuration.\(^ {22}\) Pentoxifylline was also reduced mainly to the (S)-enantiomer of M1. A Lineweaver-Burke plot of the in vitro data was not linear and the enzyme kinetics could only be explained by the action of two enzymes; one low \(K_m\) (1.1 mM), low capacity enzyme, which would account for most of the reaction at therapeutic concentrations, and a second enzyme with a higher \(V_{max}\) but a very high \(K_m\) (132 mM). Reduction of pentoxifylline to (R)-M1, on the other hand, appeared to be catalyzed by one enzyme with a comparatively high \(K_m\) (11 mM).

Significant interindividual variance was found for the \(V_{max}\) values of the enzymes, presumably reflecting different amounts of enzyme in the erythrocytes of the subjects. No significant variance in \(K_m\) is expected if the enzymes are of identical structure in the different subjects, and none was found.

In accordance with most of the literature,\(^ {27,28,30-32}\) the reversible metabolism of pentoxifylline and M1 was investigated using hemolysed erythrocytes instead of whole blood. The same metabolic reactions were found also in similar incubations with fresh blood (data not shown). However, the extent of biotransformation in blood samples at room temperature was far too modest to bias the results of the in vivo pharmacokinetic study.

From the in vitro data we estimated a 15-fold faster formation of (S)- than of (R)-M1 in erythrocytes at therapeutic concentrations of pentoxifylline. This seems to be the main reason for the marked difference in plasma concentration and AUC of the two enantiomers. Complete kinetic characterization of the in vitro conversion of (R)- and (S)-M1 to pentoxifylline was not feasible because of the limited amounts of pure enantiomer that could be prepared by means of the chiral column chromatography. We could, however, demonstrate that the formation of pentoxifylline was 3–4-fold faster from (S)-M1 than from the (R)-enantiomer at concentrations encountered in vivo. The data were thus adequate to reject the alternative hypothesis of a very fast back-conversion of the (R)-enantiomer. In addition, the total CL of (R)-M1 (lisofylline) has been determined by intravenous infusion in healthy volunteers\(^ {16}\) and proved to be around 1.3 L/min in a 70-kg person. This is considerably less than the CL\(_{app}\) of pentoxifylline.

The plasma concentrations of M4 and M5 after intravenous infusion of pentoxifylline have not been reported previously. The comparison of the AUC ratio of each metabolite to pentoxifylline between oral and intravenous administration shows that these ratios are higher after oral administration for the oxidative metabolites M4 and M5 but not for total concentration of M1. Enhanced formation of M4 and M5 but not M1 during the first pass metabolism of orally absorbed pentoxifylline further confirms the difference in sites of the two different metabolic pathways. The slight difference in M1 enantiomeric ratio after intravenous and oral administration does, however, show some influence of first-pass metabolism, possibly suggesting a contribution of the liver\(^ {12}\) to the formation of (R)-M1.

In the four cancer patients who received pentoxifylline by intravenous infusion during treatment with ciprofloxacin and interleukin-2, the RS concentration ratios of M1 ranged between 0.06–0.21 at the \(C_{max}\) of pentoxifylline.\(^ {18}\) Apparently, (R)-M1 could not be measured at the trough concentrations. These concentration ratios are considerably higher than those reported here. Whether this is due to a drug interaction, and if so with which drug, or to other factors is not clear. No details about the chiral HPLC assay are given in the article.\(^ {18}\)

The dose-dependency of plasma AUC/dose of pentoxifylline that we found appeared at least in part to be due to the concentration-dependence in \(\lambda\). The plasma CL\(_{app}\) could thus become nonlinear even if the true CL from the blood is not. This same explanation might be invoked for the observation of a more than dose-proportional increase in plasma AUC of pentoxifylline after administration of 100, 200, and 400 mg as an oral solution.\(^ {6}\) Since absorption from this solution was very rapid, the mean plasma \(C_{max}\) after the 400-mg dose (1607 ng/ml) was much higher than after a similar dose given as a slow-release tablet, and also higher than any \(C_{max}\) in our study (cf. Table 3). This would augment the influence of the concentration-dependent \(\lambda\).

The CL\(_{app}\) of pentoxifylline, 2.6 L/min, found here is similar to data from two other studies.\(^ {10,13}\) In comparison, total liver blood flow can be estimated at around 1.5 L/min in young, healthy volunteers.\(^ {30}\) A plasma CL of "only" 1.3 L/min was reported in a third study.\(^ {5}\) The reason for this discrepancy is not clear. More to the point, however, is that the true CL of pentoxifylline is even higher. When reversible metabolism occurs, calculation of CL\(_{app}\) by the standard formula Dose/AUC underestimates CL\(_{app}\) since some of the drug that has been cleared reversibly will return to the circulation. Measurement of this reverse CL of M1 to pentoxifylline would require infusion of (S)-M1 to the subjects, which is not feasible since a pharmaceutical-grade substance is not available. However, that the true CL of pentoxifylline is actually greater than its already large CL\(_{app}\) even further emphasizes the mainly extrahepatic metabolism of the drug.

In addition, the volume of distribution at steady state (V\(_{des}\)) is overestimated by the standard procedure (moment analysis).\(^ {30,37}\) The true V\(_{des}\) value, which refers to distribution of unchanged drug, is confounded by an apparent "distribution" of pentoxifylline by reversible metabolism (i.e., disappearance and reappearance of pentoxifylline in the circulation due to reversible metabolism cannot be distinguished from disappearance and reappearance due to distribution). We therefore refrained from any calculation of V\(_{des}\).

The terminal half-lives of pentoxifylline and its metabolites and the observation of formation rate-limited metabolite pharmacokinetics are in general agreement with pre-
vious findings.\textsuperscript{5,6,10,13} The longer terminal $t_{1/2}$ of pentoxifylline and its metabolites seen after oral administration does not reflect elimination but instead slow absorption from the controlled release tablet.

In vitro, M1 and M5 improve erythrocyte flexibility with potencies similar to that of pentoxifylline, and in some tests of platelet aggregation the two metabolites are more potent than pentoxifylline.\textsuperscript{14} In some tests of in vitro, M1 and M5 improve erythrocyte flexibility with potencies similar to that of pentoxifylline, and in some tests of platelet aggregation the two metabolites are more potent than pentoxifylline.\textsuperscript{14} It has not been ascertained whether (R)-M1, (S)-M1, or both, is the active species. There were 2–5-fold interindividual variations in AUC$_{0-\infty}$ of these metabolites after intravenous, and 2–4-fold variations after oral administration of pentoxifylline (see Table 3). It remains to be investigated to what extent this could underlie interindividual variation in clinical effects.

ACKNOWLEDGMENT

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REFERENCES


A placebo-controlled study of retinal blood flow changes by pentoxifylline and metabolites in humans

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Aim
To investigate the possible effects of pentoxifylline metabolites on retinal blood flow in humans.

Methods
A randomized, placebo-controlled, four-period cross-over study that was observer blinded and partly blinded for the eight participants. On one occasion a placebo was given as an intravenous (i.v.) infusion over 100 min. On the other three occasions pentoxifylline was administered as i.v. infusions over 100 min at a rate of 3 mg min$^{-1}$. Before two of the pentoxifylline infusions the subjects were pretreated with either ciprofloxacin or rifampicin. Retinal blood flow was measured by scanning laser doppler flowmetry (SLDF) in a selected area of the central temporal retina before, during and until 5 h after the end of infusion. Blood samples for concentration analyses of pentoxifylline, R-M1, S-M1, M4 and M5 were taken serially and areas under the curves (AUCs) were calculated. Linear mixed models were used for the statistical analyses.

Results
Mean AUCs (ng h ml$^{-1}$) were significantly increased for pentoxifylline (1964 vs. 1453) and S-M1 (5804 vs. 4227), but not R-M1 when pentoxifylline was co-administered with ciprofloxacin. The mean AUC for M5 was significantly reduced when subjects were pretreated with rifampicin (2041 vs. 3080). Pentoxifylline with and without pretreatment with rifampicin significantly increased retinal blood flow assessed as mean flow, pulsation (i.e. 1-systole/diastole), and diastolic flow (but not during systole), compared with placebo. The increases over placebo were more pronounced on diastolic flow, 9.7% (95% confidence interval 4.2, 15.5) than on mean flow, 4.6% (1.1, 8.3) after pentoxifylline administration. With pentoxifylline after rifampicin pretreatment the corresponding differences were 11.7% (5.8, 17.9) and 5.1% (1.4, 7.8) over placebo, respectively. After co-administration of pentoxifylline and ciprofloxacin we saw only a nonsignificant trend towards increased flow during diastole, but a significant decrease in pulsation. When AUCs for pentoxifylline and its metabolites were used as regressor variables to retinal mean flow we found that pentoxifylline, R-M1 and M5 had coefficients with a positive sign indicating that they enhanced the retinal blood flow. In contrast, S-M1 and M4 had coefficients with negative sign and thus appeared to decrease the blood flow in subjects treated with pentoxifylline.

Conclusion
The R-M1 and M5 metabolites of pentoxifylline contributed significantly to the effects of pentoxifylline on retinal blood flow.
Introduction
Pentoxifylline, 3,7-dimethyl-1(5′-oxo-hexyl)xanthine, is a haemorheological drug widely used for the treatment of intermittent claudication [1–3]. However, the clinical efficacy of the drug for this indication is still controversial. It has also been suggested that pentoxifylline may be used in diseases affecting retinal blood flow, such as diabetic retinopathy [4–7] or macular degeneration [8]. The therapeutic effect of pentoxifylline in these conditions would be to increase capillary blood flow by increasing deformability of both erythrocytes and leucocytes as well as by a possible direct vasodilatory effect.

Pentoxifylline is metabolized in humans into at least seven metabolites, denoted M1–M7 [9–12]. Reduction of the 5′-oxo group gives the hydroxy metabolite M1, 3,7-dimethyl-1(5′hydroxyhexyl)xanthine. This creates a chiral centre in the molecule, yielding S-M1 as a major and R-M1 as minor metabolite [13, 14]. This metabolism takes place both in the liver [13] and in the erythrocytes [14, 15] and is reversible [10, 13, 14]. Thus, during treatment with pentoxifylline the plasma concentrations of the parent drug, R-M1 and S-M1 rapidly attain equilibrium. Hepatic metabolism produces the carboxylic acid metabolites M4 and M5; M5 is the major excreted form [9–12]. Both M1 (as the racemic form) and M5 have been shown to influence erythrocyte deformability and platelet aggregation in vitro and may thus contribute to the in vivo haemorheological effects of pentoxifylline [16].

The aim of this study was to investigate the possible contributions of the metabolites (M1, M4 and M5) of pentoxifylline to its haemorheological effect, assessed as retinal blood flow, in humans. Scanning laser doppler flowmetry (SLDF) [17] in a selected area of the central temporal retina was used to investigate drug effects on blood flow in small capillaries. When pentoxifylline is administered there is considerable co-variation between the concentrations of pentoxifylline and its metabolites [14, unpublished]. This makes it very difficult to separate any effects of the metabolites from that of the parent compound. In this study we therefore co-administered pentoxifylline and an inhibitor of CYP1A2 (ciprofloxacin) on one occasion [18, 19], and gave pentoxifylline after pretreatment with an inducer of several enzymes of the cytochrome P450 system (rifampicin) on another occasion [20], in order to create different blood concentration ratios between the metabolites and pentoxifylline in the same subject. The study was placebo controlled with a cross-over design using four treatment arms.

Methods
Study design
The study was approved by the Ethics Committee of Lund University and by the Swedish Medical Products Agency. After giving written informed consent, eight healthy, nonsmoking volunteers (six males, 24–42 years, 73–100 kg, and two females, 21–24 years, 65–66 kg), who were free of medication and had no history of allergy to drugs, were included in the study. Each subject passed a prestudy ophthalmic examination, where inclusion criteria were normal ocular findings, no previously known eye pathology, full visual acuity and ametropia less than 3 diopters. The study was randomized, placebo controlled, observer blinded, and partly blinded for the subject in a four-period cross-over design.

All subjects were given the four treatments in random order. During one session they were given placebo (0.9% saline solution) by intravenous (i.v.) infusion over 100 min. During the three other sessions they were given pentoxifylline (Trental, Hoechst Marion Roussel) by i.v. infusion over 100 min at a rate of 3 mg min⁻¹. The volumes infused and the concentrations of pentoxifylline in the solution were measured. In one session the subjects were pretreated with a 750-mg ciprofloxacin tablet (Ciproxin, Bayer) 1 h before start of the infusion. Before another they took one rifampicin 600-mg tablet (Rimactan, Swedish Orphan) daily for 7 days, the last dose being taken approximately 24 h before the start of the pentoxifylline infusion.

The pretreatments with ciprofloxacin and rifampicin could not be blinded but the pentoxifylline and placebo administrations were.

The subjects had fasted since 22.00 h the evening before the study day and were not allowed to take any other medication except for occasional paracetamol 48 h preceding a study day and during the pretreatment with rifampicin. They were given a light meal 4 h after the start of the infusion. The study periods were separated by at least 1 week and after the pretreatment with rifampicin by 2 weeks. Adverse events were assessed before and during the study days by open-ended questions.

Analysis of pentoxifylline and its metabolites
Blood was sampled from an indwelling venous catheter (in the opposite arm from the infusion) into sodium heparin Venoject® tubes. The sampling times were: before and at 20, 45 and 85 min during the infusion period and at 0.5, 1, 2, 3, 4 and 5 h after its termination (in the first four subjects also at 6 h). In order to minimize ex vivo interconversion of pentoxi-
pentoxifylline gave an increase by 17 subjects [7] an i.v. infusion over 90 min of 200 mg of
lowing assumptions. In an earlier study on 10 healthy
The number of subjects recruited was based on the fol-
1-diastole/systole).
variables used were mean blood flow, blood flow during
vessels with simultaneous evaluation of blood flow
SLDF provides a high-definition tomographic image of perfused retinal
effect analyser program) [21]. The variables used were mean flow velocity and mean quantity of blood cells in
retina, and one picture included an area of 2.7 × 0.7 mm,
at baseline, at 45 and 85 min during the infusion period
and at 0.5, 1, 2, 3, 4 and 5 h after termination (in the
first four subjects also at 6 h). At all the time points for
measurements four pictures of the same area were taken
and the three technically best ones were assessed.
Quantification of capillary retinal blood flow was
stated in arbitrary units (AUs) describing the product of
mean flow velocity and mean quantity of blood cells in
a standardized volume. The mean values from each time
point were used in the calculations. The readings were
analysed using the AFFPIA software (automatic full
field perfusion image analyser program) [21]. The vari-
ables used were mean blood flow, blood flow during
systole, blood flow during diastole, and pulsation (i.e.
1-diastole/systole).

Data presentation and statistical considerations
The number of subjects recruited was based on the fol-
lowing assumptions. In an earlier study on 10 healthy
subjects [7] an i.v. infusion over 90 min of 200 mg of
pentoxifylline gave an increase by 17 ± 9% (mean ± SD
between subjects) and the 400-mg dose gave an increase
by 27 ± 12% in ocular fundus pulsation. We anticipated
that a 300-mg dose over 100 min would give a 23 ± 10% increase in ocular fundus pulsation between subjects,
compared with placebo. Since, in the current study, we
compare the effects within the same subject, we made a
series of power calculations assuming different within-
subject correlations, number of subjects, and detectable
differences, e.g. using eight subjects we would be able
to detect a difference of 8% with a power of at least
80%, at a two-tailed P-value <0.05, if the intrasubject
correlation was 0.75. This was calculated for a single
measurement of maximum effect. When calculations
were based on integrated effects (i.e. several measure-
ments over time) the power to detect differences were
anticipated to be even better.
The volumes infused and the concentrations of pen-
toxifylline in the solution were used for calculation of
the actual doses given. The resulting concentrations
were re-calculated to a nominal dose of 300 mg. In the
pharmacokinetic analyses we similarly used AUCs
recalculated to a nominal dose of 300 mg. However,
AUCs calculated from the observed plasma concentra-
tions were used as input in the concentration–effect
analyses described below. For statistical analysis the
MIXED procedure in SAS (version 8.2; SAS Institute,
Cary, NC, USA) was used. In the analysis of the AUCs
we used the treatments given as fixed effects and sub-
jects were entered as random effects.
We found that there were no significant changes over
time within each study day. Thus, we used all observa-
tions after dose in a repeated measures design. Further,
the observed effects were rather low, limiting the possi-
bility for detection of deviations from linear relations
between effect and regressor variables. Two main statis-
tical models were tested after an initial check for lack
of period effects. The first one comprised only the treat-
ments given as fixed effects, thus disregarding the
obtained plasma concentration data. In the second
model treatments were not included; instead, the AUCs
of pentoxifylline and its metabolites were used as
regressor variables. Such a model can be successfully
used only if the correlations between the regressors are
low. Both models used subjects as random effects, and
a repeated measures spatial exponential covariance
structure design.
Since one subject dropped out, the study was not
completely balanced and for both the kinetic and the
dynamic analyses least-square means were obtained
from the mixed model, thus providing the marginal
means that would be expected had the study been com-
pletely balanced with no missing data. Arithmetic least-
square means and 95% confidence limits are given,
extcept when relative changes in the effect model com-
prising treatments as fixed effects are presented, in
which case geometric least-square means and 95% con-
fidence limits are given. Statistical significance was
accepted at P < 0.05 (two-tailed).
Results
The treatments were generally well tolerated, but with some exceptions. Thus, in one subject (with no pretreatment) we stopped the infusion of pentoxifylline after 50 min due to nausea. All observations have been included in the analysis as appropriate (i.e. actual AUCs in the effect analysis). In another subject the rifampicin pretreatment was stopped after only one dose due to diarrhoea, and the planned infusion of pentoxifylline was therefore not given. Yet another subject did not receive placebo due to logistic reasons. All other drug treatments were administered as scheduled.

Plasma concentration curves of pentoxifylline, R-M1, S-M1, M4 and M5 after i.v. administration of the drug alone and after pretreatment with ciprofloxacin or rifampicin are shown in Figure 1. Observed plasma concentrations were used for the calculation of the AUC values that are given in Table 1. AUC for pentoxifylline and S-M1 were significantly higher after pretreatment with ciprofloxacin compared with pentoxifylline administration alone. The effect on R-M1 was not statistically significant. However, the R:S plasma concentration ratio remained unchanged at mean values of 0.019 after pentoxifylline alone and 0.020 after pentoxifylline and ciprofloxacin (P = 0.72 for the difference between ratios). The AUC for M5 was significantly lower after pretreatment with rifampicin compared with pentoxifylline alone.

The correlation matrix for the AUCs of pentoxifylline and its metabolites is given in Table 2. Generally, the absolute values of the correlations were low, with the exception of that between M4 and M5.

Retinal blood flow measured as mean flow, flow during systole and diastole, as well as pulsation, before, during and after the four treatments are shown in Figure 2. The corresponding mean values during and after the treatments are given in Table 3, as are least-square means of absolute and relative estimates after the different treatments. Least-square mean values of relative differences between the treatments are given in Table 4. In comparison with placebo, pentoxifylline alone and after pretreatment with rifampicin significantly increased retinal blood flow during diastole (P = 0.0014 and P = 0.0004, respectively) but not during systole (P = 0.1476 and P = 0.4366, respectively). This was reflected in a rise of mean flow and decreased pulsations. Pentoxifylline after pretreatment with ciprofloxacin resulted only in a nonsignificant trend (P = 0.0604) towards increased flow during diastole, but caused a significant (P = 0.0096) decrease in pulsation.

The estimated intercepts and coefficients from the mixed model where the AUCs are used as regressor variables are shown in Table 5. Overall, for the flow parameters, pentoxifylline, R-M1 and M5 had coefficients with positive signs, indicating that they enhanced the retinal blood flow. In contrast, S-M1 and M4 had...
### Table 1

Mean [min–max] observed AUC of pentoxifylline (Ptx) and its metabolites (R-M1, S-M1, M4 and M5) after administration of pentoxifylline 300 mg intravenous infusion alone or in combination with a ciprofloxacin (PtxC) 750-mg tablet or pretreatment with rifampicin (PtxR) 600-mg tablets once daily for 7 days.

<table>
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<tr>
<th></th>
<th>AUC Ptx, ng h ml(^{-1})</th>
<th>AUC RM1, ng h ml(^{-1})</th>
<th>AUC SM1, ng h ml(^{-1})</th>
<th>AUC M4, ng h ml(^{-1})</th>
<th>AUC M5, ng h ml(^{-1})</th>
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<td>4227</td>
<td>406</td>
<td>3221</td>
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<td>[2402–5911]</td>
<td>[193–833]</td>
<td>[1048–6226]</td>
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<td>117</td>
<td>5804</td>
<td>484</td>
<td>3080</td>
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<td></td>
<td>[698–2556]</td>
<td>[53–173]</td>
<td>[4322–7470]</td>
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<td>4134</td>
<td>281</td>
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<td>4370</td>
<td>430</td>
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<td></td>
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<td>(2531, 4201)</td>
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<td>116</td>
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<td>488</td>
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<td>(4864, 6698)</td>
<td>(350, 625)</td>
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<td>78</td>
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<td>PtxC-Ptx</td>
<td>431*</td>
<td>31</td>
<td>1411*</td>
<td>58</td>
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<td></td>
<td>(56, 809)</td>
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<td>(858, 1964)</td>
<td>(−132, 247)</td>
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<td>−1716*</td>
<td>−200*</td>
<td>−1000</td>
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<td>(−73, 4)</td>
<td>(−2297, 1135)</td>
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<td>(−2020, 19)</td>
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<tr>
<td>PtxC/Ptx</td>
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<td>1.48*</td>
<td>1.36*</td>
<td>1.19</td>
<td>1.01</td>
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<td>(1.05, 1.64)</td>
<td>(1.02, 2.14)</td>
<td>(1.18, 1.57)</td>
<td>(0.79, 1.78)</td>
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<td>0.938</td>
<td>1.00</td>
<td>0.96</td>
<td>0.73</td>
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<td>(0.74, 1.19)</td>
<td>(0.68, 1.46)</td>
<td>(0.83, 1.12)</td>
<td>(0.48, 1.12)</td>
<td>(0.50, 0.90)</td>
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<tr>
<td>PtxR/PtxC</td>
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<td>0.68</td>
<td>0.71*</td>
<td>0.62</td>
<td>0.66*</td>
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<tr>
<td></td>
<td>(0.56, 0.90)</td>
<td>(0.46, 1.00)</td>
<td>(0.61, 0.83)</td>
<td>(0.40, 0.93)</td>
<td>(0.49, 0.89)</td>
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Least-square estimates of means (95% confidence intervals) and their differences and ratios for AUC of pentoxifylline and its metabolites after administration of pentoxifylline 300 mg intravenous infusion alone or in combination with a ciprofloxacin 750-mg tablet or pretreatment with rifampicin 600-mg tablets once daily for 7 days. Additionally, for the least-square estimates the AUCs have been re-calculated to correspond to a nominal dose of 300 mg pentoxifylline. *P < 0.05.

### Table 2

Correlation matrix between observed AUC values for pentoxifylline (Ptx) and its metabolites (R-M1, S-M1, M4, and M5) after administration of pentoxifylline 300 mg intravenous infusion alone and in combination with ciprofloxacin 750-mg tablet and pretreatment with rifampicin 600-mg tablets once daily for 7 days, on three separate occasions.

<table>
<thead>
<tr>
<th></th>
<th>Intercept</th>
<th>AUC Ptx</th>
<th>AUC R-M1</th>
<th>AUC S-M1</th>
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<th>AUC M5</th>
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</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1</td>
<td>−0.04013</td>
<td>0.02184</td>
<td>−0.00039</td>
<td>0.02069</td>
<td>−0.02392</td>
</tr>
<tr>
<td>AUC Ptx</td>
<td>−0.04013</td>
<td>1</td>
<td>−0.4204</td>
<td>−0.5214</td>
<td>−0.4152</td>
<td>0.2249</td>
</tr>
<tr>
<td>AUC R-M1</td>
<td>0.02184</td>
<td>−0.4204</td>
<td>1</td>
<td>−0.3811</td>
<td>0.07453</td>
<td>−0.04717</td>
</tr>
<tr>
<td>AUC S-M1</td>
<td>−0.00039</td>
<td>−0.5214</td>
<td>−0.3811</td>
<td>1</td>
<td>0.2451</td>
<td>−0.3722</td>
</tr>
<tr>
<td>AUC M4</td>
<td>0.02069</td>
<td>−0.4152</td>
<td>0.07453</td>
<td>0.2451</td>
<td>1</td>
<td>−0.8062</td>
</tr>
<tr>
<td>AUC M5</td>
<td>−0.02392</td>
<td>0.2249</td>
<td>−0.04717</td>
<td>−0.3722</td>
<td>−0.8062</td>
<td>1</td>
</tr>
</tbody>
</table>
coefficients with negative signs and thus appeared to decrease the blood flow in subjects treated with pentoxifylline. The results were consistent for each molecular species as regards systolic, diastolic and mean flows, even though some of the coefficients had 95% confidence intervals that included zero.

**Discussion**

This study was planned to investigate the possible contributions of the metabolites of pentoxifylline to the haemorheological effect in humans, and measurement of retinal blood flow was chosen as a convenient experimental model. However, the findings in the retina may also be of interest in themselves in view of the proposed use of pentoxifylline in retinal vascular disease. The covariation in plasma concentration between pentoxifylline and its metabolites was successfully diminished through pretreatment of the subjects with ciprofloxacin and rifampicin, allowing the use of a multiple regression concentration–effect model.

Pharmacokinetic interactions between ciprofloxacin and methylxanthines [18], and later between ciprofloxacin and pentoxifylline [19], have been described in the literature. Ciprofloxacin is an inhibitor of cytochrome P450 (CYP) 1A2 [18, 19]. Indirect evidence for metabolism of M1 by CYP1A2 was obtained from a comparative study on pentoxifylline pharmacokinetics in smokers and nonsmokers [22]. The smokers had a significantly lower mean AUC for M1 compared with nonsmokers, with a trend in the same direction observed for the parent drug. Smoking is known to induce CYP1A2 [23]; thus, these differences were probably due to increased metabolism of M1 by this isoenzyme. Since the metabolism of pentoxifylline to M1 is reversible, depletion of M1 also lowered the AUC of the parent drug. In addition, since M1 in the circulation is >96% S-form [14] it is evident that the effect involved this enantiomer, while no conclusions can be drawn about the metabolism of R-M1. Direct evidence for a CYP1A2-mediated inhibitory interaction between ciprofloxacin and pentoxifylline was presented more recently [19]. It seems likely that CYP1A2 catalyses xanthine 7-demethylation of pentoxifylline to M6 and of M1 to M7.

In agreement with the cited findings we found that the AUCs for pentoxifylline and S-M1 were significantly increased by pretreatment with ciprofloxacin, with a trend in the same direction for R-M1. The apparent lack of effect on the R : S-M1 concentration ratio contrasts with an earlier finding [24] that the plasma concentration of R-M1 was 6–17% of total M1 when pentoxifylline was co-administered with ciprofloxacin to patients.
with renal cell carcinoma. This is much higher than the 1–4% in this and in our previous study [14]. However, the cancer patients also received interleukin-2. Treatment with interleukins may inhibit or downregulate CYP450 [25] and can thus also interfere with drug metabolism. Effects of the disease and of treatment with other drugs (ranitidine, paracetamol and indomethacin) cannot be ruled out either.

Rifampicin is a known inducer of several of the CYP450 enzymes [20] and was therefore used as pretreatment in this study. The effects of smoking and probable induction of CYP1A2 (see above) on the plasma concentrations of pentoxifylline and M1 were not reproduced after treatment with rifampicin, however. The reason may be that rifampicin is a poor inducer of CYP1A2, in particular in comparison with its effects on the CYP2 and CYP3 families [20]. Instead, the mean AUC of M5 was significantly reduced by the treatment with rifampicin. Normally, M5 is excreted without further metabolism [9–12]. The lower AUC could tentatively be explained by activation of metabolic pathways (e.g. 7-demethylation) that enhance the clearance of M5 and/or compete with its formation.

Pentoxifylline alone and after rifampicin pretreatment significantly increased retinal blood flow measured as mean flow, diastole and pulsation compared with placebo. The increases over placebo were more pronounced in diastolic flow than in mean flow. After pentoxifylline alone diastolic flow increased by 9.7% (4.2, 15.5), and mean flow by 4.6% (1.1, 8.3). The corresponding increases from pentoxifylline after rifampicin pretreatment were 11.7% (5.8, 17.9) and

<table>
<thead>
<tr>
<th>Table 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean [min–max] observed retinal blood flow (arbitrary units, AU), measured as mean flow, systole, diastole and pulsation after administration of placebo or pentoxifylline (Ptx) 300 mg intravenous infusion alone or in combination with a ciprofloxacin (PtxC) 750-mg tablet or pretreatment with rifampicin (PtxR) 600-mg tablets once daily for 7 days</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Geometric least-square estimates</th>
<th>Placebo</th>
<th>Ptx</th>
<th>PtxC</th>
<th>PtxR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulsation</td>
<td>0.50 [0.23–0.72]</td>
<td>0.46 [0.21–0.74]</td>
<td>0.47 [0.17–0.71]</td>
<td>0.45 [0.16–0.70]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Arithmetic least-square estimates</th>
<th>Placebo</th>
<th>Ptx</th>
<th>PtxC</th>
<th>PtxR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulsation</td>
<td>0.50 [0.46, 0.55]</td>
<td>0.47 [0.439, 0.51]</td>
<td>0.47 [0.43, 0.51]</td>
<td>0.45 [0.41, 0.50]</td>
</tr>
</tbody>
</table>

Geometric and arithmetic least-square estimates of means (95% confidence intervals) of retinal blood flow, measured as mean flow, systole, diastole and pulsation after administration of placebo or pentoxifylline 300 mg intravenous infusion alone or in combination with a ciprofloxacin 750-mg tablet or pretreatment with rifampicin 600-mg tablets once daily for 7 days.
5.1% (1.4, 7.8) compared with placebo. Pentoxifylline in combination with ciprofloxacin did not affect retinal blood flows compared with placebo. Thus, pentoxifylline seems to increase diastolic low-velocity flow more than the higher flow rate occurring during systole.

The increases in ocular blood flow observed in this study are smaller than those reported by Schmetterer and co-workers [7]. In their study, 200 mg and 400 mg pentoxifylline increased ocular fundus pulsation by 17% and 27% in the macula, by 15% and 26% in the peripheral region and by 11% and 13% in the optic disk, over baseline. Some of the differences might be explained by different methods of measurement [17, 26]. The main differences between the methods is that SLDF measures retinal blood flow in the temporal area of retina whereas laser interferometer measures blood flow as fundus pulsation amplitude in the macula, peripheral region and optic desk. Fundus pulsations in the macula and peripheral region are predominantly influenced by choroidal blood flow, whereas fundus pulsation in the optic desk is a mixture of choroidal and retinal blood flow. Retinal blood flow is lower than choroidal blood flow (15% compared with 85% of total chorioretinal flow), but has a higher level of oxygen extraction compared with choroidal blood flow [27]. The retinal but not the choroidal blood flow is subject

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**Table 4**

Relative differences between least-square estimates of means (95% confidence intervals) of retinal blood flow, measured as mean flow, systole, diastole and pulsation after administration of placebo or pentoxifylline (Ptx) 300-mg intravenous infusion alone or in combination with a ciprofloxacin (PtxC) 750-mg tablet or pretreatment with rifampicin (PtxR) 600-mg tablets once daily for 7 days.

<table>
<thead>
<tr>
<th></th>
<th>Mean flow</th>
<th>Systole</th>
<th>Diastole</th>
<th>Pulsation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ptx–placebo</td>
<td>4.6*</td>
<td>2.7</td>
<td>9.7*</td>
<td>−7.8*</td>
</tr>
<tr>
<td></td>
<td>(1.1, 8.3)</td>
<td>(−1.0, 6.7)</td>
<td>(4.2, 15.5)</td>
<td>(−12.3, 3.1)</td>
</tr>
<tr>
<td>PtxC–placebo</td>
<td>0.4</td>
<td>−1.1</td>
<td>5.0</td>
<td>−6.6*</td>
</tr>
<tr>
<td></td>
<td>(−3.0, 3.9)</td>
<td>(−4.6, 2.7)</td>
<td>(−0.2, 10.6)</td>
<td>(−11.2, 1.8)</td>
</tr>
<tr>
<td>PtxR–placebo</td>
<td>5.1*</td>
<td>1.5</td>
<td>11.7*</td>
<td>−10.2*</td>
</tr>
<tr>
<td></td>
<td>(1.4, 7.8)</td>
<td>(−2.4, 5.6)</td>
<td>(5.8, 17.9)</td>
<td>(−14.8, 5.4)</td>
</tr>
<tr>
<td>Ptx–Ptx</td>
<td>−4.1*</td>
<td>−3.6</td>
<td>−4.2</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(−7.1, −0.9)</td>
<td>(−7.0, 0.01)</td>
<td>(−8.8, 0.6)</td>
<td>(−3.5, 6.3)</td>
</tr>
<tr>
<td>PtxR–Ptx</td>
<td>0.5</td>
<td>−1.2</td>
<td>1.8</td>
<td>−2.6</td>
</tr>
<tr>
<td></td>
<td>(−2.9, 3.9)</td>
<td>(−4.8, 2.6)</td>
<td>(−3.3, 7.2)</td>
<td>(−7.4, 2.4)</td>
</tr>
<tr>
<td>PtxR–PtxC</td>
<td>4.7*</td>
<td>2.6</td>
<td>6.3*</td>
<td>−3.8</td>
</tr>
<tr>
<td></td>
<td>(1.2, 8.4)</td>
<td>(−1.2, 6.5)</td>
<td>(0.9, 11.9)</td>
<td>(−8.5, 1.1)</td>
</tr>
</tbody>
</table>

*P < 0.05.

**Table 5**

Estimates (95% confidence intervals) of the intercept and gradients from the regression analysis of the influence from pentoxifylline (Ptx) and its metabolites (R-M1, S-M1, M4, and M5) on retinal blood flow measured as mean flow, systole, diastole and pulsation after administration of placebo and pentoxifylline 300 mg intravenous infusion alone and in combination with ciprofloxacin 750-mg tablet and pretreatment with rifampicin 600-mg tablets once daily for 7 days, on four separate occasions.

<table>
<thead>
<tr>
<th></th>
<th>Mean flow</th>
<th>Systole</th>
<th>Diastole</th>
<th>Pulsation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>245</td>
<td>327</td>
<td>167</td>
<td>490</td>
</tr>
<tr>
<td>AU†</td>
<td>(186, 304)</td>
<td>(256, 398)</td>
<td>(122, 211)</td>
<td>(440, 540)</td>
</tr>
<tr>
<td>AUC Ptx</td>
<td>171.1*</td>
<td>36.4*</td>
<td>3.47</td>
<td>33.9</td>
</tr>
<tr>
<td>AU†/µg⁻¹ h⁻¹ ml</td>
<td>(4.31, 29.9)</td>
<td>(176, 55.3)</td>
<td>(−10.6, 17.6)</td>
<td>(−0.37, 68.1)</td>
</tr>
<tr>
<td>AUC R-M1</td>
<td>303*</td>
<td>168</td>
<td>301*</td>
<td>367</td>
</tr>
<tr>
<td>AU†/µg⁻¹ h⁻¹ ml</td>
<td>(147, 459)</td>
<td>(−62.6, 399)</td>
<td>(128, 474)</td>
<td>(−804, 71.0)</td>
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<tr>
<td>AUC S-M1</td>
<td>−9.58*</td>
<td>−12.1*</td>
<td>−4.66*</td>
<td>−751</td>
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<tr>
<td>AU†/µg⁻¹ h⁻¹ ml</td>
<td>(−13.6, 5.52)</td>
<td>(−18.1, 6.05)</td>
<td>(−9.15, 0.158)</td>
<td>(−18.7, 3.7)</td>
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<tr>
<td>AUC M4</td>
<td>−54.5*</td>
<td>−96.1*</td>
<td>−280</td>
<td>−49.7</td>
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<tr>
<td>AU†/µg⁻¹ h⁻¹ ml</td>
<td>(−93.4, 15.5)</td>
<td>(−154, 38.4)</td>
<td>(−71.2, 15.2)</td>
<td>(−159, 59.3)</td>
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<tr>
<td>AUC M5</td>
<td>6.66*</td>
<td>9.62*</td>
<td>3.52</td>
<td>4.04</td>
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<tr>
<td>AU†/µg⁻¹ h⁻¹ ml</td>
<td>(0.675, 12.6)</td>
<td>(0.762, 18.5)</td>
<td>(−3.12, 10.2)</td>
<td>(−12.7, 20.8)</td>
</tr>
</tbody>
</table>

*P < 0.05. †Except for pulsation.
to autoregulation [27], therefore making the retinal blood flow less affected by systemic factors than the choroidal blood flow. Another advantage with the SLDF is that it scans an area of $2.7 \times 0.7$ mm and measures all flow in this area. Thus, more capillaries are found and the influence of erroneous values is smaller. With SLDF it is also easier to find the same area for all the measurements compared with laser interferometer that measures in one point. Pentoxifylline increases both choroidal and retinal blood flow but the increase is more pronounced in choroidal blood flow, possibly because retinal blood flow is autoregulated.

This study was performed in healthy volunteers partly because pentoxifylline is not approved for general use in Sweden and partly because we needed to co-administer pentoxifylline with ciprofloxacin and rifampicin in order to create different concentration ratios between pentoxifylline and its metabolites. It is difficult to make assumptions about the magnitude of the effect in a patient group with reduced retinal flow after administration to healthy volunteers, but we have shown that an increase in retinal blood flow is obtainable. This has not been shown previously either in patients or in healthy volunteers. In a study by Kruger et al., ocular fundus pulsation and retinal blood flow were measured by laser interferometry and SLDF in patients with age-related macular degeneration after administration of pentoxifylline or placebo, 400 mg three times daily for 3 months [8]. They found that pentoxifylline increased ocular fundus pulsation amplitude up to 28% after 3 months’ treatment but could not find any effects on retinal blood flow. In the present study we investigated the effects of pentoxifylline and metabolites on healthy eyes. Thus, our findings are not dependent on pathophysiological changes of the retinal vascular bed. In addition, SLDF combined with the AFFPIA software analysis used in the present study resulted in better reproducibility and less bias compared with the technique in Krueger’s study. Our results are in agreement with previous studies, using the subjective blue field entoptic phenomenon computer simulation technique, that have shown an increase in capillary blood flow velocity in healthy volunteers and diabetes patients [4, 5].

A previous study has shown a clear time dependency of the effects on blood flow after a single dose of pentoxifylline [7]. We found no such time dependency (Figure 2). We cannot explain this, but we measured retinal blood flow, whereas other investigators have measured a total of retinal and the more abundant choroidal blood flow. In order to study the contributions from pentoxifylline and its metabolites we needed to diminish the naturally occurring high correlations between these compounds. The correlation matrix (Table 2) shows that we were successful, possibly with the exception of a remaining rather high correlation between M4 and M5. Applying a simple linear AUC–effect model, we found that pentoxifylline, R-M1 and M5 had coefficients with positive signs indicating that they all contribute to the effects. It is particularly noticeable that R-M1 exerts a significant effect in spite of being present in concentrations that are two orders of magnitude lower than those of pentoxifylline and M5. The high potency of this compound is also reflected in the values of the coefficients (Table 5), which are accordingly one to two orders of magnitude greater than those of the other compounds. Two of the metabolites, S-M1 and M4, showed negative coefficients. This should not necessarily be interpreted as these substances, per se, having a negative effect on blood flow, only that they, in the mix of substances obtained after pentoxifylline administration, tend to modify the effects in a direction opposite that of pentoxifylline.

Our results showing biological activity of R-M1 and M5 are to some extent in agreement with those of previous studies. The haemorheological effects of pentoxifylline and its metabolites have been investigated in vitro [16]. As regards enhancement of erythrocyte filterability, there was little difference between pentoxifylline, racemic M1 and M5. In a test for adenosine diphosphate-induced platelet aggregation M5 was not quite as effective as the parent drug; however, racemic M1 was approximately 10 times more potent than pentoxifylline. R-M1 has been investigated as an immuno-modulatory drug, under the generic name of lisofylline. In this context R-M1 was shown to be 800 times more potent than pentoxifylline for inhibiting release of inflammatory mediators from monocytic leukaemia cells [28]. All these results, in conjunction with our own, point to R-M1 as a biologically very active molecule.

In conclusion, using a linear multiple-regression model we were able to demonstrate that R-M1 and M5 metabolites of pentoxifylline contribute significantly to the haemorheological effects of pentoxifylline in humans.

Johnny Ring, Department of Ophthalmology, Malmö University Hospital, obtained the SLDF registrations and Louise Goldberg, Department of Ophthalmology, Malmö University Hospital analysed the SLDF registrations. The project has received funds from the Anna and Edwin Berger Foundation and the Faculty of Medicine, Lund University.
Retinal blood flow changes by pentoxifylline in humans

References

Effects of pentoxifylline and its metabolites on platelet aggregation in whole blood from healthy humans

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Abstract

It is known that pentoxifylline inhibits platelet aggregation in vitro, but the effects from pentoxifylline and its main metabolites: 3,7-dimethyl-1(5′-hydroxyhexyl)xanthine (R-M1 and S-M1), 3,7-dimethyl-1(4-carboxybutyl)xanthine (M4), 3,7-dimethyl-1(3-carboxypropyl)xanthine (M5), on platelet aggregation in whole blood in vitro and in vivo have not been studied. We found that pentoxifylline, rac-M1, R-M1, S-M1, and M4 significantly inhibit ADP induced platelet aggregation in whole blood in vitro in a concentration-dependent manner, R-M1 being the most potent followed by rac-M1, S-M1, pentoxifylline, and M4. In this series of experiments the effects on aggregation induced ATP-release were less pronounced and were only significant after treatment with pentoxifylline, rac-M1 and R-M1, but the potency order appears to be the same. Since the metabolites are not available for use in humans, and also since each substance would be extensively metabolised in vivo, we made an attempt to estimate the relative contribution of each substance to the total effect of pentoxifylline in vivo. Previously published concentrations of pentoxifylline and these metabolites in humans, after administration of pentoxifylline, were used in combination with the potency ratios from this study. The findings from these calculations were that the main effect in vivo comes from S-M1 followed by pentoxifylline, the other metabolites contribute less than 10% each. In conclusion: in the following potency order R-M1, rac-M1, pentoxifylline, S-M1 and M4 all have significant effects on platelet aggregation in whole blood in vitro. However, it appears that the main effects in vivo are caused by S-M1 and pentoxifylline.

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Keywords: Pentoxifylline; Metabolite effect; Platelet aggregation in whole blood

1. Introduction

Pentoxifylline 3,7-dimethyl-1(5′-oxo-hexyl)xanthine is a haemorheologic drug used in the treatment of peripheral vascular disease and other conditions with insufficient regional microcirculation (Moher et al., 2000; Samlaska and Winfield, 1994; Ward and Clissold, 1987). Pentoxifylline acts primarily by increasing erythrocyte flexibility, by reducing blood viscosity, and by decreasing the potential for platelet aggregation and thrombus formation. In addition, pentoxifylline inhibits platelet aggregation in patients with peripheral vascular disorders (Angelkort, 1979), and also prevents shunt thrombosis formation in dialysis patients (Radmilović et al., 1987). Pentoxifylline is metabolised in humans to at least seven metabolites (Hinze et al., 1972). The major metabolites in humans are the hydroxy metabolite 3,7-dimethyl-1-(5′hydroxyhexyl)xanthine (M1), and the two carboxylic acid metabolites 3,7-dimethyl-1-(4-carboxybutyl)xanthine (M4) and 3,7-dimethyl-1(3-carboxypropyl)xanthine (M5). After oral administration of pentoxifylline to healthy volunteers the areas under the plasma concentration curves (AUCs) of M5 and racemic-M1 (rac-M1) exceeded that of pentoxifylline while that of M4 was lower (Beermann et al., 1985; Bryce and Burrows, 1980; Nicklasson et al., 2002; Smith et al., 1986). Using chiral separation we found that S-M1 is the major (>96%) and R-M1 the minor metabolite (Nicklasson et al., 2002). Metabolism of
pentoxifylline to M1 takes place both in the liver (Lillibridge et al., 1996) and in the erythrocytes (Ings et al., 1982; Nicklasson et al., 2002) and is reversible (Hinze et al., 1972; Lillibridge et al., 1996; Nicklasson et al., 2002).

Antiplatelet effects of pentoxifylline only were studied by De la Cruz et al. (1993). They found pentoxifylline to be more potent in whole blood than in platelet rich plasma. A comparison of the haemorheologic effects of pentoxifylline, rac-M1, and six other metabolites in vitro showed pronounced effects of the two former species and M5 on erythrocyte filterability and platelet aggregation (Ambrus et al., 1995). We have previously investigated the effect of pentoxifylline and its metabolites on another of its modes of action, namely retinal blood flow, in a randomised, placebo controlled, four-period cross over study in healthy volunteers (Magnusson et al., 2005). Pentoxifylline was administered as intravenous infusions alone or after pre-treatment of the subjects with ciprofloxacin or rifampicin, and a placebo infusion was used as negative control. Retinal blood flow was measured by scanning laser Doppler flowmetry in a selected area of the central temporal retina. The pre-treatments with rifampicin (an inducer of several enzymes of the cytochrome P450 system) and ciprofloxacin (an inhibitor of CYP1A2) were used in order to create different blood concentration ratios between pentoxifylline and its metabolites within the same subject. This enabled a comparison of blood flow effects of pentoxifylline and its metabolites in vivo by means of a linear multiple-regression model. Pentoxifylline, R-M1 and M5 significantly increased retinal blood flow while S-M1 and M4 appeared to counteract this effect. Thus, although the mechanisms of action differ, it is interesting to note that the findings on filterability in vitro and blood flow in vivo were in general agreement as regards the activity of the metabolites. However, the influence of the enantiomers of M1 was not investigated in the in vitro study on platelet aggregation.

The aim of this study was to investigate the relative potencies of pentoxifylline and metabolite M1, M4 and M5 to inhibit platelet aggregation in whole blood, and in particular to clarify contributions of the two enantiomers of M1, to this effect.

2. Material and methods

2.1. Materials

Pentoxifylline and rac-M1, M4 and M5 were kindly supplied by Aventis Pharma (Stockholm, Sweden). The R enantiomer of M1 was a gift from Cell Therapeutics (Seattle, WA, USA). ATP standards and luciferin luciferase reagents from Chrono-Lumereagents, Trio-lab (Göteborg, Sverige) were used and ADP from Sigma (Stockholm, Sverige).

Fig. 1. Box plots of ADP induced platelet aggregation in whole blood in vitro, measured as impedance, after incubation with a series of concentrations of: (A) pentoxifylline, (B) rac-M1, (C) M4, and (D) M5. The lower, middle and upper lines of each box are the first, second and third quartiles, respectively. The bars extend from the lowest to the highest non-outlier observation of the data set. Outliers are defined as values outside 1.5 times the box width from either end of the box, and indicated by open dots.
2.2. Platelet aggregation

Measurement of platelet aggregation were done in whole blood from 8 healthy volunteers (2 males and 6 females) by impedance technology (electrical resistance between two electrodes immersed in whole blood), using whole blood lumi-aggregometer, (Chrono log modell 560 Ca, Chrono Log Corp, Havertown, PA, USA) (Podczasy et al., 1996; Vucenik and Podczasy, 1998).

The subjects were informed about the study and gave oral informed consent prior to blood sampling. The subjects had not been taking any drugs 24 h prior to blood donation and had not been taking drugs containing acetylsalicylic acid within one week prior to blood donation. The study was performed according to the declaration of Helsinki and approved by the Ethics committee of Lund University.

Blood was sampled at two occasions from each subject by venepuncture and collected into vacutainer tubes containing 1:10 (volume:volume) 0.129 mol/L citrate. All tests were completed within four hours after the blood was drawn. At one occasion (n=8) pentoxifylline, rac-M1, M4 and M5 were investigated and on another occasion (n=8) rac-M1 and R-M1.

Platelet aggregation was studied in whole blood diluted in a 1:1 ratio with 0.9% saline (450 µL each). The samples were placed in plastic cuvettes containing a magnetic stir bar and were incubated in 37 °C for at least 5 min prior to testing and stirred at 1200 rpm.

The test procedure was started by adding 0.008 mg luciferin luciferase to the sample; the samples were incubated in 37 °C for 1 min prior to addition of the test substances. Pentoxifylline, rac-M1, R-M1, M4, M5 or saline (vehicle control) was added to the sample and incubated in 37 °C for 1 min prior to addition of ADP (10 µM final concentration), aggregation was monitored for 6 min. Pentoxifylline and its metabolites were diluted in saline 0.9%, 60 µL of the stock solutions were added to the samples, giving the final concentrations 0.375 mM, 0.75 mM, 1.5 mM or 3 mM. Saline 0.9% was used as control. The effects of S-M1 were calculated from a comparison between R-M1 and rac-M1 since we did not have access to pure S-M1.

In the same specimens ATP-release from the platelets was observed from the luciferin luciferase reaction. In these observations the ATP-release was calculated based on an ATP standard (2 nmol).

2.3. Statistics

For statistical analysis the mixed procedure in SAS (version 8.2; SAS Institute, Cary, NC, USA) was used. In the analysis of ADP induced platelet aggregation and ATP-release we used logarithmically transformed observations as dependent variables. Concentrations of the studied substances were used in the mixed model as fixed effects whereas subjects were entered as random effects. Least square
estimates and 95% confidence limits after back transformation are given. Statistical significance was accepted at P<0.05 (two tailed).

### 3. Results

Pentoxifylline, rac-M1 and M4 inhibited platelet aggregation, measured as impedance, in a concentration-dependent manner (Fig. 1). Concentration–effect relationships were also found for rac-M1 and R-M1 with a more pronounced effect for R-M1. In all experiments we could see that the effects on aggregation were mirrored by changes in ATP-release. Simultaneously with aggregation, secretion of dense granule M. Magnusson et al. / European Journal of Pharmacology 581 (2008) 290–295

<table>
<thead>
<tr>
<th></th>
<th>Fractional effect at 1 mM estimate</th>
<th>Fractional effect at 1 mM 95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentoxifylline/ rac-M1</td>
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<td>1.1727–1.5482</td>
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<tr>
<td>Pentoxifylline/ M4</td>
<td>0.64</td>
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<tr>
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<td>rac-M1/M4</td>
<td>0.48</td>
<td>0.41–0.55</td>
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<tr>
<td>rac-M1/M5</td>
<td>0.42</td>
<td>0.37–0.49</td>
<td>&lt;0.0001</td>
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<tr>
<td>M4/M5</td>
<td>0.89</td>
<td>0.77–1.02</td>
<td>0.094</td>
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Ratios of the fractional effects at 1 mM for platelet aggregation in whole blood, mean and 95% CI. P-values against the null hypothesis of unity (i.e. saline) are given.

Fig. 2B (rac-M1, R-M1, and S-M1). Aggregation was reduced in a concentration-dependent manner for rac-M1, R-M1, S-M1, pentoxifylline and M4. The intercept and slopes for the impedance–concentration curves were used for calculation of fractional effect at 1 mM and are shown in Table 1 (pentoxifylline, rac-M1, M4 and M5) and Table 2 (rac-M1, R-M1, and S-M1). The most potent inhibitor of aggregation was rac-M1 followed by pentoxifylline and M4. However, when the R-enantiomer of M1 was compared with rac-M1 we found that this enantiomer inhibited aggregation 23% more efficiently than rac-M1 and 41% more than S-M1.

Simultaneously with aggregation, secretion of dense granule ATP was measured in the same sample. Predicted values from the mixed model for ATP-release are shown in Fig. 2C (pentoxifylline, rac-M1, M4 and M5) and 2D (rac-M1, R-M1, and S-M1). Pentoxifylline, rac-M1 and R-M1 significantly decreased ATP-release, Tables 3 and 4.

<table>
<thead>
<tr>
<th></th>
<th>Fractional effect at 1 mM estimate</th>
<th>Fractional effect at 1 mM 95% CI</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Racoxifylline</td>
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<td>Rac-M1</td>
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<td>M4</td>
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<tr>
<td>M5</td>
<td>0.87</td>
<td>0.66–1.16</td>
<td>0.34</td>
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</table>

Ratios of the fractional effects at 1 mM for ATP-release in whole blood, mean and 95% CI. P-values against the null hypothesis of unity (i.e. saline) are given.
4. Discussion

The aim of this study was to investigate the relative potencies of pentoxifylline and metabolite M1, M4 and M5 to inhibit platelet aggregation in whole blood, and in particular to clarify contributions of the two enantiomers of M1, that are formed to very different extents in vivo, to this effect.

We found that pentoxifylline, rac-M1, R-M1, S-M1 and M4 significantly inhibit platelet aggregation in a concentration-dependent manner, R-M1 being the most potent followed by rac-M1, S-M1, pentoxifylline, and M4. The effects on ATP-release were less pronounced and were only significant after treatment with pentoxifylline, rac-M1 and R-M1, and the potency order appears to be the same, but these measurements exhibit more scatter and thus less explained variability. Thus, in the following we focus on the effect on platelet aggregation.

Previously Ambrus et al. (1995) studied the effects of pentoxifylline, rac-M1, M4 and M5 on aggregation in platelet rich plasma using approximately similar concentration to ours. They found that rac-M1, pentoxifylline and M5 but not M4 inhibited aggregation and that rac-M1 was more potent than pentoxifylline. Our results are in agreement with the findings of Ambrus and co-workers regarding the most potent substances, but not regarding M4 and M5. A direct comparison between studies is always difficult. Further, the methodologies differ: Ambrus et al. (1995) used aggregation in platelet rich plasma, and De la Cruz et al. (1993) used both platelet rich plasma and whole blood, whereas we studied platelet aggregation in whole blood. Our method should be more relevant for in vivo situations since we studied aggregation in whole blood instead of aggregation in platelet rich plasma. Aggregation in whole blood is preferable, both since it evaluates the platelets in a physiologic milieu in the presence of red and white blood cells, which are known to modulate platelet function, and also since aggregation in platelet rich plasma require centrifugation that causes injury to the platelets and loss of giant thrombocytes (Dyskiewicz-Korpanty et al., 2005). In addition, Ambrus et al. (1995) could not distinguish between the enantiomers of M1, which, as we now show, differ significantly in their potencies.

In vivo there will always be a mixture of the parent compound and its metabolites after administration of pentoxifylline, which allows pharmacological interactions between the different species. An aspect that has not been investigated in vitro, where only one specimen is added at the time, with the obvious exception of the racemate. Even if there is an in vitro metabolism in red blood cells, the extent of this phenomenon should be negligible taken into account the abundance of the species added and the short duration of the experiment (Nicklasson et al., 2002). In addition, the relative plasma concentrations of pentoxifylline and the metabolites will be very different from those in the in vitro experiments, so that activities are compared for different regions of the underlying concentration-effect relationships.

In an attempt to estimate the relative contribution of each substance to the total effect of pentoxifylline on platelet aggregation in vivo we use our in vitro data from this study together with our previously published concentrations of pentoxifylline and its metabolites in humans after administration of pentoxifylline (Nicklasson et al., 2002). In that study we found that the relative mean concentrations (calculated from ratios of AUCs of the metabolites over the AUC of pentoxifylline) were 0.058, 2.91, 0.28, and 2.22 for R-M1, S-M1, M4, and M5, respectively. If we combine these results with the potencies found in this study, we then conclude that the main effect on platelet aggregation in vivo should actually be brought about by S-M1 and pentoxifylline, and that the remaining metabolites would contribute by less than 10% each. This approximation holds true also if we take the inter-subject variability of the metabolism of pentoxifylline into account. Thus, even if R-M1 is twice as potent as S-M1 in vitro the low concentration achieved after administration of pentoxifylline results in only a small contribution to the total effect on platelet aggregation in vivo.

In conclusion, we found that in the following potency order R-M1, rac-M1, pentoxifylline, S-M1 and M4 all have significant effects on platelet aggregation in whole blood in vivo. When combining our findings in this study with previously known concentration data in humans after administration of pentoxifylline it appears that the main effects in vivo are caused by S-M1 and pentoxifylline. However, further studies are needed in order to confirm this.

References