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Development and application of sensitive HPLC assays for NK_3 antagonists in rat plasma[1]

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Abstract

CAM 5500 and CAM 5187 are new nonpeptide tachykinin NK_3 receptor antagonists with different lipophilicity and solubility. We have developed and validated two separate, simple HPLC methods for quantitation of these two compounds in plasma to support oral pharmacokinetic/bioavailability studies in rats. The two compounds in plasma were extracted on cyano SPE cartridges with different washing schemes to optimize extraction efficiency and chromatographic specificity. The analytes and internal standard in the resulting extracts were chromatographed on a C18 HPLC column, using mobile phases containing different phosphate buffer strengths and acetonitrile concentrations. Both compounds were detected using UV. Peak area ratios were proportional over the concentration range of 50–3000 ng ml^{-1} for CAM 5500, and 100–1500 ng ml^{-1} for CAM 5187. Stability profiles of both drugs and internal standard in rat plasma at 37°C and in injection solvent at ambient temperature were good. Assay precision, based on quality controls, was $< 5.6\%$ and 13.4% (%RSD) for CAM 5500 and CAM 5187, respectively. Similarly, assay accuracy for both compounds was within $\pm 7.1\%$ and $\pm 6.0\%$ (%RE), respectively. The HPLC methods were successfully applied to assay samples from two oral bioavailability studies. Oral bioavailability studies were conducted for each compound in rats receiving a PO dose of 20 mg kg^{-1} or an IV dose of 5 mg kg^{-1}. Despite their difference in lipophilicity and solubility, the absolute oral bioavavilability of CAM 5500 ($5.3 \pm 4.8\%$) is similar to that of CAM 5187 ($8.8\% \pm 3.2\%$). Published by Elsevier Science B.V. All rights reserved.

Keywords: HPLC assay; Tachykinin NK_3 antagonists; CAM 5500; CAM 5187; Pharmacokinetics; Bioavailability; Rats

1. Introduction

CAM 5500 and CAM 5187 (Fig. 1) are new nonpeptide tachykinin NK_3 receptor antagonists with different lipophilicity and solubility. The tachykinins (also referred to as neurokinins), substance P (SP), neurokinin A (NK_A), and neurokinin B (NK_B), are a family of neuropeptides

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(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 [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 NK_1 and NK_2 receptors [6–9], with potential therapeutic use in the areas of migraine, emesis, schizophrenia, rheumatoid arthritis, asthma and parkinsonism [6,10,11]. Like other NK_3 receptor antagonists [12,13], CAM 5500 and CAM 5187 exhibit potent selective receptor binding and were rationally designed based on the chemical structure of the target peptide, neurokinin B, of the NK_3 receptor [1,2].

Discovery of tachykinin antagonists has been previously reviewed [6,10]. The goal of developing 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. CAM 5500 and CAM 5178 are different in their lipophilicity: the former is a water-soluble maleate salt with a log P of 2.9; and the latter is water insoluble with a log P of 3.5. 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 the present investigation we describe two separate validated HPLC methods for quantitation of CAM 5500 and CAM 5178 in rat plasma. Animal studies have shown a similar oral bioavailability of less than 10% for these two prelead compounds, despite that their lipophilicity and solubility are different.

2. Materials and methods

2.1. Chemicals, reagents, instrumentation

CAM 5500 maleate, [2-(2,3-difluoro-phenyl)-1-methyl-1-(2-morpholin-4-yl-ethylcarbamoyl)-ethyl]-carbamic acid 2-methyl-1-phenyl-propyl ester, (E)-2-butenedioate (salt) [*S*-(*R**,*S**)]; (E) (mol. wt., 503.8 g mol^{-1} free base, 619.7 g mol^{-1} salt) and CAM 5187 [2-(2,3-difluoro-phenyl)-1-methyl-1-(7-ureido-heptylcarbamoyl)-ethyl]-carbamic acid 2-methyl-1-phenyl-propyl ester [*S*-(*R**,*S**)] (mol. wt. 546.8 g mol^{-1} free base) and their analog as internal standard (IS), CAM 4659, D-phenylalaninamide, *N*-[(1,1-dimethylethoxy)-carbonyl-L-phenylalanyl-*N*-[7-[(aminocarbonyl) amino]heptyl]-α-methyl, were obtained from the Parke–Davis Neuroscience Research Center (Cambridge, UK). Acetonitrile, water and methanol are HPLC grade and obtained from Mallinckrodt (Paris, KY). Analytical reagent grade acetone, isopropyl alcohol, TEA and 85% phosphoric acid, were obtained from Mallinckrodt. Reagent grade dibasic sodium phosphate was obtained from EM Science (Cherry Hill, NJ).

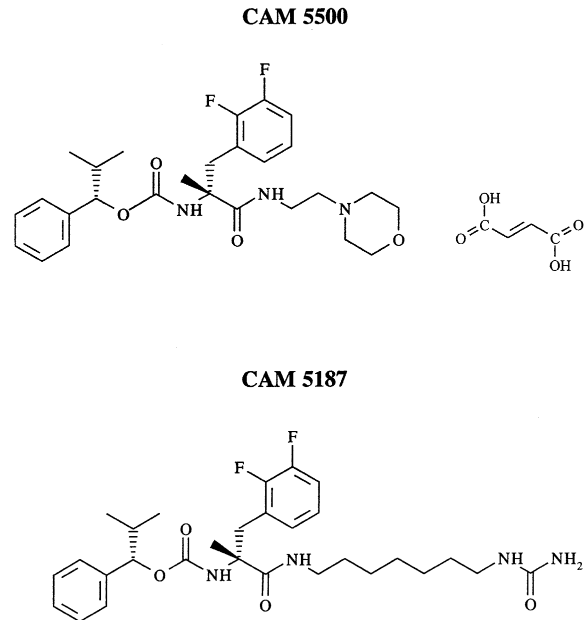

Fig. 1. Chemical structures of CAM 5500 and CAM 5187.

CN-E Encapped Solid Phase Extraction (SPE) cartridges (100 mg packing, 1 ml cartridge volume) were from Varian Analytichem (Harbor City, CA). Absolute ethanol was obtained from Aaper Alcohol and Chemical (Shelbyville, KY) and PEG 400 was from Union Carbide (Danbury, CT). Heparinized rat plasma was obtained from Pel-Freez Biologicals (Rogers, AR). The HPLC system was composed of a Perkin-Elmer series 410 pump, a Perkin-Elmer ISS 200 autosampler (Norwalk, CT), and a Milton Roy Spectromonitor 3100 variable wavelength UV detector (Riviera Beach, FL). Data were collected with a Spectra Physics SP 4400 integrator interfaced to a ChromNet/2 data acquisition system (San Jose, CA). Sample evaporation was performed with a Turbovap LV evaporator from Zymark Corp. (Hopkinton, MA).

2.2. Standard and quality control preparation

Fresh CAM 5500 or CAM 5187 stock solutions were prepared for each validation run. Stock solution (1 mg ml^{-1}) in acetonitrile was prepared and diluted with water:acetonitrile (90:10) to prepare 5 and 50 μg ml^{-1} stocks of CAM 5500 or 20 and 100 μg ml^{-1} stocks of CAM 5187. These stock solutions were further diluted with water:acetonitrile (90:10) to prepare working standards; 50, 100, 250, 500, 1000, 1500 and 3000 ng ml^{-1} for CAM 5500 or 100, 250, 500, 750, 1000 and 1500 ng ml^{-1} for CAM 5187. A 100-μl aliquot of each working standard was added to 100 μl of blank rat plasma to prepare calibration standards for validation runs. Rat plasma quality control samples containing 50, 200, 500 and 1500 ng ml^{-1} CAM 5500 were prepared by diluting 40 and 160-μl aliquots of a 5 μg ml^{-1} stock and 40 and 120-μl aliquots of a 50 μg ml^{-1} stock into 4 ml of control rat plasma. Likewise, rat plasma quality controls containing 100, 250, 500 and 1000 ng ml^{-1} CAM 5187 were prepared by diluting 10 and 20-μl aliquots of a 20 μg ml^{-1} stock and 20 and 40-μl aliquots of a 100 μg ml^{-1} stock into 4 ml of control rat plasma. Quality control samples were subdivided into 500-μl aliquots, stored frozen, and used for up to 1 month. Internal standard, CAM 4659, was prepared by diluting a 100 μg ml^{-1} stock in acetonitrile to 500 ng ml^{-1} in water:acetonitrile (95:5).

2.3. Assay procedure

2.3.1. CAM 5500

In 12 × 75 mm disposable glass tubes, 100 μl of plasma standards, quality control samples or unknown samples were mixed with 100 μl of CAM 4659 (IS) working standard and 750 μl of water. CN-E Encapped solid phase extraction (SPE) cartridges were conditioned, under vacuum, with 1 ml of methanol and 2 × 1 ml water taking care not to dry cartridges. Samples were applied to cartridges and drawn through under low vacuum (10–15 in Hg). Cartridges were then washed; 2 × 1 ml water:isopropanol (75:25), 2 × 1 ml water:acetonitrile (75:25) and 2 × 1 ml water:acetone (75:25), finally drying the cartridges for 10 min under full vacuum (25–30 in Hg). Cartridges were eluted with 500 μl methanol and evaporated to dryness at 50°C. The residues were reconstituted in 200 μl mobile phase which consisted of 5 mM sodium phosphate with 0.02% TEA buffer (pH 7.5) and acetontirile (56:44) and 150 μl injected into the HPLC.

2.3.2. CAM 5187

Work-up and extraction of CAM 5187 samples were similarly performed by SPE extraction with some minor modifications in the cartridge wash step. In 12 × 75 mm disposable glass tubes, 100 μl of plasma standards, quality control samples or unknown samples were mixed with 100 μl of CAM 4659 (IS) working standard and 750 μl of water. CN-E Encapped SPE cartridges were conditioned as for CAM 5500. Samples were applied to cartridges and drawn through under low vacuum. Cartridge washes consisted of 2 × 1 ml water, 2 × 1 ml water:acetone (80:20) and 2 × 1 ml water:acetonitrile (80:20), finally drying cartridges for 10 min under full vacuum. Cartridges were eluted with 500 μl methanol and evaporated to dryness at 50°C. The residues were reconstituted in 200 μl mobile phase (0.02 M sodium phosphate with 0.08% TEA buffer pH 7.5:acetonitrile [53:47]), and 175 μl injected into the HPLC.

2.4. Chromatographic conditions and data reduction

2.4.1. CAM 5500

CAM 5500 and IS were analyzed using a 4.6 × 150 mm, 5 μm Ultrasphere ODS column from Beckman Instruments (Fullerton, CA) with a 3.2 × 15 mm, 7 μm RP-18 Newguard cartridge from Applied Biosystems (San Jose, CA). Both CAM 5500 and IS were eluted isocratically with a mobile phase consisting of 0.005 M sodium phosphate with 0.02% TEA buffer pH 7.5:acetonitrile (56:44) at a flow rate of 1.5 ml min^{-1} for 12 min. Late eluting peaks were flushed from the column with a gradient, 0.005 M sodium phosphate with 0.02% TEA buffer pH 7.5:acetontirile (50:50) at a flow rate of 1.5 ml min^{-1} for 3 min. The column was returned to initial conditions over a 3 min interval at 1.5 ml min^{-1} and allowed to equilibrate for an additional 7 min before the next injection. CAM 5500 and IS were detected by UV absorbance at 218 nm.

2.4.2. CAM 5187

CAM 5187 and IS were analyzed using a 4.6 × 150 mm, 3 μm Supelcosil LC-18-DB column from Supelco, (Bellefonte, PA) with a guard cartridge as for the CAM 5500 assay. Drug and IS were eluted isocratically with a mobile phase consisting of 0.02 M sodium phosphate with 0.08% TEA buffer pH 7.5:acetonitrile (53:47) at a flow rate of 1.5 ml min^{-1} for 10 min. Similar to the CAM 5500 assay, late eluting peaks were flushed from the column with a gradient, 0.02 M sodium phosphate with 0.08% TEA buffer pH 7.5:acetonitrile (40:60) at a flow rate of 1.5 ml min^{-1} for 3 min. The column was returned to initial conditions over a 3 min interval at 1.5 ml min^{-1} and allowed to equilibrate for an additional 5 min before the next injection. CAM 5187 and IS were detected by UV absorbance at 210 nm.

Chromatographic responses of calibration standards were integrated and peak area ratios, CAM 5500/IS or CAM 5187/IS, calculated using weighted ($1/\text{concentration}^2$) linear regression. CAM 5500 and CAM 5187 concentrations in quality controls and unknown samples were calculated using the regression parameters of their respective standard curves.

2.5. Stability tests

Stabilities of CAM 5500, CAM 5187, and IS were tested by incubation in rat plasma at 37°C for 24 h. Stability in reconstitution solvent was tested at room temperature for up to 35 h, a time interval encompassing the duration of a 60 sample batch analysis run. In both tests a drug concentration of 500 ng ml^{-1} was used. Peak area response of CAM 5500, CAM 5187, and IS, expressed as percent relative error, %RE, from the initial chromatographic response, served as an index of stability.

2.6. Bioavailability study protocol

CAM 5500 and CAM 5187 were each given to fasted male Wistar rats as single 20 mg kg^{-1} PO doses by oral gavage or 5 mg kg^{-1} IV doses by jugular vein (four rats per route of administration). All doses (IV and PO) were administered as a solution of PEG-400:ethanol:water (40:15:35). Blood samples were drawn from jugular vein cannulae into syringes containing heparin before dosing and at 5, 15, 30 min, 1, 2, 4, 6, 8, 12 and 24 h after dosing. Plasma was harvested by centrifugation and stored at −20°C until analysis. After analysis, pharmacokinetic parameters were calculated as follows: $t^{1/2} = 0.693/k$ where $t^{1/2}$ is the terminal elimination half life of the drug and k 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 ($AUC_{0\text{-}4}$) of the drug compounds was calculated by the trapezoidal rule with extrapolation to 4 h; and %F (oral bioavailability) is the ratio of oral AUC to intravenous AUC corrected for the doses ($\%F = [AUC_{PO}/AUC_{IV}] \times [\text{dose}_{IV}/\text{dose}_{PO}]$).

3. Results and discussion

3.1. Chromatographic results

Good chromatograms were generated when CAM 5500 and CAM 5178 were extracted from plasma using a 1-ml cyano column. Fig. 2 (CAM

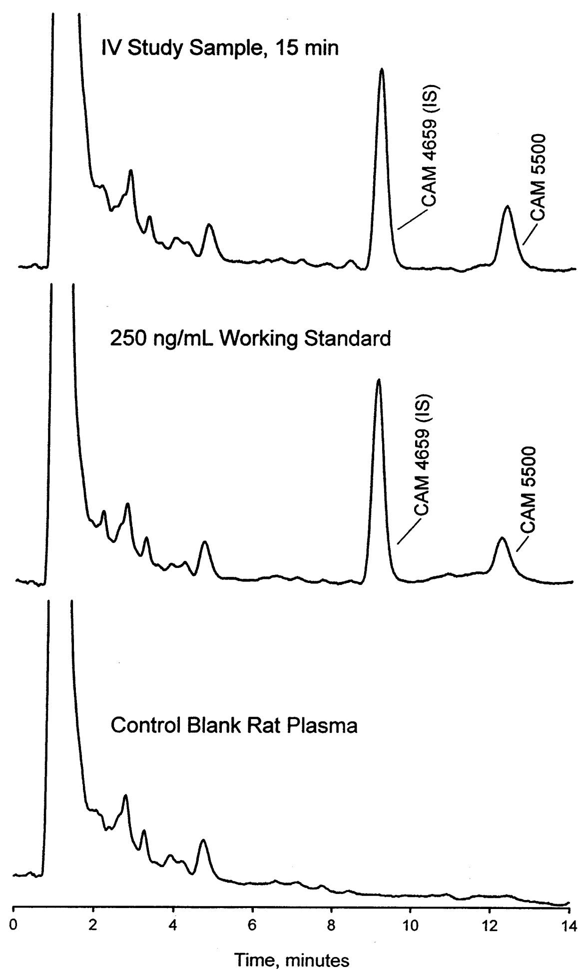

Fig. 2. Representative chromatograms of plasma sample collected 15 min following an IV dose of CAM 5500, plasma standard and control rat plasma.

5500) and Fig. 3 (CAM 5178) are chromatograms for extracts from blank plasma, a plasma-based standard, and an in vivo plasma sample. No interference from plasma constituents was seen for either assay system. Due to different physicochemical properties, different washing schemes were used for the two compounds to optimize extraction efficiency (> 85% for both compounds) and chromatographic specificity.

3.2. Stability of drug compounds

Compared with the mean value of duplicate peak areas at time zero (33 362) from plasma incubated at 37°C, CAM 5500 has a peak area of 29 530 (11.5%RE) for at least 24 h which is considered stable. Similar results were also observed for CAM 5187 and the internal standard CAM 4659 (< 10%RE). These results indicate that degradation of drug compounds in blood circulation (37°C) would not contribute substantially to the in vivo clearance. Both CAM 5500 and CAM 5187 including the internal standard were also stable for at least 24 h in mobile phases.

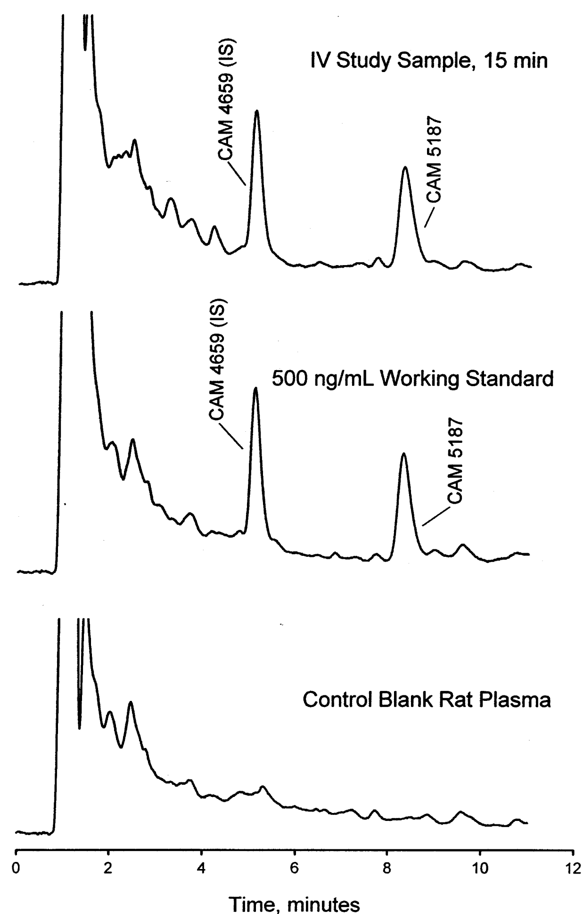

Fig. 3. Representative chromatograms of plasma sample collected 15 min following an IV dose of CAM 5187, plasma standard and control rat plasma.

Table 1
Predicted and observed plasma concentrations (ng ml^{-1}) to obtain intra- and inter-assay precision (%RSD) and accuracy (%RE) of quality control samples of CAM 5500 and CAM 5178 in rat plasma

	Intra-assay ($n=3$)			Inter-assay ($n=6$)		
Predicted	Observed	%RSD	%RE	Observed	%RSD	%RE
CAM 5500						
200	180	4.9	−10.0	186	5.6	−7.1
500	482	4.2	−3.7	481	3.9	−3.7
1500	1383	3.7	−7.8	1446	5.6	−3.6
CAM 5178						
250	220	7.0	−12.1	241	13.4	−3.8
500	467	8.3	−6.7	470	8.8	−6.0
1000	1027	3.7	2.7	1016	6.7	1.6

3.3. Linearity and sensitivity

The peak area ratios were linearly related to concentrations over the range of 50–3000 ng ml− 1 for CAM 5500 and 100–1500 ng ml^{-1} for CAM 5187. The best-fit line was determined by least squares linear regression of the calibration data using 1/$concentration^2$ weighting. The lowest concentration that could be precisely (%RSD < 9.4% for CAM 5500 and < 6.4% for CAM 5187, $n=3$) and accurately (%RE < 2.0% for CAM 5500 and < 12% for CAM 5187, $n=3$) quantitated was 50 ng ml^{-1} for CAM 5500 and 100 ng ml^{-1} for CAM 5187 using 100 μl plasma. At the limit of quantitation, the chromatographic signal-to-noise ratio was > 10 for both compounds.

3.4. Precision and accuracy

Validation for 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). Standard, quality control, and unknown samples were randomized just prior to injection. For CAM 5500, the intra-assay precision and accuracy, which were observed on one occasion with three replicates of each quality control, ranged from 3.7 to 4.9 %RSD and from − 3.7 to − 10 %RE, respectively. The inter-assay precision and accuracy obtained from two occasions for CAM 5500, ranged from 3.9 to 5.6 %RSD and from − 3.6 to 7.1 %RE, respectively. Similar excellent intra- and inter-assay precision and accuracy were also observed for CAM 5187. These data demonstrate that both assay methods are highly reproducible.

3.5. In vivo application

3.5.1. CAM 5500

The present two HPLC methods have been applied to oral bioavailability studies in rats. Following IV dosing, CAM 5500 plasma concentrations declined rapidly in a multiexponential manner (Fig. 4) with a mean ± SD terminal elimination half-life of 0.44 ± 0.06 h. Following PO administration, the mean ± SD terminal elimination half-life was 1.0 ($n=2$) h. In both routes of administration, plasma CAM 5500 concentrations were not quantifiable beyond 4 h postdose. Mean ± SD absolute PO bioavailability based on a ratio of dose normalized mean PO and IV AUC_{0-4} values was only 5.3 ± 4.8%.

3.5.2. CAM 5187

For comparison, the CAM 5187 dosing solutions for both routes of administration were prepared in the same co-solvent mixture. Following IV dosing, CAM 5178 plasma concentrations declined multiexponentially (Fig. 4) with a mean ± SD terminal elimination $t^{1/2}$ of 0.28 ± 0.04 h. After PO dosing, CAM 5178 exhibited a relatively fast but prolonged absorption pattern in plasma.

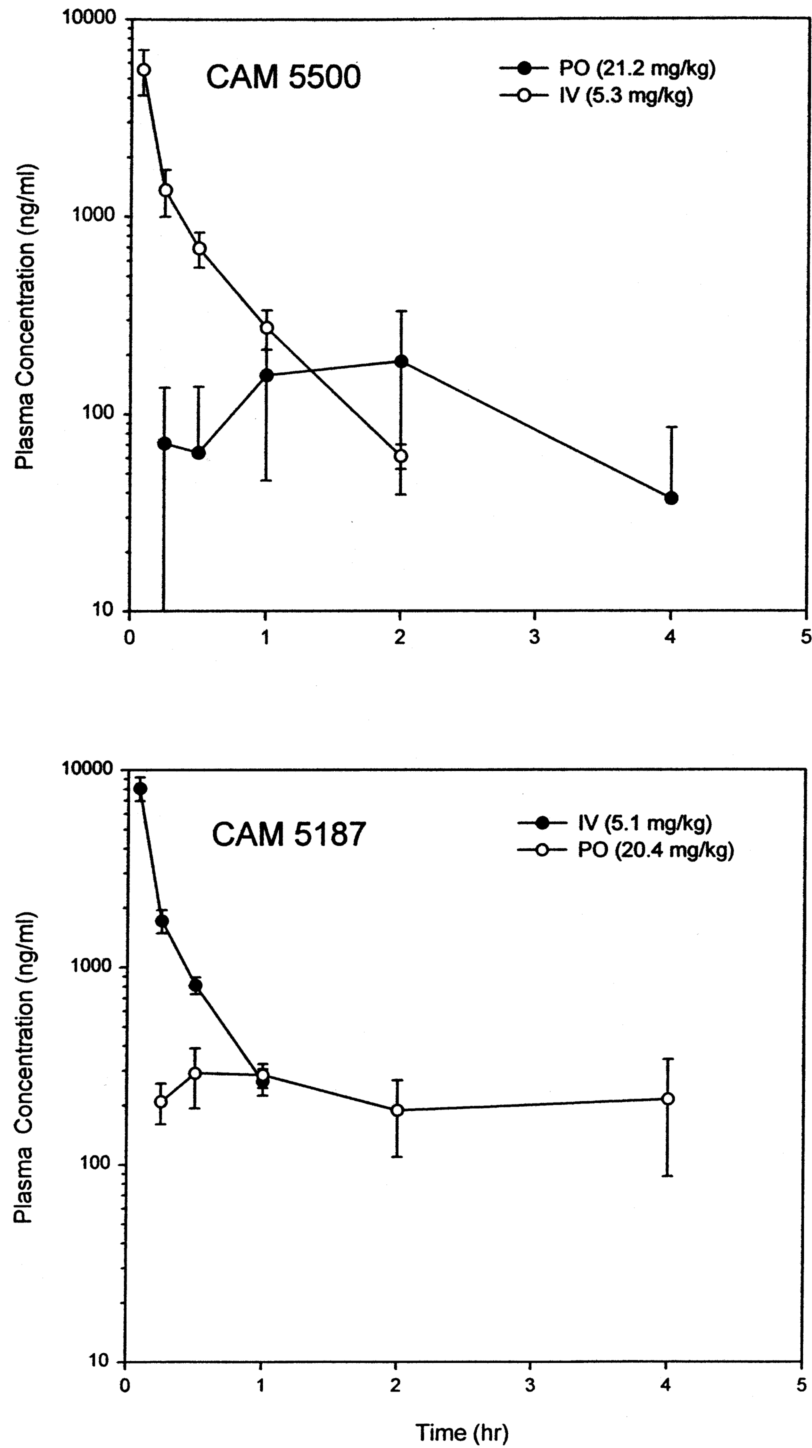

Fig. 4. Plasma profiles of CAM 5500 and CAM 5187 in rats after an IV and a PO dose.

The oral $t^{1/2}$ could not be calculated because the terminal elimination phase data were not available. Mean ± SD absolute PO bioavailability, based on a ratio of dose normalized mean PO and IV AUC_{0-4} values was 8.8 ± 3.2%.

CAM 5500 and CAM 5178 are different in their lipophilicity: the former is a water-soluble maleate salt with a log P of 2.9; and the latter is water insoluble with a log P of 3.5. The present investigation has shown a similar oral bioavailability of less than 10% for the two prelead compounds, despite their difference in lipophilicity and solubility. This result is different from a previous report where a difference in lipophilicity had led to a wide different oral bioavailability for two NK_1 receptor antagonists [14].

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