# Development of HPLC plasma assays for CAM 4515 and CAM 4750, two new nonpeptide tachykinin antagonists, and application to bioavailability studies[1]

Ted Van Noord, D. Scott Wright, Be-Sheng Kuo*

*Department of Pharmacokinetics and Drug Metabolism, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105, USA*

## Abstract

CAM 4515 and CAM 4750 are new nonpeptide tachykinin $NK_1$ receptor antagonists with different lipophilicities. Two separate, simple, and sensitive HPLC methods for the quantitation of these two compounds in plasma and the evaluation of their oral bioavailability in rats were developed and validated. Extraction of CAM 4515 from plasma involved protein precipitation with acetonitrile, while that for CAM 4750 involved a one-step liquid–liquid extraction with methylene chloride. The analytes in extracts were chromatographed on a C18 column using two different separation buffers, 47% 0.02 M sodium citrate (pH 3.5)–53% acetonitrile for CAM 4515 and 59% 0.02 M potassium phosphate dibasic (pH 7.0)–41% acetonitrile for CAM 4750, and both compounds were detected by fluorescence (excitation 278 nm; emission 342 nm). Stability profiles of both drugs at $-20°C$ or room temperature in plasma and in reconstituted buffers were good. The limit of quantitation for both drugs was 5 ng ml$^{-1}$ with good linearity from 5 to 1000 ng ml$^{-1}$ using 100–200 $\mu$l of plasma. Excellent precision (relative standard deviation <8.3%) and accuracy (relative error $\pm 9.2\%$) were observed for both CAM 4515 and CAM 4750. Oral bioavailability studies were conducted for each compound in rats receiving a p.o. dose of 20 mg kg$^{-1}$ and an i.v. dose of 5 mg kg$^{-1}$. The absolute oral bioavailability of CAM 4750 (80%) was estimated to be 40-fold greater than that of CAM 4515 (2%). The experimental results suggest that incorporation of a pyridine group into the structural backbone may greatly improve bioavailability.

Keywords: Bioavailability; CAM 4515; CAM 4750; HPLC assay; Pharmacokinetics; Rats; Tachykinin antagonist

## 1. Introduction

CAM 4515 and CAM 4750 (Fig. 1) are new nonpeptide tachykinin $NK_1$ receptor antagonists

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* Corresponding author.
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with different lipophilicities. The tachykinins (also referred to as neurokinins), substances P (SP), neurokinin A ($NK_A$) and neurokinin B ($NK_B$), are a family of neuropeptides (10–11 amino acids) widely distributed in the mammalian central and peripheral nervous system [1,2]. The actions of tachykinins are mediated by three distinct receptors, $NK_1$, $NK_2$ and $NK_3$ [1,3,4], which bind preferentially SP, $NK_A$ and $NK_B$, respectively
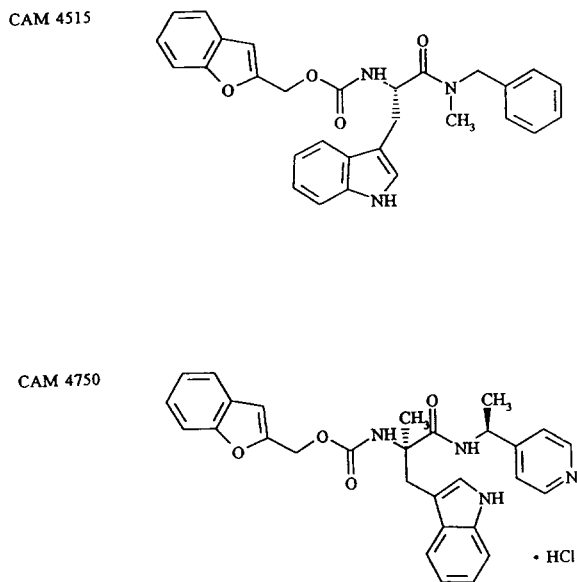
Fig. 1. Structures of CAM 4515 and CAM 4750.

[5,6] and all belong to the superfamily of G-protein coupled receptors with seven putative transmembrane spanning segments [5]. Tachykinins are involved in the regulation of pain and some autonomic reflexes and behavior, and they are mediators of neurogenic inflammation and motility in the gut [1,2]. In recent years, a number of potent and selective antagonists of both peptide and nonpeptide nature have been developed for the NK₁ and NK₂ receptors [6–9], with potential therapeutic use in the areas of migraine, emesis, schizophrenia, rheumatoid arthritis, asthma and parkinsonism [6,10,11]. Like CAM 4261 reported earlier [8], CAM 4515 and CAM 4750 exhibit sub-nanomolar selective receptor binding and were rationally designed based on the chemical structure of the target peptide, substance P, of the $Nk_1$ receptor [8,11].

The discovery of tachykinin antagonists has been reviewed previously [6,10]. The goal of the development of nonpeptide tachykinin antagonists has been to eliminate potential problems of poor oral absorption (bioavailability), short half-life, poor brain penetration, immunogenicity and enzymatic/chemical instability and thus fluctuating pharmacokinetic behavior associated with peptide drugs. Both nonpeptide CAM 4515 and

CAM 4750 contain a D-trytophan moiety imparting protease-resistant and antagonistic properties to their chemical structures, but CAM 4515, which contains a phenyl ring, has a lower solubility than that of the latter, which has a corresponding pyridine ring. In order to examine the oral bioavailability of these two drug compounds for further development, it was essential to develop a simple, sensitive assay to determine the drug concentrations in plasma of experimental animals. In this paper, we describe two separate validated HPLC methods for the quantitation of CAM 4515 and CAM 4750 in rat plasma. The two assay methods are different and have a sensitivity of 5 ng ml$^{-1}$ for either compound using 100 $\mu$l of rat plasma for CAM 4515 and 200 $\mu$l of plasma for CAM 4750. The methods involved protein precipitation by acetonitrile for CAM 4515 or liquid–liquid extraction with methylene chrloride for CAM 4750, use of internal standards, separation of the analytes on a C18 column with different mobile phases and quantitation by fluorescence. Animal studies have shown a markedly higher oral bioavailability for CAM 4750, consistent with its structure with lower lipophilicity.

## 2. Materials and methods

### 2.1. Chemicals, reagents and apparatus

CAM 4515 {carbamic acid, [1(1H-indol-3-ylmethyl)-2-[methyl(phenylmethyl)amino]-2-oxoethyl], 2-benzofuranylmethyl ester, (S)} (M.W. 482) and CAM 4750 {carbamic acid, [1-(1H-indol-3-ylmethyl)-1-methyl)-2-oxo-2-[[1(4-pyridinyl)ethyl]amino]ethyl]amino]ethyl], 2-benzofuranylmethyl, ester, monohydrochloride [R-(R*, S*)]} (M.W. 533), and their respective analogues as internal standards (IS), CAM 2688 (IS₁) {carbamic acid, [1 - (1H - indol - 3 - ylmethyl) - 1 - methyl - 2 - oxo - 2 - [(1 -phenylethyl)amino]ethyl]-, (4-ethylphenyl) methyl ester, [R-(R*, S*)} and CAM 4451 (IS₂) {carbamic acid, [2 - [(2 - hydroxy - 1 - phenylethyl) amino]-1-phenylethyl)amino]-1-(1H-indol-3-ylmethyl)-1-methyl-2-oxoethyl]-, 2-benzofuranylmethyl ester, [R-(R*, R*)]}, were obtained from

the Parker-Davis Neuroscience Research Center (Cambridge, UK). Acetonitrile (HPLC grade) and sodium phosphate (AR grade) were obtained from Mallinkrodt (Paris, KY). Citric acid, sodium hydroxide, methylene chloride (all GR grade) and water (HPLC grade) were obtained from EM Science (Gibbstown, NY). Absolute ethanol was obtained from Aaper Alcohol (Shellyville, KY) and PEG 400 (polyethylene glycol) from Union Carbide (Danbury, CT). Heparinized rat plasma was obtained from Pel Freez Biologicals (Rogers, AK). The HPLC system from Waters (Milford, MA) consisted of a model 600 multisolvent delivery system, a Model 712 WISP autosampler and a Model 470 fluorescence detector. Data were collected using a ChromJet integrator interfaced with a Chrom Station/2 data system from Spectra-Physics. Evaporations were done with a Turbovap LV from Zymark (Hopkinton, MA).

## 2.2. Standard and quality control preparation

Plasma standards of CAM 4515 or CAM 4750 were prepared freshly for each analysis run by diluting the stock solutions (0.5 mg ml$^{-1}$ in acetonitrile) with rat plasma to concentrations of 5, 10, 25, 50, 100, 250, 500 and 1000 ng ml$^{-1}$. Quality control samples for assay method validation were prepared by diluting CAM 4515 or CAM 4750 (0.5 mg ml$^{-1}$ in acetonitrile) with rat plasma to three different concentrations (14.9, 124.5 and 597.6 ng ml$^{-1}$ for CAM 4515; 24.4, 121.9 and 812.8 ng ml$^{-1}$ for CAM 4750). Quality control samples were stored frozen at $-20°C$ and used for up to 2 months. Internal standards (CAM 2688 and CAM 4451) were prepared by diluting the respective stock solutions to 70 ng ml$^{-1}$ in 50% acetonitrile–50% water for CAM 2688 and 2000 ng ml$^{-1}$ in 25% acetonitrile–75% water for CAM 4451.

## 2.3. Sample processing

### 2.3.1. CAM 4515
A simple one-step precipitation with acetonitrile was used for the extraction of CAM 4515. In 12 × 75 mm polypropylene tubes, 100 μl of plasma standards, quality control samples or un-

known samples were mixed with 50 μl of the CAM 2688 internal standard working solution. A 200 μl volume of acetonitrile was added to precipitate the plasma proteins and the samples were vortex mixed and centrifuged for 10 min. The supernatants were transferred to 12 × 75 mm glass tubes for evaporation to dryness under a steady stream of nitrogen in a Turbovap evaporator set at 40°C. The residues were reconstituted in 200 μl of 50% acetonitrile–50% water and 150 μl were injected with the autosampler using 200 μl glass inserts.

### 2.3.2. CAM 4750
Whereas a one-step protein precipitation was used for extraction of CAM 4515, a liquid–liquid extraction procedure was used for CAM 4750 to ensure optimal chromatography. In 13 × 100 mm screw-capped borosilicate tubes, 200 μl of plasma standards, quality control samples or unknown samples were mixed with 50 μl of the CAM 4451 internal standard working solution, 100 μl of 1 M KOH, and 6 ml of methylene chloride. The tubes were capped with Teflon-lined caps and rocked for 20 min on a Labquake shaker, then centrifuged for 15 min. The top aqueous layer and the protein interface layer were aspirated to waste and the lower methylene chloride layer was transferred into 12 × 75 mm borosilicate tubes and evaporated to dryness under a steady stream of nitrogen in a Turbovap evaporator set at 40°C. The residues were reconstituted in 200 μl 40% acetonitrile–60% water and 150 μl were injected with the autosampler using 200 μl glass inserts.

## 2.4. Chromatographic separation, detection and data acquisition

CAM 4515 and CAM 4750 along with their respective internal standards were analyzed using a 150 × 4.6 mm i.d., 5 μm Ultrasphere ODS column from Beckman Instruments (Fullerton, CA) protected by a Brownlee 15 × 3.2 mm i.d., 7 μm RP-18 Newguard guard column from Applied Biosystems (Foster City, CA). For the CAM 4515 separation, the mobile phase consisted of a mixture of 47% 0.02 M citric acid, pH adjusted to 3.5 with sodium hydroxide, and 53% acetonitrile

(47:53, v/v) and was run at a flow rate of 2.0 ml min$^{-1}$. CAM 4750 required a different mobile phase consisting of a mixture of 59% 0.02 M potassium phosphate dibasic, pH adjusted to 7.0 with phosphoric acid, and 41% acetonitrile (59:41, v/v) and was also run at a flow rate of 2.0 ml min$^{-1}$. All analytes were detected by fluorescence monitoring with excitation at 278 nm and emission at 342 nm, which were found to be the optimal excitation and emission wavelengths for both compounds. Chromatographic peak responses were integrated and peak height ratios (drug/internal standard) calculated using weighted (1/concentration) linear regression. Drug concentrations in unknown samples and quality control samples were calculated using a calibration curve.

## 2.5. Stability tests

The stability of CAM 4515 and 4750 and their respective internal standards was tested by incubation in rat plasma at room temperature and 37°C for different durations up to 8 h. The stability of these compounds reconstituted in the acetonitrile–water injection mixtures at the end of sample preparation was also tested for different durations up to 16 h, a time span equivalent to an overnight analysis of 60 samples. The long-term stability of the drug compounds in rat plasma stored at −20°C was also tested for a duration of up to 2 months. The change in the absolute peak height of the same sample, compared with the peak height value at time zero, in all experiments was used as an index of stability.

## 2.6. Bioavailability study protocol

CAM 4515 and CAM 4750 were each given to fasted male Wistar rats as single 20 mg kg$^{-1}$ p.o. doses by gavage or 5 mg kg$^{-1}$ i.v. doses by jugular vein (four rats per route of administration). All doses (i.v. and p.o.) were administered as a solution of PEG-400–ethanol–water (50:25:25). Blood samples were drawn from jugular vein cannulae into syringes containing heparin before dosing and at 5, 15 and 30 min and 1, 2, 4, 6, 8 and 24 h (and at 12 h in the case of CAM 4750) after dosing. Plasma was harvested by cen-
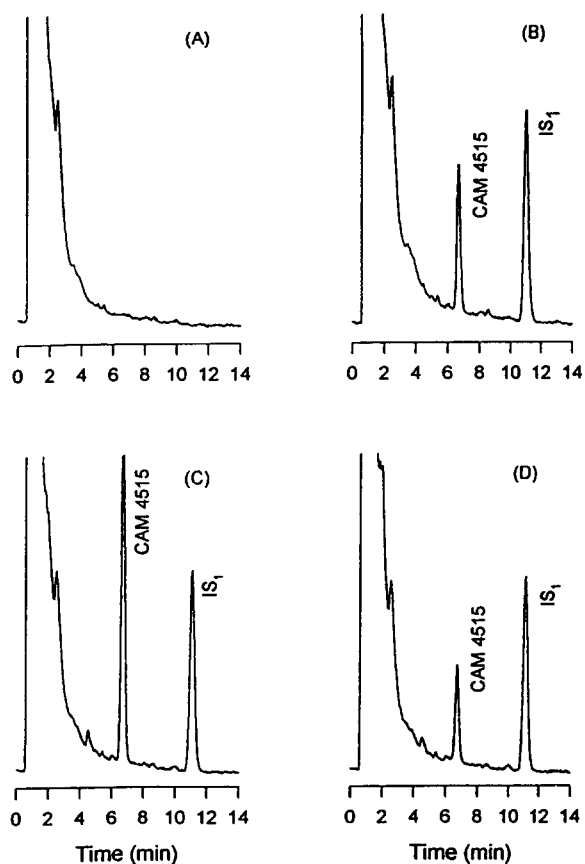


Fig. 2. Representative chromatograms of CAM 4515 and the internal standard (IS$_1$) from rat plasma: (A) blank plasma; (B) plasma spiked with CAM 4515 (100 ng ml$^{-1}$) and IS$_1$; (C) plasma sample 30 min after intravenous dosing (5 mg kg$^{-1}$); and (D) plasma sample 30 min after oral dosing (20 mg kg$^{-1}$).

trifugation and stored at −20°C until analysis. After analysis, pharmacokinetic parameters were calculated as follows: $t_{1/2} = 0.693/\lambda$, where $t_{1/2}$ is the terminal elimination half-life of the drug and $\lambda$ is the rate constant determined by nonlinear regression of the terminal phase of the plasma concentration–time profiles; the area under the plasma concentration–time curves (AUC$_{0-\infty}$) of the drug was calculated by the trapezoidal rule with extrapolation to infinity (or, in the case of CAM 4750, AUC was calculated 0–12 h); and %$F$ (oral bioavailability) is the ratio of oral AUC to intravenous AUC corrected for the doses [%$F$ = (AUC$_{p.o.}$/AUC$_{i.v.}$) × (dose$_{i.v.}$/dose$_{p.o.}$)].

## 3. Results and discussion

### 3.1. Chromatographic results

Good chromatograms were generated when CAM 4515 was extracted from plasma using a one-step precipitation with acetonitrile and CAM 4750 was extracted using a one-step liquid–liquid partitioning with methylene chloride. Figs. 2 (CAM 4515) and 3 (CAM 4750) are chromatograms for extracts from blank plasma, a plasma-based standard, and plasma samples from i.v. and p.o. studies. No interference from plasma constituents was seen for either assay system, and the lifetime of the analytical column was excellent.
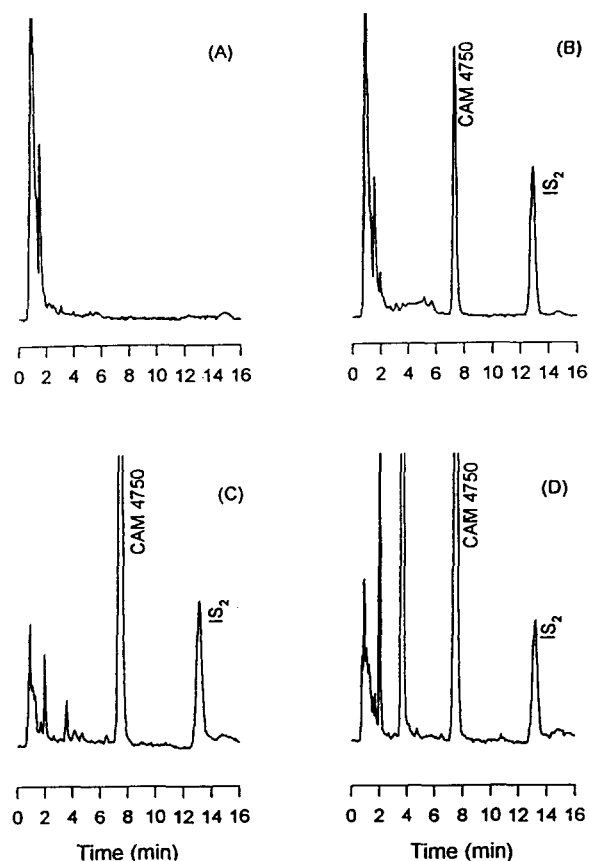


Fig. 3. Representative chromatograms of CAM 4750 and the internal standard (IS₂) from rat plasma: (A) blank plasma; (B) plasma spiked with CAM 4750 (250 ng ml⁻¹) and IS₂; (C) plasma sample 2 h after intravenous dosing (5 mg kg⁻¹); and (D) plasma sample 2 h after oral dosing (20 mg kg⁻¹).

With either mobile phase, the retention time of CAM 4750 was 3.5-fold shorter than that of CAM 4515. This is consistent with their difference in solubility, for example, in alcohol: $>250$ mg ml⁻¹ for CAM 4750 and $<1$ mg min⁻¹ for CAM 4515. It is the pyridine ring of CAM 4750 that renders the compound less lipophilic, in contrast with the phenyl ring at the same position of CAM 4515 (Fig. 1). Under the mobile phase conditions used for CAM 4515, CAM 4750 would elute in the solvent front.

### 3.2. Stability of drug compounds

Compared with the peak height at time zero ($1022 \pm 26$), CAM 4515 was found to be stable in rat plasma at room temperature for up to 4 h (peak height: $982 \pm 31$). The internal standard in plasma at room temperature was also stable for up to 4 h. After 8 h of incubation at 37°C and without the presence of sodium fluoride (NaF), only 33% of CAM 4515 remained in the plasma, whereas with NaF present (3.75 mg ml⁻¹), 89% of CAM 4515 remained in the plasma. Note that sample preparation (60 samples for each analytical run overnight) was normally completed within 2 h at room temperature. These results indicate that degradation in blood circulation (37°C), a process of hydrolysis that could be retarded by NaF, would contribute substantially to the in vivo clearance. However, no decomposition products(s) were detectable with the existing fluorescence set-up. Both CAM 4515 and its internal standard were found to be stable for at least 16 h in the reconstituting solution (50% acetonitrile–50% water) at room temperature. On the other hand, CAM 4750 was found to be more stable than CAM 4515 in plasma. CAM 4750 was stable for up to 8 h at both room temperature and 37°C. Both CAM 4750 and its internal standard were stable in the reconstitution solution (40% acetonitrile–60% water) at room temperature. Note that both compounds were stable in plasma for at least 2 months at $-20$°C.

### 3.3. Linearity and sensitivity

The peak height ratios were linearly related to concentrations over the range 5–1000 ng ml⁻¹ for

Table 1
Intra- and inter-assay precision (RSD) and accuracy (RE) of quality control samples of CAM 4515 and CAM 4750 in rat plasma

| Predicted | Intra-assay ($n = 3$) | | | Inter-assay[a] | | |
|---|---|---|---|---|---|---|
| | Observed | RSD (%) | RE (%) | Observed | RSD (%) | RE (%) |
| Cam 4515 | | | | | | |
| 14.9 | 13.8 | 3.6 | −7.4 | 14.6 | 6.2 | −2.0 |
| 124.5 | 119.7 | 3.2 | −3.9 | 119 | 3.7 | −4.4 |
| 597.6 | 612.0 | 4.1 | 2.4 | 596 | 3.8 | −0.3 |
| CAM 4750 | | | | | | |
| 24.4 | 23.4 | 6.8 | −4.1 | 23.4 | 7.7 | −4.1 |
| 121.9 | 130.7 | 2.7 | 7.2 | 125.7 | 3.8 | 3.1 |
| 812.8 | 812.8 | 2.0 | 0.0 | 737.7 | 8.3 | −9.2 |

[a] For CAM 4515, $n = 9$ in five assays; for CAM 4750, $n = 11$ in five assays.

CAM 4515 ($r = 0.9993$ for $y = 0.00734x − 0.00388$, $n = 5$) and CAM 4750 ($r = 0.9996$ for $y = 0.00817x + 0.00761$, $n = 5$). The best-fit line was determined by least-squares linear regression of the calibration data using a weighting function of 1/concentration. The lowest concentration that could be precisely [RSD < 9.4% for CAM 4515 and <6.4% for CAM 4750, $n = 3$) and accurately relative error (RE) <2.0% for CAM 4515 and <12% for CAM 4750, $n = 3$] quantitated was 5 ng ml$^{-1}$ for both compounds using 100 $\mu$l plasma for CAM 4515 and 200 $\mu$l for CAM 4570. At the limit of quantitation, the chromatographic signal-to-noise ratio was >10 for both compounds.

Under the mobile phase conditions used for CAM 4515, the fluorescence intensity of CAM 4750 was four times lower than that of CAM 4515 for the same excitation and emission spectra. The sensitivity of CAM 4750 was improved with several modifications: a methylene chloride extraction method was used, which reduced the size of the void peak (Fig. 3); a 200 $\mu$l plasma sample was used in the methylene chloride extraction procedure, compared with 100 $\mu$l in the acetonitrile precipitation procedure; and the fluorescence signal of CAM 4750 was increased by using a mobile phase buffer pH of 7.0. A greater than two-fold increase in fluorescence signal was observed when using a mobile phase buffer pH of 7.0, than when using a mobile phase

buffer pH of 3.5. as was used in the method for CAM 4515. Since CAM 4750 has a basic pyridine group, this fluorescence signal increase may correspond to a change in ionization of CAM 4750.

3.4. Precision and accuracy

Validation of the present two assay methods was evidenced by the excellent intra- and inter-assay precision and accuracy obtained for the quality control samples at three different concentrations (Table 1). These samples were stored at −20°C for up to 2 months. Standard, quality control and unknown samples were randomized just prior to injection. Quality control and unknown samples were calculated using the linear regression equation from the calibration standards. For CAM 4515, the intra-assay precision and accuracy, which were observed on one occasion with three replicates of each quality control, ranged from 3.2 to 4.1% RSD and from −7.4% to 2.4% RE, respectively. The inter-assay precision and accuracy for CAM 4515, which were observed on five occasions (up to 2 months), ranged from 3.7 to 6.2% RSD and from −0.3 to −4.4% RE, respectively. Similar excellent intra- and inter-assay precision and accuracy were also observed for CAM 4750. These data demonstrate that both assay methods are highly reproducible, and that the two compounds in plasma were stable for at least 2 months at −20°C.

## 3.5. In vivo application

### 3.5.1. CAM 4515

The present two HPLC methods were applied to oral bioavailability studies in rats. Owing to the high lipophilicity and low solubility (<1 mg min$^{-1}$ in alcohol) of CAM 4515, the dosing solutions had to be prepared in a co-solvent mixture (ethanol–PEG 400–water, 25:50:25) for both i.v. and p.o. routes of administration. Following i.v. dosing, CAM 4515 plasma concentrations declined rapidly in a multi-exponential manner (Fig. 4, top) with a mean ±SD terminal elimination half-life of $4.2 \pm 1.1$ h. Following p.o. administration, the mean ±SD terminal elimination half-life was $1.4 \pm 0.2$ h. The mean ±SD absolute p.o. bioavailability was only $2.0 \pm 0.7\%$.

### 3.5.2. CAM 4750

For comparison, the CAM 4750 dosing solutions for both routes of administration were prepared in the same co-solvent mixture. Following i.v. dosing, the CAM 4750 plasma concentrations declined multi-exponentially (Fig. 4, bottom) with a mean ±SD terminal elimination $t_{1/2}$ of 2.6 ($\pm$ 1.5) h. After p.o. dosing, CAM 4750 exhibited a relatively fast but prolonged absorption pattern in plasma. The oral $t_{1/2}$ could not be calculated because the terminal elimination phase data were not available. CAM 4750 concentrations were below the limit of quantitation at 24 h after both i.v. and p.o. doses. The mean ±SD absolute p.o. bioavailability, based on a ratio of dose normalized mean p.o. and i.v. $AUC_{0-12}$ values, was $80 \pm 23\%$, which is 40-fold higher than that for CAM 4515. This suggests that incorporation of a pyridine group into the structure may greatly improve bioavailability. In addition, the methyl group on the carbon adjacent to the amide bond may hinder metabolism at this position and thereby account for part of the improved bioavailability of CAM 4750.
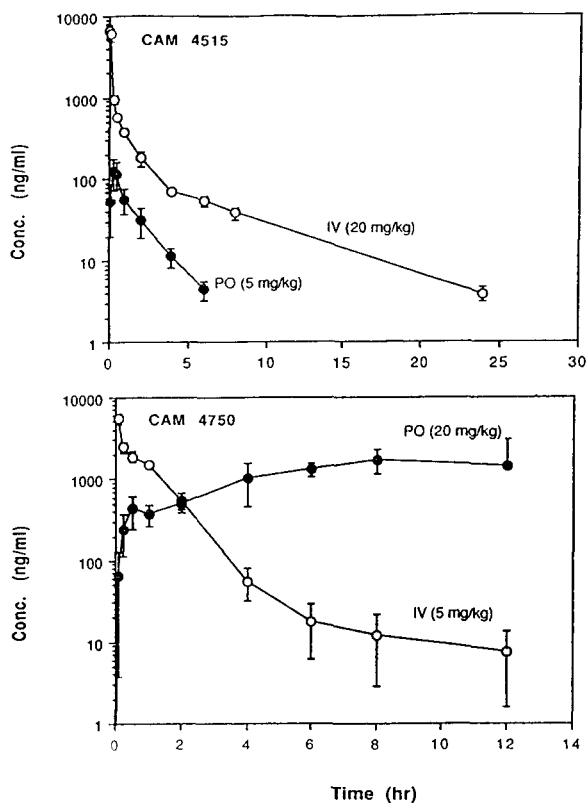
Fig. 4. Plasma profiles of CAM 4515 and CAM 4750 in rats following a 5 mg kg$^{-1}$ i.v. or 20 mg kg$^{-1}$ p.o. dose of each compound. Mean ±SD, $n = 4$.

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