CLINICAL PHARMACOLOGY & THERAPEUTICS
VOLUME 80 NUMBER 3 SEPTEMBER 2006

PHARMACOKINETICS AND DRUG DISPOSITION

Use of microdosing to predict pharmacokinetics at the therapeutic dose: Experience with 5 drugs

Objectives: **A volunteer trial was performed to compare the pharmacokinetics of 5 drugs—warfarin, ZK253 (Schering), diazepam, midazolam, and erythromycin—when administered at a microdose or pharmacologic dose. Each compound was chosen to represent a situation in which prediction of pharmacokinetics from either animal or in vitro studies (or both) was or is likely to be problematic.**

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Supported by the Consortium for Resourcing and Evaluating AMS Microdosing (CREAM) trial consisting of Xceleron, Schering, Institut de Recherches Internationales Servier, F. Hoffmann-La Roche, Eli Lilly, and Pharma Bio-Research Group.

Received for publication Dec 22, 2005; accepted May 1, 2006.

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Clin Pharmacol Ther 2006;80:203-15.
0009-9236/$32.00

doi:10.1016/j.clpt.2006.05.008

Methods: **In a crossover design volunteers received (1) 1 of the 5 compounds as a microdose labeled with radioactive carbon (carbon 14) (100 μg), (2) the corresponding ^{14}C-labeled therapeutic dose on a separate occasion, and (3) simultaneous administration of an intravenous ^{14}C-labeled microdose and an oral therapeutic dose for ZK253, midazolam, and erythromycin. Analysis of ^{14}C-labeled drugs in plasma was done by use of HPLC followed by accelerator mass spectrometry. Liquid chromatography–tandem mass spectrometry was used to measure plasma concentrations of ZK253, midazolam, and erythromycin at therapeutic concentrations, whereas HPLC–accelerator mass spectrometry was used to measure warfarin and diazepam concentrations.**

Results: **Good concordance between microdose and therapeutic dose pharmacokinetics was observed for diazepam (half-life [$t_{1/2}$] of 45.1 hours, clearance [CL] of 1.38 L/h, and volume of distribution [V] of 90.1 L for 100 μg and $t_{1/2}$ of 35.7 hours, CL of 1.3 L/h, and V of 123 L for 10 mg), midazolam ($t_{1/2}$ of 4.87 hours, CL of 21.2 L/h, V of 145 L, and oral bioavailability [F] of 0.23 for 100 μg and $t_{1/2}$ of 3.31 hours, CL of 20.4 L/h, V of 75 L, and F of 0.22 for 7.5 mg), and development compound ZK253 (F = <1% for both 100 μg and 50 mg). For warfarin, clearance was reasonably well predicted (0.17 L/h for 100 μg and 0.26 L/h for 5 mg), but the discrepancy observed in distribution (67 L for 100 μg and 17.9 L for 5 mg) was probably a result of high-affinity, low-capacity tissue binding. The oral microdose of erythromycin failed to provide detectable plasma levels as a result of possible acid lability in the stomach. Absolute bioavailability for the 3 compounds examined yielded excellent concordance with data from the literature or data generated in house.**

Conclusion: **Overall, when used appropriately, microdosing offers the potential to aid in early drug candidate selection. (Clin Pharmacol Ther 2006;80:203-15.)**

New drugs for the treatment of unmet medical needs are of great importance, but unfortunately, over the past 20 years, development costs have been rising progressively while the number of new drugs receiving marketing approval has fallen to a historically low level.[1,2] The situation has become so serious that the Food and Drug Administration published the "Critical Path" document highlighting problems in drug development and encouraging novel approaches to be incorporated into the current drug development paradigm.[3]

One important attribute of a drug is its pharmacokinetic (PK) profile. If poor, it can severely limit the utility of the compound. Over the past 10 to 15 years, there have been many improvements in predicting human pharmacokinetics based on a combination of animal, in vitro, and in silico models, but failures still occur.

A new approach using big physics instrumentation to obtain human PK information, before the usual expensive phase 1 safety program is conducted, has been proposed, known as human phase 0 microdosing.[4] In microdosing studies PK data are obtained after administration of a trace subpharmacologic quantity to human subjects. This approach has been the subject of guidance documents from the European Medicines Agency[5] and, more recently, the Food and Drug Administration.[6] Both documents permit a reduced safety testing package before microdosing studies in humans.[7]

To assess the concern of predictivity of pharmacokinetics of a pharmacologic dose from a microdose, we have compared pharmacokinetics at both doses in human volunteers for 5 compounds—4 marketed drugs and 1 drug that was rejected during development because of inadequate PK characteristics.[8] Each compound was selected to represent a situation in which prediction of human pharmacokinetics from nonclinical data might be considered problematic. The ultrasensitive analytic technique of accelerator mass spectrometry (AMS) was used to quantify the low plasma concentrations anticipated after microdose administration. AMS is an isotope ratio technique requiring the administration of an isotopically enriched drug labeled with radioactive carbon (carbon 14).[9]

Microdosing has been investigated previously in dogs,[10] and there is also an account of microdosing in humans as part of a general review.[4] Human microdosing has been conducted by use of positron emission tomography with relevance to tissue distribution but without classical pharmacokinetics.[11] This report presents the first comprehensive human study comparing microdose and therapeutic dose pharmacokinetics.

METHODS

Test substances and reagents. Diazepam was supplied by Centrafarm (Rotterdam, The Netherlands), and midazolam was supplied by Hameln Pharmaceuticals (Hameln, Germany). [^{14}C]Diazepam and [^{14}C]midazolam (both with a specific activity of 2.0 GBq/mmol and radiopurity of 99.5%) were supplied by F. Hoffmann-La Roche (Basel, Switzerland). ZK253 and [^{14}C]ZK253 (with a specific activity of 1.9 GBq/mmol and radiopurity >98%) were supplied by Schering (Berlin, Germany). Warfarin was obtained from

Table I. Treatment regimens for each drug

	Treatment regimen		
Drug	*1*	*2*	*3*
Warfarin	Oral, 100 μg	Oral, 5 mg	
ZK253	Oral, 100 μg	Intravenous, 100 μg	Simultaneous: Intravenous, 100 μg, and oral, 50 mg*
Diazepam	Intravenous, 100 μg	Intravenous, 10 mg	
Midazolam	Oral, 100 μg	Intravenous, 100 μg	Simultaneous: Intravenous, 100 μg, and oral, 7.5 mg*
Erythromycin	Oral, 100 μg	Simultaneous: Intravenous, 100 μg, and oral, 250 mg*	

*Simultaneous dose of 100 μg of ^{14}C-labeled drug intravenously and therapeutic nonradiolabeled oral dose.

Table II. Solvents used for extraction of plasma

Drug	*Extraction solvent*	*Wash solvent*
Warfarin	Methanol	Methanol/water (1:1 [vol/vol])
ZK253	Acetonitrile	Acetonitrile/water (1:1 [vol/vol])
Diazepam	Acetonitrile plus 5 μL ammonium hydroxide	Acetonitrile/water (1:1 [vol/vol])
Midazolam	Chilled 0.1% (vol/vol) hydrochloric acid in acetonitrile	0.1% Hydrochloric acid (vol/vol) in acetonitrile/water (1:1 [vol/vol])
Erythromycin	Acetonitrile	Acetonitrile/ammonium acetate, 2 mol/L (pH 7)/water (35:5:60 [vol/vol/vol])

Bristol-Myers Squibb (New York, NY) as sodium-lyophilized powder, and [^{14}C]warfarin (with a specific activity of 2.07 GBq/mmol and radiopurity of 99.8%) was obtained from Amersham Biosciences (Buckinghamshire, United Kingdom). Erythromycin was supplied by Abbott (Hoofddorp, The Netherlands) as Erythrocine-ES 250 granulate to produce a suspension for oral administration and Erythrocine powder for intravenous administration. [^{14}C]Erythromycin (with a specific activity of 1.9 GBq/mmol and radiopurity >99%) was obtained from Metabolic Solutions (Nashua, NH). Nonradiolabeled components for intravenous administration were provided in sterile form. ^{14}C-Labeled components for intravenous administration were sterilized by filtration (0.22-μm pore size). All chemicals were of HPLC grade or equivalent.

Dose formulation and administration. The clinical study was performed by Pharma Bio-Research (Zuidlaren, The Netherlands). Intravenous doses, comprising the test substance dissolved in 50 mL of physiologic saline solution (diazepam and midazolam) or 5% (wt/vol) glucose (ZK253 and erythromycin), were infused over a period of 30 minutes. Oral doses comprised the test substance dissolved in 50 mL of water (25 mL for ZK253), except for the erythromycin therapeutic dose (250 mg), which was prepared as a 50-mL suspension. The complete oral dose was swallowed with an additional 150 mL of water as quickly as possible, within approximately 2 minutes. All radioactive doses were 7.4 KBq (200 nCi) per volunteer. Actual doses administered, determined by weight, were as follows for microdoses and therapeutic doses, respectively: warfarin, 97.4 μg (SD, 6 μg) and 5.3 mg (SD, 74 μg); ZK253, 102.1 μg (SD, 5.7 μg) and 50.0 mg; diazepam, 96.0 μg (SD, 1.7 μg) and 10.0 mg (SD, 41.7 μg); midazolam, 95.4 μg (SD, 2.9 μg) and 7.5 mg; and erythromycin, 96.1 μg (SD, 2.8 μg) and 250 mg.

Nonspecific binding. Nonspecific binding of the microdose to the dosing apparatus was assessed before dose administration. Mock dose preparations were passed through the dosing apparatus, and the radioactive recovery was measured by liquid scintillation counting. Recoveries were greater than 90%.

Volunteers. We enrolled 30 subjects, 24 men (age range, 18-55 years) and 6 postmenopausal women (age range, 45-80 years), in this study. All had a body mass index between 18 and 30 kg/m^2, did not smoke more than 10 cigarettes per day, and abstained from smoking during the study. Their test results were negative for drugs of abuse, and they had not had an illness within 5 days before dosing. The study received independent ethical approval, and all subjects provided informed

consent, as defined in the Declaration of Helsinki (1964). Volunteers fasted overnight before drug administration. Six different subjects were administered single doses of each test substance in a randomized crossover design (Table I). There was at least a 14-day period between each dose administration (washout period). Men were given doses of all drugs except ZK253, which only female subjects received. The levels of radioactivity used were extremely low and did not require regulatory approval for administration to human volunteers in this study.

Sample collection. Blood samples (10 mL) were taken, via an indwelling catheter or by direct venipuncture (sodium heparin anticoagulant), approximately a half hour before and at a range of times after dosing (times were chosen based on the known therapeutic dose pharmacokinetics of the drug). The exact time of each blood collection was recorded. For intravenous administrations, the time of sample collection was taken from the start of the infusion. Blood samples were centrifuged to produce plasma, which was stored between −20°C and −80°C and transported from the clinic to the AMS facility on solid carbon dioxide.

^{14}C-Labeled parent drug measurement by HPLC and AMS. AMS analysis was conducted at Xceleron (York, United Kingdom). The concentration of each parent drug was determined by HPLC-AMS by extraction of 500 μL of plasma with an equal volume of extraction solvent, followed by a wash solvent (Table II). After each solvent extraction, the sample was centrifuged and the 2 supernatants were combined. The weight of the combined supernatant was recorded.

Extraction efficiencies were determined by spiking of 0.5 mL of control plasma with approximately 5000 disintegrations per minute (dpm) of ^{14}C-labeled drug and extraction via the respective method. Radioactivity in the spiked plasma and extracts was measured by use of a Packard Tricarb 3100TR Liquid Scintillation Counter (Packard Instrument Co, Pangbourne, Berkshire, United Kingdom). Extraction efficiencies were between 92.2% and 97.3%.

HPLC-AMS analysis of parent drug was performed on all plasma samples. In each case 100 μL of the extract was injected onto a Shimadzu HPLC system with a Shimadzu or ISCO Foxy fraction collector (Cole-Parmer Instrument Co Ltd, London, United Kingdom). The HPLC eluent was collected as a series of 30-second fractions, typically 10 to 20 fractions across the relevant retention time for the drug being assayed. For each analysis, the corresponding nonradiolabeled drug was coinjected onto the relevant HPLC system (as discussed later), and fractions corresponding to the appropriate retention time were pooled and analyzed by AMS as a single sample (typically 3-5 fractions pooled). The radioactivity measured by AMS in this fraction was used to calculate the concentration of the parent drug in the sample. For brevity, this method is referred to as HPLC-AMS. In addition, for selected samples, each fraction in the region of the parent drug was analyzed to reconstruct a chromatogram to confirm that only parent drug was present in the peak of interest and was not coeluting with any putative metabolites.

The plasma concentration of each drug was determined by use of the following HPLC conditions. For warfarin, we used a Waters Xterra MS C18 column (4.6 × 250 mm, 5-μm particle size, 40°C) (Waters, Elstree, United Kingdom) eluted isocratically with water plus 0.1% (vol/vol) triethylamine in methanol (3:1 [vol/vol]) at a flow rate of 1 mL/min. For ZK253, we used a Luna C18 column (2 × 100 mm, 3-μm particle size, 25°C) (Phenomenex, Torrance, Calif) eluted with 0.1% (vol/vol) acetic acid in water (A) and 0.1% (vol/vol) acetic acid in acetonitrile (B) (85% A and 15% B with a linear gradient to 5% A and 95% B over a period of 20 minutes) at a flow rate of 0.3 mL/min. For diazepam, we used a Waters Symmetry Shield RP18 column (4.6 × 100 mm, 3.5-μm particle size, 40°C) eluted with 0.33% (vol/vol) aqueous acetic acid containing 0.002% (vol/vol) triethylamine (A) and 0.002% (vol/vol) triethylamine in methanol (B) (49% A and 51% B for 13.5 minutes and then a linear gradient to 5% A and 95% B over a period of 10 minutes) at a flow rate of 1 mL/min. For midazolam, we used a Waters Xterra MS C18 column (4.6 × 250 mm, 5-μm particle size, 40°C) eluted isocratically with acetonitrile–ammonium acetate, 0.01 mol/L, in water (1:1) at a flow rate of 1 mL/min. Finally, for erythromycin, we used a Waters Xterra MS C18 column (40°C) eluted isocratically with acetonitrile–ammonium acetate, 2 mol/L (pH 7)–water (7:1:12) at a flow rate of 1 mL/min.

Sample preparation for AMS analysis. HPLC fractions were "graphitized" according to the method of Vogel and Turteltaub.[12] In addition to HPLC fractions, 5 to 7 mg ANU sugar, 2 to 3 mg graphite, and 2.5 μL of liquid paraffin controls were graphitized according to the same method. ANU is a certified AMS standard, and the graphite was a negative control (ie, depleted of ^{14}C). Liquid paraffin was used as a carbon carrier and, like the graphite, was depleted in ^{14}C. Each batch of samples (134 in total) (see "AMS analysis" section) had at least 4 ANU, 2 graphite, and 5 liquid paraffins included. For an analysis batch to pass, 3 of the 4 ANU were 150.61 ± 22 percent modern carbon (pMC), graphite was less than or equal to 8 pMC, and 3 of the

5 liquid paraffins were less than or equal to 8 pMC. (pMC is defined in the "AMS data processing" section.)

AMS analysis. Cathodes containing graphite were placed into the ion source of a 5-MV tandem Pelletron AMS instrument (National Electrostatics, Madison, Wis). Conditions for AMS analysis were the same as those previously reported.[13]

AMS data processing. Results from the HPLC-AMS data were used to calculate the disintegrations per minute per fraction pool and nanograms of drug per milliliter of plasma from the specific activity. The AMS results were expressed as pMC, where 100 pMC = 98 fmol ^{14}C/g carbon or 0.1356 dpm ^{14}C/g carbon.[9] To convert to units of radioactivity per volume of sample, the percentage of carbon in the sample (based on 84.17% carbon in the 2.5-μL carrier used) is accounted for as follows: dpm ^{14}C/mL = (dpm ^{14}C/g carbon) × (% wt/vol carrier carbon). Concentrations of parent drug in plasma were calculated from the concentration in the HPLC fraction, the weight of the plasma extract, and the amount of plasma extracted.

Liquid chromatography–tandem mass spectrometry analysis. Liquid chromatography (LC)–tandem mass spectrometry (MS/MS) analysis was conducted by use of validated methods by Xendo Laboratories (Leiden, The Netherlands), which included determination of precision, accuracy, and linearity. LC-MS/MS analysis of parent drug in plasma after therapeutic doses was undertaken for ZK253, midazolam, and erythromycin by use of an Applied Biosystems API 3000 LC-MS/MS system (Foster City, Calif) in positive ionization mode with multiple reaction monitoring. Typical LC-MS/MS conditions were as follows: nebulizing gas (nitrogen) at a flow rate of 8 L/min and at 250°C. Nitrogen was used in the collision cell. The MS/MS was coupled to an Agilent Technologies HPLC system (Amstelveen, The Netherlands).

For analysis of ZK253, plasma (500 μL), to which 25 μL of methanol-water (1:1 [vol/vol]) and 20 μL of 85% (vol/vol) orthophosphoric acid had been added, was loaded onto an Oasis column (Waters), conditioned with 1 mL of methanol and then 1 mL of water, and washed with 1 mL of methanol/water (1:19 [vol/vol]), and ZK253 was eluted with 1 mL of methanol. HPLC separation was achieved by use of a Zorbax SB C8 column (2.1 × 50 mm, 5-μm particle size, 30°C) (Agilent Technologies), eluted with 10-mmol/L ammonium acetate (pH 5.0) (A) and methanol (B) (50% A and 50% B for 0.5 minute and then a linear gradient to 5% A and 95% B over a period of 2.5 minutes) at a flow rate of 0.3 mL/min. The ZK253-related compound ZK208819 was used as an internal standard, and

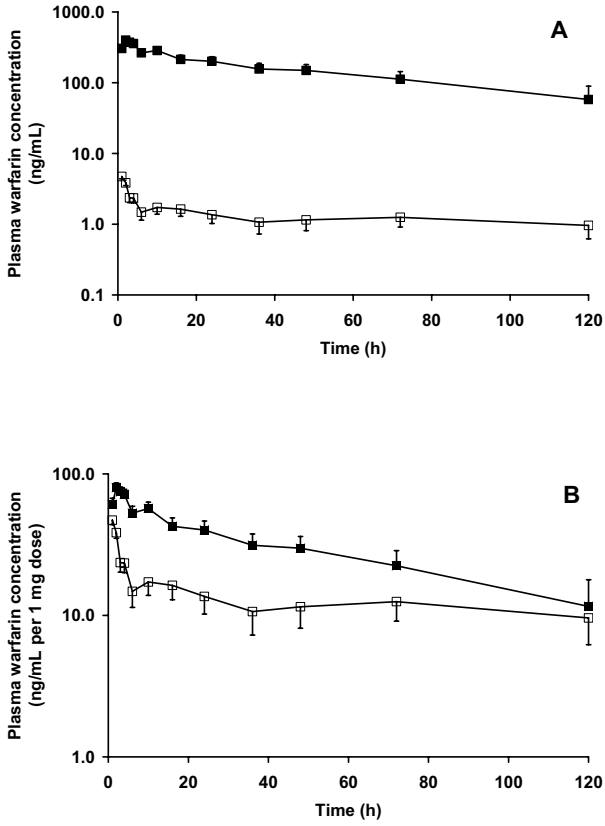

Fig 1. **A**, Semilog plot of plasma warfarin concentrations (geometric mean, SE) versus time for 100-μg (*open squares*) and 5-mg (*solid squares*) oral doses. **B**, Data shown in A normalized to 1-mg dose.

ZK253 was measured by use of a transition of mass-to-charge ratio (*m/z*) 692.3 → 334.1.

Midazolam was extracted from 100 μL of plasma with 3 mL of diethyl ether after the addition of 10 μL of methanol/water (1:1 [vol/vol]) and 100 μL of 0.7-mol/L sodium bicarbonate (pH 9.6). The diethyl ether was evaporated and the residue redissolved in 200 μL of water/acetonitrile (1:1 [vol/vol]). Separation was achieved with a Zorbax SB Phenyl column (150 × 3 mm, 3.5-μm particle size, 60°C) (Agilent Technologies), eluted with 10-mmol/L ammonium formate (pH 5.0) (A) and acetonitrile (B) (70% A and 30% B for 1 minute and then a linear gradient to 10% A and 90% B over a period of 1 minute) at a flow rate of 0.5 mL/min. Midazolam-d4 was used as an internal standard and midazolam measured by use of a transition of *m/z* 326.2 → 291.0.

Erythromycin was extracted from 250 μL of plasma with 1 mL of n-hexane/ethyl acetate (1:1 [vol/vol]) after the addition of 25 μL of methanol/water (1:1

Table III. Pharmacokinetic parameters for test drugs

Drug	*Treatment*	t_{max} *(h)*	C_{max} *(ng/mL)*	$AUC_{0\text{-}t}$ *(ng · h/mL)*
Warfarin	Oral, 100 μg	1.12*† (35.0)	5.04*† (29.2)	157*† (20.2)
	Oral, 5 mg	1.70 (41.1)	493 (23.8)	16,152 (38.9)
ZK253	Intravenous, 100 μg	0.71 (72.7)	0.91 (61.6)	5.53 (31.9)
	Oral, 100 μg	ND	ND	ND
	Intravenous, 100 μg	0.50 (0)	1.52 (56.3)	5.78 (13.1)
	Oral, 50 mg	0.50 (0)	2.84 (34.1)	4.4# (21.2)
Diazepam	Intravenous, 100 μg	0.56 (35.0)	4.70 (28.7)	55.9 (33.3)
	Intravenous, 10 mg	0.79 (77.5)	322 (45.6)	4791 (40.9)
Midazolam	Intravenous, 100 μg	0.40 (30.1)	2.56 (42.1)	3.69 (31.2)
	Oral, 100 μg	0.56 (2.86)	0.37 (62.2)	0.89 (87.8)
	Intravenous, 100 μg	0.50 (0)	2.97 (59.4)	4.40 (35.6)
	Oral, 7.5 mg	0.63# (139.5)	34.0 (79.2)	81.8# (79.0)
Erythromycin	Intravenous, 100 μg	0.56 (90.4)	3.35 (57.4)	4.27 (80.0)
	Oral, 250 mg	0.45 (22.3)	717 (38.0)	1510# (38.6)

Data are presented as mean and percent coefficient of variation. Comparisons of C_{max} and area under the curve are dose-normalized data.

t_{max}, Time to maximum concentration; C_{max}, maximum concentration; $AUC_{0\text{-}t}$, area under curve for period studied; $AUC_{0\text{-}\infty}$, area under curve extrapolated to infinity; $t_{½}$, half-life; CL, clearance; V, volume of distribution; F, oral bioavailability; ND, not determined because data were below limit of detection.

*n = 6 (all subjects used in calculation, with time corresponding to 120 hours).

†ANOVA between microdose and corresponding therapeutic dose: $P < .01$.

‡n = 3 (ie, the 3 subjects receiving the microdose on the first dosing occasion used for calculation). For warfarin, CL and V refer to CL/F and V/F, respectively (with time corresponding to 528 hours).

§Determined on the basis of dose/$AUC_{0\text{-}\infty}$ and with the assumption of 100% oral bioavailability.

‖ANOVA between microdose and corresponding therapeutic dose: $.01 < P < .05$.

¶Determined on the basis of $1.44 \times CL \times t_{½}$.

#The areas under the curve for the intravenous 100-μg dose have been subtracted.

**The $t_{½}$ value was difficult to estimate because the assay was below the limit of detection at and beyond 16 hours.

[vol/vol]) and 30 μL of 1-mol/L sodium carbonate. The n-hexane/ethyl acetate was evaporated and the residue redissolved in 100 μL of water/acetonitrile (1:4 [vol/vol]). Separation was achieved by use of a Synergi MAX 80A column (50 × 2 mm, 4-μm particle size, 30°C) (Phenomenex, Macclesfield, United Kingdom), eluted with 5-mmol/L ammonium acetate (pH 3.0) (A) and acetonitrile (B) (85% A and 15% B for 0.5 minute and then a linear gradient to 10% A and 90% B over a period of 2.5 minutes) at a flow rate of 0.5 mL/min. Roxithromycin was used as an internal standard, and erythromycin was measured by use of a transition of *m/z* 734.4 → 158.1.

Limits of detection. Limits of detection (LODs) for AMS analysis were derived by analysis of predose plasma samples and measurement of ^{14}C in fractions corresponding to the retention time of the parent compound.[9] Twice the mean value, across subjects, per dose regimen was deemed to equal the LOD (ie, 2× background). With the exception of ZK253, the LOD for HPLC-AMS of the microdose was approximately 10 pg/mL plasma. This LOD was based on fractions pooled across the parent peak (see "^{14}C-Labeled parent drug measurement by HPLC and AMS" section) and could be considered a routine LOD, that is, without undue method development or peak optimization (although peak resolution was important to separate parent from any potential metabolites). For ZK253, a lower LOD was necessary because of the low oral bioavailability and large volume of distribution for this drug. In this case the HPLC peak shape was optimized, and individual fractions were analyzed across the parent peak, which achieved an LOD of 300 fg/mL plasma. LODs for LC-MS/MS were determined by standard curves and were 100 pg/mL plasma.

Pharmacokinetics. PK parameters were calculated via a noncompartmental model with WinNonlin software, version 4.1 (Pharsight, Mountain View, Calif). Input consisted of plasma drug concentrations (in nanograms per milliliter), recorded times of sample collection (in hours), and doses administered. Output consisted of maximum concentration, time to maximum concentration, terminal half-life, area under the curve (AUC) for the period studied, and AUC extrapolated to infinity (for both intravenous and oral doses), as well as clearance and volume of distribution for intravenous doses. AUC was calculated by use of a combination of linear and log-linear trapezoid approximations. Oral bioavailability was calculated from the ratio of dose-normalized AUC values after oral and intravenous doses, in the usual manner. For ZK253, midazolam, and erythromycin, the oral and intravenous doses were ad-

$AUC_{0-\infty}$ (ng · h/mL)	$t_{1/2}$ (h)	CL (L/h)	V (L)	F (%)
571†‡ (82)	274†‡ (80.6)	0.17‡§‖ (54.8)	67.3†‡¶ (41.4)	
20,801 (44.6)	48.6 (48.6)	0.26§ (48.1)	17.9¶ (19.9)	
7.42 (51.4)	61.4 (67.3)	9.29 (54.1)	1207 (40.6)	
ND	ND	ND	ND	<1
7.15 (16.3)	56.2 (32.4)	14.8 (18.8)	1201 (31.2)	
6.55# (58.8)	7.42** (116)			0.16
65.5 (34.5)	45.1 (51.2)	1.38 (36.8)	90.1 (35.8)	
5577 (77.7)	35.7 (49.1)	1.30 (66.5)	123 (45.6)	
4.53 (31.1)	4.87 (38.8)	21.2 (30.6)	145 (29.6)	
1.02 (87.6)	3.95 (24.7)			22.8
4.68 (37.5)	2.55 (38.6)	20.4 (34.3)	75.1 (48.7)	
87.0# (83.2)	3.31 (33.7)			22.1
4.38 (78.8)	2.52 (34.6)	21.9 (76.4)	79.8 (68.0)	
1556# (40.3)	2.50 (17.6)			14.0

ministered simultaneously. To remove the error in the oral measurements resulting from the contribution of the intravenous microdose, the concentrations achieved by the intravenous dose (measured by HPLC-AMS) were subtracted from those obtained for the oral therapeutic dose (measured by LC-MS/MS). Differences in the PK parameters after the oral microdose and therapeutic dose were evaluated by use of ANOVA.

RESULTS

All subjects completed the study, and treatments were well tolerated. On the basis of the radioactive concentrations of plasma samples taken before each dose administration, there was no evidence of any carryover from a previous dose, with a minor exception in the case of warfarin (see Discussion section).

Warfarin. Fig 1, *A*, shows a semilog plot of plasma warfarin concentration versus time after oral administration of the microdose (100 μg) and therapeutic dose (5 mg). Only 1 concentration value was excluded as an outlier (subject 1, 3 hours, 5-mg dose). The percent coefficient of variation for plasma concentrations achieved across subjects was approximately 31% for the microdose and 36% for the therapeutic dose. In all subjects the plasma concentration at the last sampling time (120 hours) was above the LOD for both assays. Fig 1, *B*, shows the same data as presented in Fig 1, *A*, but with normalization to a 1-mg dose. Subsequent plots for the other 4 compounds are of the same 1-mg dose–normalized format to facilitate graphic comparison of the kinetics after the 2 sized doses. The PK parameters for warfarin are summarized in Table III. As shown in Fig 1, *B*, there is no overlap in the dose-normalized concentrations except for those at the initial sampling time (1 hour) and last sampling time (120 hours). The time to maximum concentration occurred earlier and the plasma concentration then dropped more sharply before displaying a longer terminal phase after microdose administration than after therapeutic dose administration. For some subjects, the terminal decline in plasma concentration after microdose administration was too slow to provide a reliable estimate of half-life over the 5-day period of study. After therapeutic dose administration, the values of clearance and volume of distribution were 0.26 L/h and 17.9 L, respectively (Table III). For microdose administration, the AUC up to 120 hours was reasonably similar for all 6 subjects (percent coefficient of variation, 20%).

ZK253. Fig 2, *A*, shows semilog plots of the dose-normalized plasma ZK253 concentration versus time after the simultaneous administration of 50 mg orally and the microdose intravenously. There were no outliers excluded from the concentration data. The percent coefficient of variation for plasma concentrations achieved across subjects was approximately 51% for the microdose (measured by HPLC-AMS) and 59% for the therapeutic dose (measured by LC-MS/MS). Table III lists the PK parameters after the intravenous dose. Despite 50 mg being administered orally, the plasma concentration fell below the LOD by 16 hours of this 120-hour study, making an accurate estimate of oral bioavailability problematic. However, when we assume that absorption ceases after 16 hours and that the terminal half-life observed after the intravenous microdose, which was well characterized with concentrations well above the LOD up to 72 hours, applies to the oral dose, the oral bioavailability of ZK253 is in the vicinity of 0.16%. Because of this very low oral bioavailability, despite the 500-fold difference between the therapeutic

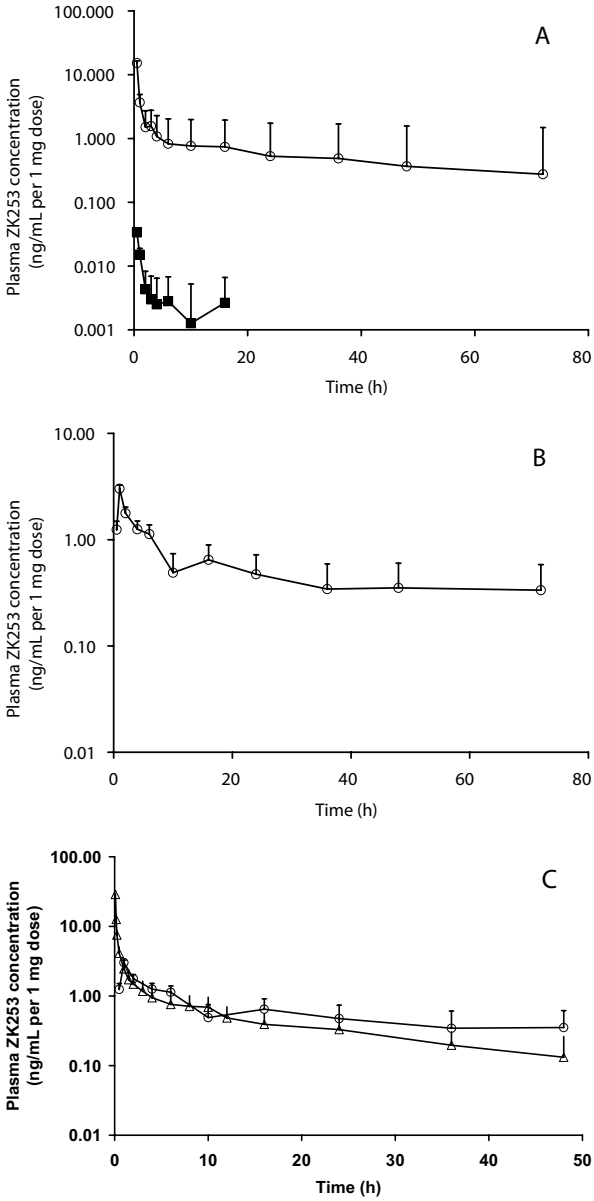

Fig 2. **A**, Semilog plot of plasma ZK253 concentrations (geometric mean, SE), normalized to 1-mg dose, versus time after simultaneous administration of 100-μg intravenous dose (*circles*) (^{14}C-labeled and measured by HPLC–accelerator mass spectrometry [AMS]) and 50-mg oral dose (*squares*) (nonradiolabeled and measured by liquid chromatography–tandem mass spectrometry [LC-MS/MS]). **B**, Semilog plot of plasma ZK253 concentrations, normalized to 1-mg dose, versus time for 100-μg intravenous dose (alone, without oral therapeutic dose) (*circles*). Data for the oral administration were all below the limit of detection (300 fg/mL). Intravenous doses were administered by infusion over a period of 30 minutes. **C**, Semilog plot of plasma ZK253 concentrations (geometric mean, SE), normalized to 1-mg dose, versus time after administration of 100-μg intravenous dose (*circles*) (^{14}C-labeled and measured by HPLC-AMS) and 5-mg intravenous dose (*triangles*). The latter dose was part of a clinical trial performed before the current study.

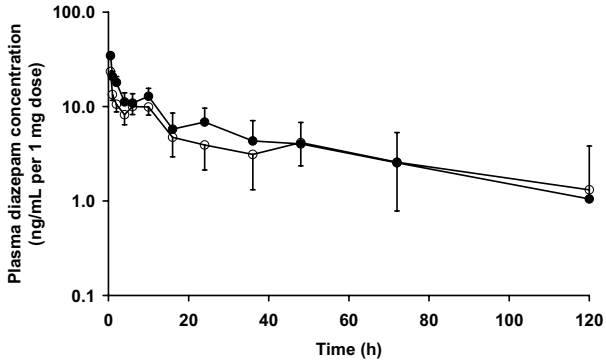

Fig 3. Semilog plot of plasma diazepam concentrations (geometric mean, SE), normalized to 1-mg dose, versus time after administration of 100-μg intravenous dose (*open circles*) and 10-mg intravenous dose (*solid circles*). Intravenous doses were administered by infusion over a period of 30 minutes.

and microtherapeutic oral doses, the plasma concentrations associated with the intravenous microdose were a substantial fraction of those after the oral dose, constituting approximately 30% of the measured oral AUC, which necessitated subtracting the contribution of the intravenous microdose from the measured oral dose when oral dose parameters were estimated.

All measurements of plasma ZK253 concentrations after the oral microdose (100 μg) were below the LOD. Fig 2, *B*, shows the semilog plot of plasma concentrations, normalized for dose, achieved for the intravenous 100-μg dose. The failure to measure any concentration after the oral microdose meant that no PK parameters could be determined.

Diazepam. Plasma diazepam concentrations, normalized for dose, achieved after intravenous administration of the microdose (100 μg) and the 10-mg therapeutic dose are shown in Fig 3. One subject had anomalously low results on HPLC-AMS analysis after the therapeutic dose and was excluded from the group data. The percent coefficient of variation for plasma concentrations achieved across subjects was approximately 50% for the microdose and 70% for the therapeutic dose. Table III lists the corresponding PK pa-

rameters. All PK parameters were very similar between the microdoses and therapeutic doses.

Midazolam. Fig 4 displays semilog plots of the dose-normalized plasma midazolam concentration versus time after the simultaneous administration of the 7.5-mg therapeutic dose orally and the 100-μg microdose intravenously. There were no outliers except 1 anomalously high predose sample, which was excluded from the calculation of the LOD. The percent coefficient of variation for plasma concentrations achieved across subjects was approximately 50% for the microdose and 90% for the therapeutic dose. The summary PK information is listed in Table III. Several points emerge. First, there was no difference in intravenous disposition kinetics of midazolam when given as the microdose alone or in the presence of the 7.5-mg oral dose. Second, the same is true for the oral dose, in that the plasma concentrations, when normalized for dose, were essentially superimposable after the 100-μg and 7.5-mg doses. Last, the mean oral bioavailabilities calculated after the simultaneous administration of the oral 7.5-mg dose and intravenous microdose, when compared with the AUCs after administration of the microdose orally and intravenously, given on separate occasions, were 23% and 22%, respectively. As with ZK253, the plasma concentrations obtained after intravenous administration were subtracted from the corresponding plasma concentration obtained for the 7.5-mg oral administration. However, because midazolam has a relatively high oral bioavailability compared with that of ZK253, subtraction of the plasma concentrations contributed by the intravenous 100-μg dose made only a marginal difference (approximately 5%) to the oral data.

Erythromycin. Semilog plots are displayed in Fig 5. There was only 1 datum point excluded as an outlier. The percent coefficient of variation for plasma concentrations achieved across subjects was approximately 85% for the microdose (HPLC-AMS) and 90% for the therapeutic dose (LC-MS/MS). A clear parallelism in the decline of the oral therapeutic and intravenous microdose curves is evident, and together, these curves provide an estimate for oral bioavailability of 14%. However, because of the 2500-fold difference in doses and the moderate oral bioavailability of erythromycin, the correction of the measured plasma concentration after the 250-mg oral dose for the intravenous microdose was minimal. Results for the oral 100-μg microdose were all below the LOD for all subjects. The LOD for erythromycin is shown in Fig 5.

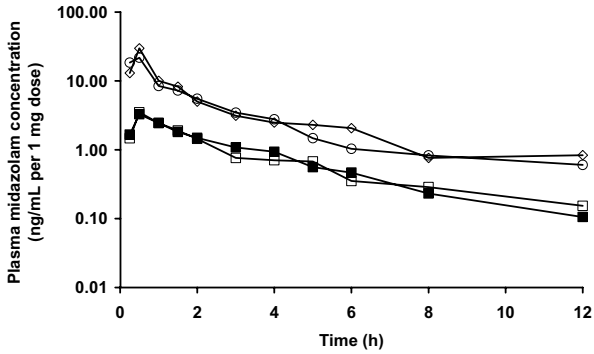

Fig 4. Semilog plot of plasma midazolam concentrations, normalized to 1-mg dose, after simultaneous administration of 100-μg intravenous dose (*diamonds*) (^{14}C-labeled and measured by HPLC-AMS) and 7.5-mg oral dose (*solid squares*) (nonradiolabeled and measured by LC-MS/MS), along with those after administration on separate occasions of 100-μg intravenous dose (*circles*) and 100-μg oral dose (*open squares*) (both measured by HPLC-AMS). Data are presented as geometric mean; error bars have been removed for clarity. Intravenous doses were administered by infusion over a period of 30 minutes.

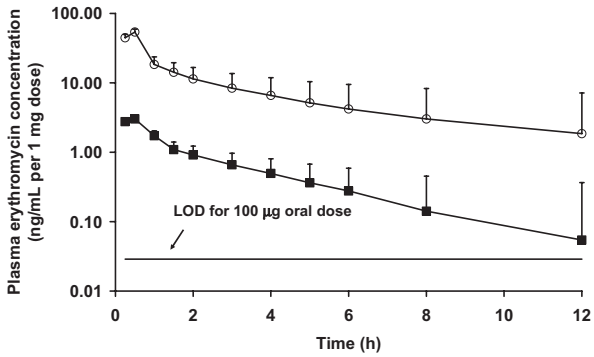

Fig 5. Semilog plots of plasma erythromycin concentrations (geometric mean, SE), normalized to 1-mg dose, versus time after simultaneous administration of 100-μg intravenous dose (*circles*) (^{14}C-labeled and measured by HPLC-AMS) and 250-mg oral dose (*squares*) (nonradiolabeled and measured by LC-MS/MS), along with limit of detection (LOD) for oral 100-μg dose. Intravenous doses were administered by infusion over a period of 30 minutes.

DISCUSSION

This collaborative trial was undertaken to investigate the ability of microdose data by use of AMS to predict human pharmacokinetics after therapeutic doses, against the general concern that failure to predict, as a result of nonlinearities in one or more PK processes,

Table IV. Values for pharmacokinetic parameters for 4 commercially available drugs and 1 in-house company drug from literature and unpublished data

Drug	*CL (L/h)*	*V (L)*	*Terminal $t_{1/2}$ (h)*	*F (%)*	*Source*
Warfarin	0.19 (1.9)	9.8 (49.0)	37 (40.0)	93 (8.6)	Reference 15
ZK253	29.5 (25.4)	1257 (19.9)	31.1 (34.1)	<1	Unpublished data (Kuhnz W, Schering, 2005)
Diazepam	1.6 (64.0)	77 (100)	43 (30.0)	100 (14.0)	Reference 16
Midazolam	27.7 (27.4)	77 (54.5)	1.9 (47.4)	44 (38.6)	References 17 and 18
Erythromycin	38.2 (45.0)	54.6 (56.4)	1.6 (43.7)	35 (78.1)*	References 19 and 20

Data are presented as mean and percent coefficient of variation. When values were given per kilogram of body weight, they were converted by use of a nominal body weight of 70 kg.
*Enteric-coated erythromycin base.

may result in falsely rejecting a drug for further development. A secondary objective was to study the usefulness of simultaneous intravenous microdosing with the therapeutic oral dose to assess absolute bioavailability. The drugs selected for this study were representative of those for which either animal models or in vitro studies (or both) failed or might reasonably be expected to be problematic in predicting human pharmacokinetics at therapeutic doses.

Microdosing is not anticipated to produce any pharmacologic or toxicologic effect or saturate enzymatic processes. As such, the nonclinical safety data required are much fewer than needed when pharmacologic doses are administered. Human studies can therefore be conducted earlier, with the potential to help assess whether further investment in a compound is worthwhile. Any sufficiently sensitive analytic technique could be used in such studies. However, given the often extremely low plasma concentrations achieved with microdosing, AMS is routinely used because it is capable of measuring a compound in the femtogram range and below.

AMS measures total ^{14}C, that is, drug plus metabolites. To measure parent drug, we have coupled offline HPLC with AMS. An additional application of ^{14}C-labeled compound provides the opportunity to determine the oral bioavailability of a therapeutic dose, by simultaneously administering a radiolabeled microdose intravenously and assaying the 2 sources of drug in plasma by use of LC-MS/MS and LC-AMS, respectively. LC-MS/MS data after a therapeutic dose also allowed direct comparison with literature data.

The low mass of a microdose will typically dissolve in the 150 to 250 mL commonly used in oral dosing studies. Accordingly, oral microdosing studies characterize the in vivo solution, and not the solid dosage form, properties of a compound. In this study, to minimize formulation effects for 4 of 5 test compounds, the therapeutic dose was administered in solution to allow direct comparison with the microdose. The exception was erythromycin; because the 250-mg therapeutic dose does not dissolve in 250 mL, a suspension was used.

Warfarin was chosen to represent a low-clearance acidic drug whose intrinsic clearance in human in vitro microsomal or hepatocyte systems could not be determined with sufficient accuracy during the viability of the preparation to predict clearance in vivo. The marketed compound is a racemate, but because the pharmacokinetics of the 2 enantiomers are similar,[14] a nonenantioselective specific assay was used. Warfarin is fully bioavailable when given orally at therapeutic doses.[14] If we assume this applies in our study, the mean PK parameters after the 5-mg therapeutic dose are broadly similar to literature values (Table IV). However, the dose-normalized microdose data were decidedly different, with the plasma concentration initially falling more rapidly and then more slowly than after the therapeutic dose. Unfortunately, the 120-hour duration of the study, chosen based on an anticipated 1.5-day half-life (Table IV), was insufficient to allow full characterization after microdose administration. However, in 3 of 6 subjects, who received the microdose first, a 528-hour (22-day) postdose sample, just before the therapeutic dose, was found to contain warfarin at approximately 3 times the LOD. This provided an estimated terminal half-life of 8 days. The resultant estimated clearance of 0.17 L/h, with complete absorption assumed, was similar to that at therapeutic doses, implicating distribution as the major source of nonlinearity in warfarin pharmacokinetics (volume of distribution of 67 L versus 10-18 L for the therapeutic dose).

When warfarin is bound to albumin,[21] which is not saturated at therapeutic concentrations, it is extremely unlikely that the difference in warfarin distribution with

dose can be attributed to plasma protein binding. The explanation probably lies in saturable tissue binding. Evidence supporting this possibility comes from tracer and tissue distribution studies in rats, in which a low-capacity, high-affinity warfarin binding site was identified within the liver, vitamin K 2,3-epoxide reductase, which may be the therapeutic target.[22] Moreover, scaling up of these data through a physiologically based model to humans indicated that this nonlinearity should be evident when one compares the plasma concentration profiles well above and below 50 ng/mL,[22] which occurred with the therapeutic doses and microdoses, respectively (Fig 1, *A*). Clearly, preclinical data can aid in the interpretation of human microdose data.

ZK253, a Schering drug development candidate, was studied because it represents a compound with great uncertainty in predicted oral absorption in humans based primarily on conflicting animal data (absolute bioavailability of 27% in rats, 73% in mice, 5% in dogs, and 0.7% in monkeys). An absolute bioavailability study was performed by the company in postmenopausal female volunteers receiving either an oral 50-mg dose or an intravenous 5-mg dose, and when a bioavailability of less than 1% was found (Table IV), the development program was stopped. A close accord in the intravenous disposition pharmacokinetics between this earlier company study and the current microdose data is clearly evident (Fig 2, *C*), suggesting that the disposition kinetics of ZK253, which extensively distributes into tissues (volume of distribution of 1200 L), is independent of dose.

A direct test of linearity between oral microdoses and therapeutic doses of ZK253 could not be made, because plasma concentrations after the microdose were all below the LOD. However, even with the assumption that the maximum oral concentration was at the LOD (300 fg/mL plasma) (see Methods section) and remained there for 16 hours, the oral bioavailability of ZK253 would still have been less than 1%, and the compound would have been dropped. Clearly, critical to this decision are the intravenous microdose data, which provide the reference AUC for a given systemic dose. The results with ZK253 also emphasize the need for an ultrasensitive analytic technique such as AMS with microdosing studies, especially for compounds with extensive distribution.

Midazolam represents the class of compounds that have a low oral bioavailability as a result of extensive first-pass cytochrome P450 (CYP) 3A4/5–catalyzed metabolic intestinal and hepatic loss. Despite the considerable potential for saturation of intestinal and hepatic metabolism, this did not occur, as evidenced by no change in the disposition kinetics of the intravenous microdose in the presence of the oral therapeutic dose, as well as the excellent linearity between the oral microdoses and therapeutic doses. A similar observation was also made by Eap et al,[23] comparing a 75-μg and a 7.5-mg oral dose using LC-MS/MS to characterize the resultant profiles.

Erythromycin was chosen for several reasons. Like midazolam, erythromycin is a substrate for CYP3A, but its pharmacokinetics is also influenced by transporters, including P-glycoprotein.[24] Coupled enzyme-efflux transporter interplay is expected to be responsible, at least in part, for its low oral bioavailability. It is also administered therapeutically at high doses that could saturate processes during absorption, The estimated 14% oral bioavailability of Erythrocine-ES (ethyl succinate) granulate suspension in this study is somewhat lower than the 35% reported for erythromycin.[25] However, its bioavailability varies considerably with pharmaceutical formulation, salt and ester form, and dose, so strict comparison is not possible.

Why erythromycin is undetectable in plasma after oral microdosing is unclear. Possibly, all of the compound is completely metabolized in the small-intestinal wall and liver during absorption of this low dose. Certainly, some drug-related material was absorbed as evidenced by appreciable plasma concentrations of total radioactivity after microdose administration (data not shown). Partial saturation of the processes involved would explain the much higher oral bioavailability of the 250-mg therapeutic dose. An alternative possibility is that the microdose of erythromycin in solution is substantially degraded in the acidic stomach before entering the small intestine, where absorption most likely occurs. Erythromycin is acid-labile, with a degradation half-life of 15 minutes at pH 3 and an even shorter half-life at lower gastric pH values.[26] With a typical mean gastric-emptying half-life of solutions of 15 minutes,[27] this would imply that at least 50% (and probably more) erythromycin could have been hydrolyzed before entering the small intestine. The granulate form of the therapeutic dose, with incomplete dissolution in the stomach, is expected to protect erythromycin from such acid hydrolysis. Irrespective of the cause of the loss of bioavailability of a microdose of erythromycin, this finding highlights the need to evaluate drug stability in gastric and intestinal fluids when one is contemplating performing oral microdosing studies.

Several general points emerge from this study. The marked difference in the volume of distribution between the microdoses and therapeutic doses of warfarin, but not of ZK253, diazepam, and midazolam, may be a result of

a chance selection of these compounds. Alternatively, it may reflect differences in their basic physicochemical properties. Warfarin is typical of the majority of acidic drugs, with small volumes of distribution, usually 0.1 to 0.3 L/kg. This occurs because acids are often predominantly bound in the body to albumin, which itself has a volume of distribution of only 0.1 L/kg.[28] Hence, any high-affinity but low-capacity tissue binding would have a noticeable impact on events in plasma, with an increase in estimated volume of distribution. In contrast, most basic compounds, which include diazepam, midazolam, and indeed ZK253, bind extensively outside of plasma, particularly to the large amount of tissue-acidic phospholipids.[29] Hence, even though bases bind to specific receptors, saturation of high-affinity, low-capacity specific tissue binding sites would not be readily reflected in plasma, because so little drug in the body is bound to the receptor even at very low, nonsaturating concentrations. Still, when one is undertaking microdosing studies, the possibility of high-affinity, low-capacity binding as a source of nonlinearity in pharmacokinetics needs to be kept in mind.

Diazepam was chosen to represent compounds that have reasonable tissue distribution, are of low clearance, and are extensively metabolized by one of the common CYP enzymes, in this case CYP2C9, so that changes in intrinsic metabolic clearance should be reflected in the observed clearance and, hence, disposition kinetics. For this compound, linear pharmacokinetics over the dose range 100 μg to 7.5 mg was evident. In practice, strict dose linearity is not critical when microdosing is used. Rather, the results should allow a correct decision as to whether developing the compound further is worthwhile. In the broad sense one would like to know that microdosing yields results in the range of the likely pharmacokinetics within the population, even though some nonlinearity of dose may exist. This view may be thought of as similar to that applied in bioequivalence testing, although the bounds would be considerably wider than the typical ±20% used in such testing.

Though not the primary objective of this study, simultaneous administration of an intravenous trace radiolabeled dose with an oral therapeutic dose is a useful way of estimating not only the disposition kinetics of a compound but also the absolute oral bioavailability of a therapeutic dose, even when, at this dose level, the kinetics is nonlinear.[30] Because the trace intravenous dose adds minimally to the exposure achieved with the therapeutic dose and the amount of radioactivity is minute, there should be minimal need for safety testing of this intravenous dose, so its use can be implemented at an early stage of phase I testing. Indeed, if the radiolabeled microdose had been given orally and intravenously on separate occasions before pharmacologic doses were given, one would have known the absolute oral bioavailability of the oral microdose, as illustrated with midazolam, and would have had some idea of the maximum value even when all plasma concentrations were undetectable after oral administration, as seen in the case for ZK253. This possibility of gaining very early PK information should be contrasted with the more limited insights obtained by having only systemic exposure data after oral pharmacologic doses.

In summary, microdose data from 3 of the 5 drug candidates tested would have predicted the therapeutic dose pharmacokinetics well (diazepam, midazolam, and ZK253). For warfarin, microdose data would have been useful in predicting clearance for a therapeutic dose, but there was a lack of linearity in the distribution pharmacokinetics. No clear conclusion could be drawn from the microdose data derived from erythromycin, except that careful consideration should be given to orally microdosing acid-labile drugs. Although there are potential limitations, this study shows that, when used intelligently, microdosing is a useful additional tool to assist in decision making during drug development. Studies with more compounds displaying different physicochemical and structural properties will help to further clarify the utility of this tool.

Drs Garner and Lappin are employees of Xceleron and hold stock in the company. Dr Oosterhuis is an employee of Pharma Bio-Research and has no conflict of interest. Dr Drijfhout is an employee of and holds stock in Pharma Bio-Research. Dr Chaudhary is an employee of Eli Lilly and has no conflicts of interest regarding consultancies, stock ownership, honoraria, paid expert testimony, patent applications, and research and travel grants with regard to this report. Dr Kuhnz is an employee of Schering and holds stock options in the company. Dr Kneer is an employee of F. Hoffman-La Roche and has no conflict of interest. Dr Jochemsen is an employee of Laboratoires Servier and has no conflict of interest. Dr Rowland has no conflicts of interest. He acted as an independent honorary advisor throughout the study. Subsequently, he accepted the position of chair of the Scientific Advisory Board of Xceleron but holds no stock in the company.

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