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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 mM), while that to (S)-M1 was best described by a two-enzyme model (K_m = 1.1 and 132 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.*

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.[1–3] 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).[4] 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.[5–7] 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.[4,6–8] However, small amounts of M1 may be excreted in conjugated form.[7]

The transformation of pentoxifylline to M1 is rapidly reversible.[8] It takes place both in erythrocytes[9–11] and liver.[12] The apparent clearance of pentoxifylline after intravenous administration has been shown to considerably exceed hepatic blood flow.[5,10,13] 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.[13] 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.[14] In addition, the (R)-enantiomer of M1 (lisofylline) inhibits the effects of tumor

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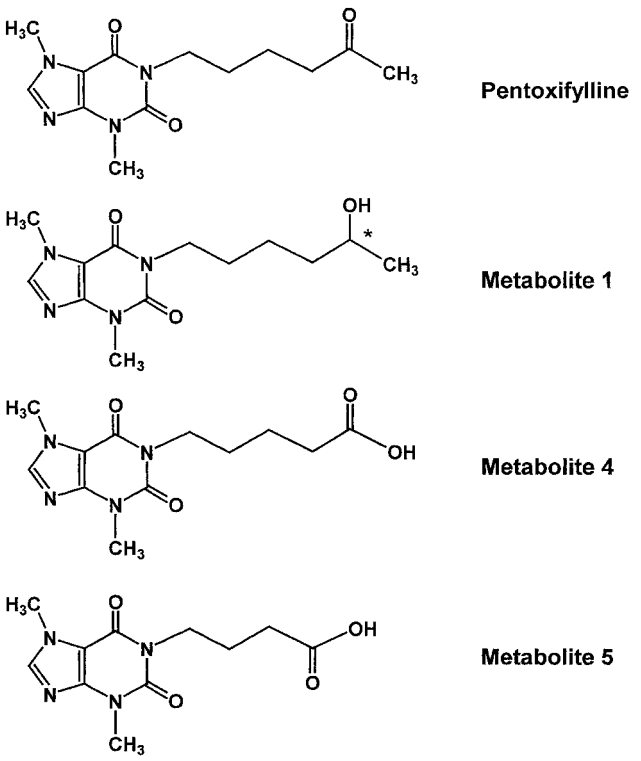

Fig. 1. The structural formulae of pentoxifylline and its metabolites M1, M4, and M5.

necrosis factor-α and suppresses serum levels of free fatty acids and is being developed as a drug in its own right.[15–17] 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,[12] 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[12] and in cancer patients (peak and trough plasma concentrations in four patients) who received pentoxifylline together with ciprofloxacin and interleukin-2.[18] 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.[19] 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 cellobiohydrolase 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.[12] 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 NaH_2PO_4 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 NaH_2PO_4 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 NaH_2PO_4 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-mg/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 stabililty 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 Metaboliltes 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 (λ), 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 1,000*g* 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 100*g* and 4°C. The supernatant was discarded. This washing was repeated three times. The erythrocytes were then centrifuged for 10 min at 1,000*g*. They were then suspended in phosphate-buffered saline solution (0.02 M K_2HPO_4, 0.08 M NaH_2PO_4, and 0.036 M NaCl, pH 7.4) in a proportion corresponding to the EVF of the originally used blood.

The λ 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 1,000*g*. 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 hemolysed 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 hemolysed 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^{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_{max} and K_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:

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

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.:

$$V_{max(1)} = V_{max(1),TV} \times (1 + \eta)$$

where $V_{max(1),TV}$ is the typical value and η is the interindividual variance. If the η of a parameter turned out to be very low, the model was rerun after deletion of this η. The criteria for selecting a final model from the investigated ones (one or two enzymes, with or without η on the V_{max} and K_m values) were the values of the NONMEM objective function and the distributions of residuals. The V_{max} is given as nmoles/min per 5×10^9 erythrocytes, which corresponds to the normal erythrocyte count in 1 ml of blood.

From the obtained values of V_{max} and K_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[21,22] by the addition of 5 mM of SKF525-A, ethacrynic acid, menadione, daunorubicin, 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 Venoject® 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, 1.5, 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 R:S 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_{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_u) was calculated from the AUC based on total plasma concentrations by means of the f_u values determined for the compounds in vitro. Finally, for comparisons of metabolite concentrations the AUC_u in weight units (ng×h/ml) was recalculated to molar AUC_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 each enantiomer. The (R)-M1 supplied by Cell Therapeutics proved to contain less than 0.4% of (S)-M1.

On the RP-18 column, pentoxifylline, thalidomide (I.S.), and derivatized (R)-M1 and (S)-M1 were separated with capacity factors (k′) of 6.8, 9.1, 10.4, and 11.0, respectively. Baseline separation was achieved for the enantiomers of M1. Underivatized M1, if present, had k′ = 6.0. With a different mobile phase, the k′ of enprofylline, M4, and M5 were 2.6, 10.3, and 5.0, respectively. Caffeine and theophylline had lower k′ values than the analytes in both systems and thus did not interfere with the assays.

The mean extraction yields of pentoxifylline and *rac*-M1 from plasma were 99 and 100%, respectively, and from blood 91 and 94%. The mean extraction yields of M4 and M5 from plasma were 79 and 76% and from blood 67 and 62%.

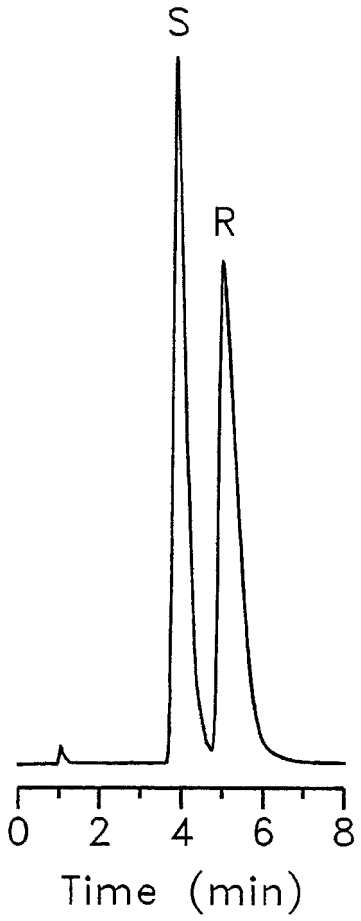

Fig. 2. The separation of the enantiomers of metabolite 1 on the cellobiohydrolase column.

When derivatization of M1 was performed as originally described,[12] 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 2 h of 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_e, and f_u of pentoxifylline and its metabolites are summarized in Table 2. The λ and r_e of pentoxifylline were concentration-dependent, as illustrated in Figure 3, while f_u was not. The difference in λ between the enantiomers of M1 was entirely due to the difference in f_u. The median and range f_u of pentoxifylline in the plasma from the subjects in the pharmacokinetic study was 0.70 (0.60–0.73) and the f_u 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_e 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

Compound	Added concentration (ng/ml)	Found concentration (ng/ml, mean ± S.D.)	C.V. (%)
Pentoxifylline	3.0	3.0 ± 0.23	5.6
	15	14.7 ± 0.43	2.9
	150	149 ± 4.31	2.9
(R)-M1	3.0*	3.0 ± 0.18	6.0
	15*	15.4 ± 0.84	5.4
	150*	158 ± 4.46	2.8
(S)-M1	3.0*	3.0 ± 0.19	6.4
	15*	15.8 ± 1.22	7.7
	150*	152 ± 4.58	3.0
M4	3.0	3.0 ± 0.16	5.3
	15	14.8 ± 0.80	5.4
	150	151 ± 6.28	4.2
M5	15	14.5 ± 0.45	3.1
	150	156 ± 4.32	2.8

*As 6.0, 30, or 300 ng/ml of racemate.

TABLE 2. Blood:plasma concentration ratio (λ), 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.

Compound	EVF	λ	r_e	f_u
Pentoxifylline	0.41 ± 0.045	—[a]	—[a]	0.65 ± 0.040
(R)-M1	0.41 ± 0.045	0.77 ± 0.059	1.09 ± 0.104	0.54 ± 0.057
(S)-M1	0.41 ± 0.045	0.84 ± 0.063	1.15 ± 0.171	0.65 ± 0.065
Ratio R:S		0.92 ± 0.004	0.96 ± 0.051	0.83 ± 0.017
M4	0.41 ± 0.027	—[b]	0.19 ± 0.17	0.62 ± 0.046
M5	0.41 ± 0.027	—[b]	0.13 ± 0.14	0.58 ± 0.058

[a]Concentration-dependent, see Figure 3.
[b]Not measured because of technical problems.

Interconversion of Pentoxifylline and M1 by Erythrocytes

The metabolism of pentoxifylline to (R)-M1 by hemolysed erythrocytes was best described by a one-enzyme reaction model. The Michaelis-Menten parameter values (population mean value and interindividual range) were V_{max} = 0.041 (0.035–0.56) nmoles/min per 5 × 10^9 erythrocytes and K_m = 11 mM. There was clear interindividual variance only in V_{max}. In contrast, the metabolism of pentoxifylline to (S)-M1 was best described by a two-enzyme model, with interindividual variance only in the V_{max} of one of the enzymes. The parameter values of the two enzymes were V_{max} = 0.054 (0.031–0.096) nmoles/min per 5 × 10^9 erythrocytes and K_m = 1.1 mM, and V_{max} = 0.86 nmoles/min per 5 × 10^9 erythrocytes and K_m = 132 mM, respectively. At a therapeutic blood concentration of pentoxifylline (0.5 μg/ml, 1.8 μM) the calculated rates of formation of (R)- and (S)-M1 were 6.8 × 10^{-6} and 1.0 × 10^{-4} nmoles/min per 5 × 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.

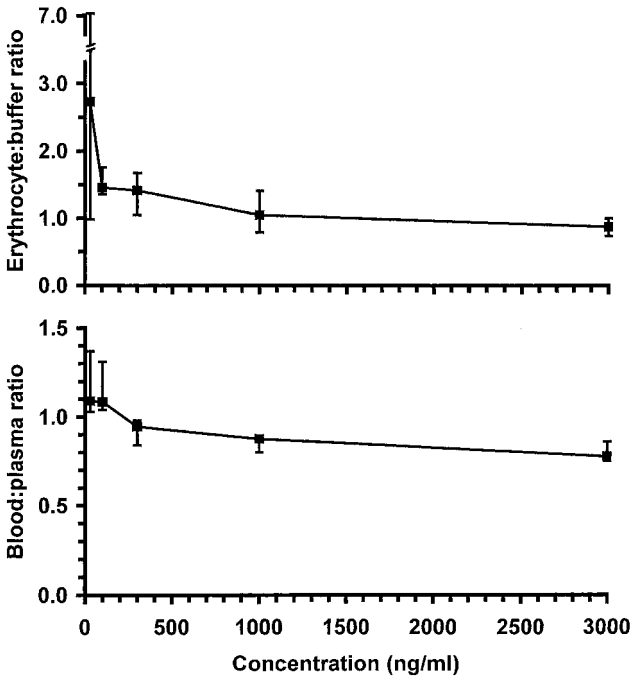

Fig. 3. Top: The erythrocyte:buffer concentration ratio (r_e; median and range) of pentoxifylline in blood from four healthy volunteers. Bottom: The blood:plasma concentration ratio (λ; median and range) of pentoxifylline in the same blood samples.

At the concentrations investigated (0.022 and 0.072 mM) of (R)- and (S)-M1, the median rates of formation of pentoxifylline from (R)-M1 were 6.5 × 10^{-4} and 1.9 × 10^{-3} nmoles/min per 5 × 10^9 erythrocytes, respectively, and from (S)-M1 2.2 × 10^{-3} and 5.0 × 10^{-3} nmoles/min per 5 × 10^9 erythrocytes. The formation from (S)-M1 was thus approximately 3 times faster at these concentrations. By extrapolation, the rate of formation of pentoxifylline from (R)-M1 would be 7.2 × 10^{-7} nmoles/min in 1 ml of blood at an in vivo concentration of 0.02 μg/ml (0.07 μM) while the formation from (S)-M1 at its 50-fold higher in vivo concentration (see below) would be 1.5 × 10^{-4} nmoles/min. If the two enantiomers were present at an equal concentration of 0.5 μg/ml (1.8 μM), the calculated rates of formation of pentoxifylline would be 1.8 × 10^{-5} and 7.4 × 10^{-5} nmoles/min per 5 × 10^9 erythrocytes from (R)- and (S)-M1, respectively, 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. Inhibition by phenobarbital could not be evaluated due to interference with the HPLC assay of M1.

Pharmacokinetics in Humans

Plasma concentration curves of pentoxifylline and its metabolites after administration of oral and intravenous pentoxifylline 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 pentoxifylline (median and range) was 35 (24–47)% when calculated 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-

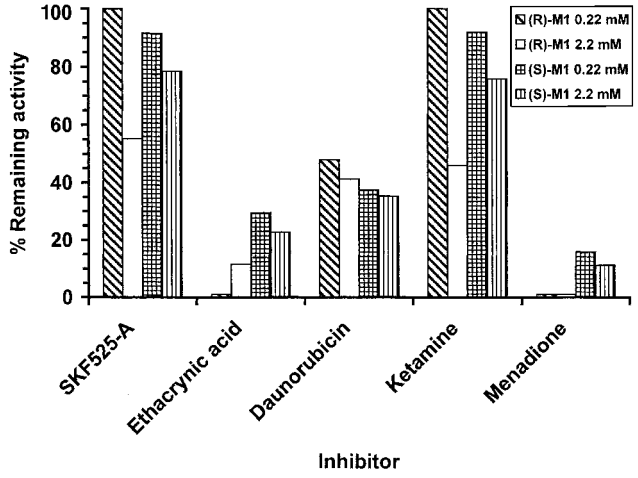

Fig. 4. Inhibition of the transformation of pentoxifylline to (R)- and (S)-M1 in vitro at two concentrations of the substrate (0.22 and 2.2 mM), expressed as the percent measured rate of formation in the presence of inhibitors as compared to control incubations.

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 λ 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 infusion and from 0.019–0.037 after 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 AUC_u 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

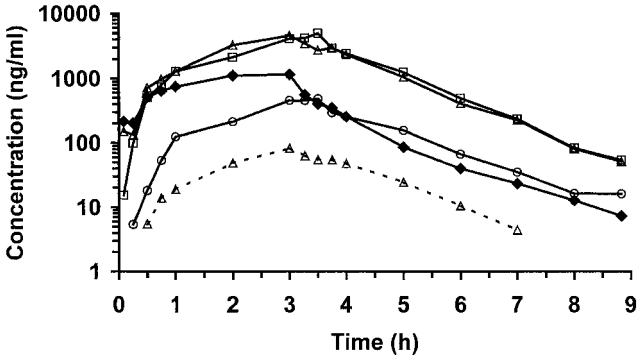

Fig. 5. The plasma concentrations of pentoxifylline and its metabolites during and after the intravenous infusion of 600 mg of the drug over 3 h in one subject. Key: filled diamonds: pentoxifylline, open triangles with dashed line: (R)-M1, with continuous line: (S)-M1, open circles: M4, and open squares: M5.

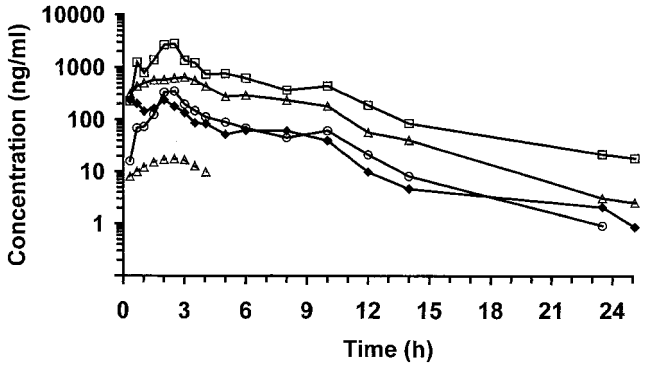

Fig. 6. The plasma concentrations of pentoxifylline and its metabolites after administration of the 600-mg controlled release tablet to the same subject as in Figure 5. Key: filled diamonds: pentoxifylline, open triangles with dashed line: (R)-M1, with continuous line: (S)-M1, open circles: M4, and open squares: M5.

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-

TABLE 3. Pharmacokinetic parameters of pentoxifylline (Ptx) and its metabolites (M1, M4, M5) after intravenous or oral administration of pentoxifylline, median (range)

Compound	Parameter	Administration		
		Intravenous, 300 mg (n = 6)	Intravenous, 600 mg (n = 5)	Oral, 600 mg (n = 6)
Pentoxifylline	AUC/dose*	4.0 (2.7–6.0)	4.1 (3.8–7.3)	1.4 (0.9–1.8)
	AUC_u/dose	2.7 (2.0–3.6)	3.0 (2.5–4.4)	0.94 (0.65–1.3)
	CL_{app} (L/min)	4.2 (2.8–6.3)	4.1 (2.3–4.6)	not applicable
	Terminal $t_{1/2}$ (h)	0.90 (0.75–1.0)	0.81 (0.75–1.1)	2.7 (1.2–3.7)
	T_{max} (h)	2.5 (1.0–3.0)	2.0 (0.5–3.0)	0.67 (0.33–2.0)
	C_{max} (ng/ml)	428 (332–625)	944 (754–1508)	194 (41–239)
(R)-M1	AUC/dose*	0.14 (0.06–0.26)	0.23 (0.19–0.40)	0.13 (0.06–0.22)
	AUC_u/dose	0.07 (0.03–0.16)	0.12 (0.11–0.24)	0.07 (0.03–0.11)
	Terminal $t_{1/2}$ (h)	0.94 (0.76–1.0)	0.99 (0.81–1.2)	1.7 (n = 1)
	T_{max} (h)	3.3 (3.0–3.3)	3.0 (2.0–3.0)	1.8 (0.32–2.5)
	C_{max} (ng/ml)	14 (7.9–27)	46 (31–83)	14 (7.2–4.6)
	Molar AUC_u ratio (R)-M1/Ptx	0.02 (0.02–0.05)	0.01 (0.01–0.02)	0.06 (0.03–0.16)
(S)-M1	AUC/dose*	12 (9.2–18)	13 (11–22)	4.9 (3.2–6.9)
	AUC_u/dose	8.4 (6.2–13)	8.6 (6.9–15)	2.9 (2.2–4.8)
	Terminal $t_{1/2}$ (h)	0.81 (0.61–0.93)	0.86 (0.67–1.2)	2.6 (2.5–3.0)
	T_{max} (h)	3.0 (2.0–3.1)	3.0 (2.0–3.0)	2.8 (2.5–3.0)
	C_{max} (ng/ml)	1222 (897–1558)	2486 (1971–4588)	475 (220–650)
	Molar AUC_u ratio (S)-M1/Ptx	3.0 (2.4–3.7)	2.8 (2.1–3.5)	3.2 (2.1–4.5)
M4	AUC/dose*	1.7 (0.77–3.3)	1.2 (0.85–2.3)	0.89 (0.70–2.1)
	AUC_u/dose	1.1 (0.47–2.0)	0.73 (0.53–1.4)	0.55 (0.44–1.3)
	Terminal $t_{1/2}$ (h)	1.0 (0.42–2.1)	0.81 (0.71–1.8)	2.8 (1.8–5.3)
	T_{max} (h)	3.0 (2.6–3.3)	3.3 (3.0–3.5)	3.5 (2.5–4.0)
	C_{max} (ng/ml)	211 (74–449)	225 (213–485)	104 (79–349)
	Molar AUC_u ratio M4/Ptx	0.35 (0.19–0.72)	0.24 (0.16–0.32)	0.69 (0.51–1.0)
M5	AUC/dose*	11 (9.6–16)	11 (8.9–21)	10 (9.5–23)
	AUC_u/dose	6.6 (5.6–9.2)	6.4 (4.1–12)	6.0 (5.5–13)
	Terminal $t_{1/2}$ (h)	0.94 (0.63–1.0)	0.85 (0.68–1.1)	2.9 (1.7–3.6)
	T_{max} (h)	3.3 (1.0–3.3)	3.3 (3.0–3.5)	2.8 (2.5–4.0)
	C_{max} (ng/ml)	1148 (924–1522)	1885 (1612–4960)	1039 (883–2779)
	Molar AUC_u ratio M5/Ptx	2.6 (1.9–3.5)	2.1 (1.5–3.3)	8.6 (5.7–10)

*Area under the curve (ng × h/ml) divided by dose of pentoxifylline 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 pentoxifylline were not different between patients with cirrhosis and healthy volunteers, even though the total clearance of pentoxifylline was reduced by 60% in the former group.[13] Renal insufficiency did not influence the AUC ratios of M1 to pentoxifylline.[26] Thus, the erythrocytes appear to be the main site of the reduction of pentoxifylline to M1. This metabolism in blood has been described previously,[9–11] 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. Human blood components reduce the antineoplastic agent daunorubicin to daunorubicinol in an NADPH-dependent reaction.[27] Erythrocytes from rats and humans reduced bunolol, a beta-adrenergic blocking agent, to dihydrobunolol in the presence of NADPH and also catalyzed the reverse reaction with NADP as cofactor.[28] Later, an NADPH-dependent ketone reductase that catalyzed the transformation of 4-nitroacetophenone to 4-nitrophenylmethylcarbinol was partially purified from human erythrocytes.[29] The K_m value was 0.39 mM. Haloperidol was transformed to reduced haloperidol in human liver cytosol, with K_m values of 0.61 and 0.50 mM in two liver samples.[21] The same reduction 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 erythrocytes. The V_{max} and K_m values in human erythrocytes were 9.2 ± 0.88 nmoles/min/g hemoglobin and 0.70 ± 0.13 mM, respectively.[32] The total enzyme activity in whole blood was calculated to be approximately 30% of the total activity in the liver.

The reduction of haloperidol in liver cytosol was completely inhibited by menadione, daunorubicin, and ethacrynic acid, which are known substrates of ketone reductase, but not by SKF525-A, an inhibitor of cytochrome P450 monooxygenases.[21] Similarly, menadione and daunorubicin, but not SKF525-A, inhibited haloperidol reduction in blood,[22] while ethacrynic acid, menadione, and daunorubicin inhibited the reduction of acetohexamide by erythrocytes.[32] The same pattern of inhibitor activities was found also for the reduction of pentoxifylline. We also investigated putative inhibition by ketamine, another ketone

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[32,33]) 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 ciprofloxaxin and interleukin-2, the R:S 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 λ. 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 λ.

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.[35] 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,[36,37] 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_{dss}) is overestimated by the standard procedure (moment analysis).[36,37] The true V_{dss} 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_{dss}.

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.[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 erytrocyte flexibility with potencies similar to that of pentoxifylline, and in some tests of platelet aggregation the two metabolites are more potent than the parent compound.[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_u/dose 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.

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