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Development of a high-performance liquid chromatographic–tandem mass spectrometric method for the determination of pharmacokinetics of Co 102862 in mouse, rat, monkey and dog plasma

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Abstract

A method for determining concentration levels of Co 102862 in mouse, rat, monkey and dog plasma was validated in the range of 5 to 2000 ng/ml using 200 μl plasma sample volume. This validation report describes the linearity, specificity, sensitivity, reproducibility, accuracy, recovery and stability of the analytical method. The inter-day RSD ranged from 3.5 to 10.1%, intra-day RSD from 0.6 to 5.7% and intra-day accuracy (mean absolute percent difference) ranged from 2.2 to 14.9% for rat, monkey and dog plasma. A mini-validation (5–2000 ng/ml) of Co 102862 was performed in mouse plasma using the same methods. Additionally, the assay range at the low end was successfully extended to 0.5 ng/ml for monkey plasma. The method was used for the routine analysis of Co 102862 in mouse, rat, monkey and dog plasma and summary of the pharmacokinetic data are presented.

Keywords: Co 102862

1. Introduction

Co 102862 (4-[4-fluorophenoxy]benzaldehyde semicarbazone) (Fig. 1) is a novel compound, structurally unrelated to the major drugs in clinical use as analgesics or antiepileptics. In radioligand binding and electrophysiological studies, Co 102862 is a potent blocker of neuronal voltage-gated sodium channels, with negligible action at other neuroeffector sites. It interacts selectively with inactivated states as opposed to resting states of the channel. Co 102862 is being developed for the treatment of neuropathic pain based upon its antinociceptive activity in the rat Chung model for pain [1].

In an experimental rat model of peripheral neuropathic pain, Co 102862 demonstrated marked antiallodynic effects, producing dose-dependent reversals of tactile sensitivity at oral doses ≥2.5 mg/kg (plasma levels of 480 ng/ml) in a 10% Tween 80

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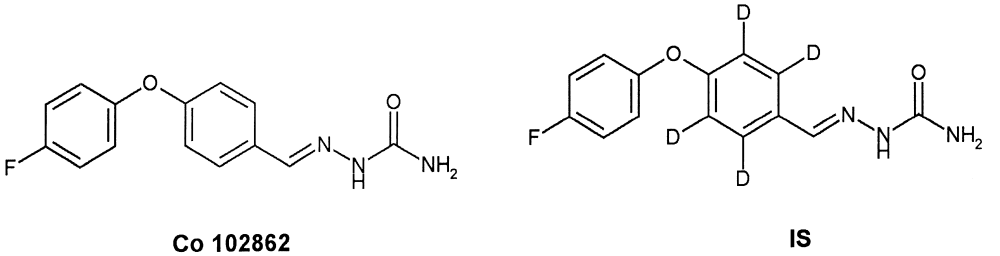

Fig. 1. Structures of Co 102862 and I.S.

formulation [2]. In the mouse formalin test, Co 102862 blocked both early and late phase nociceptive responses with oral ED_{50} values of 10.6 and 5.2 mg/kg, respectively [2]. Antiallodynic and antinociceptive effects occur at doses well below those that disrupt behavior, indicating a high therapeutic index in animals. These data suggest that Co 102862 may be of use in the treatment of neuropathic pain states as well as possessing potential utility in the treatment of mild to moderate pain of acute or persistent nature.

In experimental seizure models, Co 102862 exhibits potent oral anticonvulsant activity against the tonic hindlimb extension induced by maximal electroshock (MES) in both mice and rats, and moderately potent activity against the forelimb clonus induced by pentylenetetrazol (PTZ) in mice. Moreover, Co 102862 exhibits efficacy in rats against fully-kindled stage-5 seizures induced by corneal kindling [3]. In contrast to carbamazepine [4], and lamotrigine [5], which are proconvulsant at high doses in humans, Co 102862 does not reduce seizure threshold in mice. Thus, Co 102862 would not be expected to be proconvulsant in man and may demonstrate a broader margin of utility in therapeutic use than other clinically-employed sodium channel blockers.

In order to correlate activity with plasma levels and to understand the pharmacokinetics of Co 102862, it was necessary to develop a sensitive and specific methods for the determination of Co 102862 in animal plasma with the limit of quantification (LOQ) of at least 5 ng/ml. Due to lack of a good chromaphore and poor UV absorbance of Co 102862, the development of an assay based on high-performance liquid chromatography (HPLC) with UV absorption detection was not feasible below 25 ng/ml.

An alternative and more sensitive assay for Co 102862 in plasma was evaluated using HPLC with tandem mass spectrometry (MS–MS). The pharmacokinetics of Co 102862 were investigated in CD1 mice, Sprague–Dawley rats, Cynomolgus monkeys and Beagle dogs after intravenous (i.v.) and oral (p.o.) administration [6].

2. Experimental

2.1. Materials

Co 102862 and internal standard (I.S., Co 200222) (Fig. 1) were synthesized at CoCensys (Irvine, CA, USA). HPLC-grade solvents methanol (MeOH), methyl-*tert*.-butyl ether (TBME), methylene chloride, hexane, ethyl acetate, formic acid and K_2-EDTA Vacutainer tubes were obtained from VWR Scientific (San Diego, CA, USA) or Ville Mont-Royal (Québec, Canada). Sodium hydroxide, sodium sulfate, $CDCl_3$, DMSO-d_6 and other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA), EM Sciences (Gibbstown, NJ, USA) or J.T. Baker (Phillipsburgh, NJ, USA). Deionized water Type I, Elgastat UHQ-PS, was supplied by Elga (Northbrook, IL, USA). Nitrogen and refrigerated liquid nitrogen were obtained from Keen Compressed Gas (Wilmington, DE, USA). Blank Sprague–Dawley rat, Cyno monkey and Beagle dog plasma containing EDTA as the anticoagulant was purchased from Biological Specialties (Landsdale, PA, USA). Blank CD1 mouse plasma containing

EDTA as the anticoagulant was obtained from mice at CoCensys.

2.2. Synthesis of Co 102862

2.2.1. 4-(4-Fluorophenoxy)benzaldehyde

To a solution of 4-fluorobenzaldehyde (Aldrich 98%; 379 ml, 440 g, 3.54 mol) and 4-fluorophenol (Aldrich 99%; 396 g, 3.54 mol) in *N*,*N*-dimethylacetamide (Aldrich, anhydrous, 99.8%; 1.5 l) was added potassium carbonate (anhydrous granular, EM Sciences, 99.9%: 491 g, 3.55 mol). The mixture was mechanically stirred under nitrogen at 135°C for 7 h and then 150°C for 3 h. After cooling, the mixture was added with stirring to water (2 l). The resulting solid was collected by filtration, washed with water (3×300 ml) and dried in air to yield a crude pink solid (595 g) which was purified by vacuum distillation (1.0 mmHg, b.p. 132–133°C; 1 mmHg= 133.322 Pa) to give 4-(4-fluorophenoxy)benzaldehyde as a white solid (450 g, 2.08 mol, 59%), purity: 99.6% (HPLC), m.p.: 74–75°C. ^{1}H nuclear magnetic resonance (NMR) ($CDCl_3$): δ 9.92 (s, 1H), 7.85 (d, 2H), 7.11–7.02 (m, 6H).

2.2.2. 4-(4-Fluorophenoxy)benzaldehyde semicarbazone

To a mechanically stirred refluxing solution of 4-(4-fluorophenoxy)benzaldehyde (99.6% by HPLC; 200 g, 0.925 mol) in ethanol (anhydrous, J. T. Baker, 94.5%, containing 5% isopropanol; 1.5 l) was added a solution of semicarbazide hydrochloride (Aldrich, 99+%; 124 g, 1.11 mol) and sodium acetate (Aldrich, anhydrous, 99%; 77 g, 0.94 mol) in water (500 ml) dropwise over 1.5 h. A white solid formed after 10 min. The mixture was further refluxed for 1 h after the addition was complete and then allowed to cool to room temperature. After standing overnight the solid that had formed was collected by filtration, washed with water (4×300 ml), dried in vacuo (28 mmHg, 32°C, 2 days) to yield the title compound as a white solid (248 g, 0.907 mol, 98%), m.p. 234–235°C. ^{1}H-NMR (DMSO-d_6): δ 10.19 (s, 1H), 7.82 (s, 1H), 7.73 (d, J=8.7 Hz, 2H), 7.26 (t, J=8.8 Hz, 2H), 7.14–7.09 (m, 2H), 6.97 (d, J=8.7 Hz, 2H), 6.46 (s, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6), 158.44 (d, $J_{C,F}$=239 Hz, 1C), 157.90 (1C), 156.83 (1C), 152.06 (C=O), 138.59 (C=N), 129.99 (1C), 128.34 (2C), 121.10 (d, $J_{C,F}$=8.8 Hz, 2C), 117.85 (2C), 116.69 (d, $J_{C,F}$=23.7 Hz, 2C) ppm. ^{19}F-NMR (DMSO-d_6) Analysis calculated for $C_{14}H_{12}FN_3O_2$: C, 61.53; H, 4.43; N, 15.38. Found: C, 61.70; H, 4.45; N, 15.41. Purity was 99.6% as assayed by HPLC. Reaction using this procedure (at reflux) gives the more stable solid form, polymorph B. Reaction at room temperature was found to give less stable solid form, polymorph A. This procedure was modified from that in the literature [7].

2.3. Synthesis of I.S. (Co 200222)

2.3.1. 4-Fluorobenzaldehyde-D4 [8]

To a solution of 4-fluorobenzene-D5 (Aldrich, 97% atom; 2 ml, 2.16 g, 21.4 mmol) in methylene chloride (15 ml) was added aluminum chloride (Aldrich, 3.0 g) at 0°C and stirred. To this solution was added 1,1-dichlorodimethylether (Aldrich, 1.9 ml, 2.4 g, 21.0 mmol) dropwise with stirring and at 0°C and further stirred for another 2 h at 0°C and then at room temperature for 1 h. After cooling, the mixture was added with stirring to ice/water (100 ml) to which methylene chloride (50 ml) was added and stirred for 1 h. The organic phase was separated and aqueous phase extracted with methylene chloride (2×25 ml). The organic phases were combined, washed with water, then with saturated sodium hydrogencarbonate, water again, then brine and then dried over sodium sulfate. The product was concentrated under vacuum to yield a light yellow liquid (1.53 g) which was then purified by chromatography (hexane–ethyl acetate, 12:1) to yield a light yellow liquid of 4-fluorobenzaldehyde-D4 (0.53 g, 4.72 mmol, 23%). ^{1}H-NMR ($CDCl_3$): δ 9.98 (s, 1H).

2.3.2. 4-(4-Fluorophenoxy)benzaldehyde-D4

Using the procedures similar to that used to synthesize Co 102862; to a solution of 4-fluorobenzaldehyde-D4 (0.53 g, 4.72 mmol) and 4-fluorophenol (Aldrich 99%; 068 g, 6.1 mmol) in *N*,*N*-dimethylacetamide (Aldrich, anhydrous, 99.8%; 25 ml) was added potassium carbonate (anhydrous granular, EM Sciences, 99.9%: 0.87 g, 6.3 mmol). The mixture was mechanically stirred and refluxed under nitrogen for 3 h. After cooling, the mixture was diluted with 1:1, hexane–ethyl acetate (75 ml), washed with water, then with 2 *M* sodium hydroxide,

water again, then brine and then dried over sodium sulfate. The product was concentrated under vacuum and the residue purified by chromatography (hexane–ethyl acetate, 3:2) to yield a light yellow liquid of 4-(4-fluorophenoxy)benzaldehyde-D4 (250 mg, 1.14 mmol, 24%). ^{1}H-NMR ($CDCl_3$): δ 9.93 (s, 1H), 7.11–7.07 (m, 4H).

2.3.3. 4-(4-Fluorophenoxy)benzaldehyde semicarbazone-D4

To a mechanically stirred refluxing solution of 4-(4-fluorophenoxy)benzaldehyde-D4 (250 mg, 1.14 mmol) in ethanol (anhydrous, J.T. Baker, 94.5%, containing 5% isopropanol; 10 ml) was added a solution of semicarbazide hydrochloride (Aldrich, 99+%; 150 mg, 1.34 mmol) and sodium acetate (Aldrich, anhydrous, 99%; 114 mg, 1.39 mmol) in water (5 ml) dropwise over 1.5 h. A white solid formed after 10 min. The mixture was further refluxed for 1 h after the addition was complete and then allowed to cool to room temperature. After standing overnight the solid that had formed was collected by filtration, washed with water (50 ml), dried in vacuo (28 mmHg, 32°C, 2 days) to yield the title compound as a white solid (230 mg, 0.83 mmol, 73%), m.p. 222–223°C. ^{1}H-NMR (DMSO-d_6): δ 10.17 (s, 1H), 7.79 (s, 1H), 7.26–7.07 (m, 4H), 6.44 (s, 2H). Analysis calculated for $C_{14}H_8D_4FN_3O_2$: C, 60.64; H (incl. D), 4.36; N, 15.15; F, 6.85. Found: C, 60.57; H (incl. D), 4.48; N, 15.58; F, 6.63. Purity was 99.6% as assayed by HPLC.

2.4. Instrumentation

A Micromass Quattro II (Micromass, Beverly, MA, USA) triple quadruple mass spectrometer equipped with a pepper pot ion source and electrospray ionization interface, a HP 1100 binary pump solvent delivery system (Hewlett-Packard) along with a HP 1100 vacuum degasser and HP 1100 autoinjector was used for the HPLC–MS–MS analysis. The data were processed using MassLynx version 3.0 (Micromass) software.

2.5. Chromatographic conditions

The analysis was performed on a Columbus C_{18}, 5 μm, 100×2.0 mm analytical column (Phenomenex, Torrance, CA, USA) and a mobile phase consisting of methanol–water–formic acid (75:25:0.2) at a flow-rate of 300 μl/min. The total run time was 3 min. Under these conditions the retention time of Co 102862 and the I.S. was 1.8 min and well separated from the solvent front.

2.6. HPLC–MS–MS conditions

A Micromass Quattro II mass spectrometer was interfaced via a electrospray (ESP) probe in the positive mode with the HPLC system. The source was maintained at a temperature of 150°C with drying gas (nitrogen) at a flow-rate of 450 l/h and ionization was effected with a capillary voltage of 3.5 kV. The sample cone energy was set at 30 V, with counter-electrode at 0.5 kV, skimmer at 1.5 V, skimmer lens at 5 V, radio frequency (RF) lens at 0.2 V and nebulizing gas pressure at 15 l/h. The vacuum running pressure was maintained at ~$1.5\cdot10^{-5}$ mBar. The mass spectrometer was programmed to monitor the transitions (MRM) m/z 274→m/z 257 (for Co 102862) and m/z 278→m/z 261 (for Co 200222), with collision induced fragmentation (collision gas argon at $1.0\cdot10^{-3}$ mBar). The dwell time was 300 ms, and the collision energy was set at 16 and 15 eV for Co 102862 and Co 200222, respectively. The electron multiplier setting was 650 V.

2.7. Stock solutions

2.7.1. Primary stock solutions SS-A and SS-B for standards

The primary stock solution A (222.2 μg/ml) was prepared by carefully transferring 11.11 mg (accurately weighed on Denver Analytical Balance, Model M220-D, Denver Instrument Company, Arvada, CO, USA) of Co 102862 to a 50-ml volumetric flask and diluting to volume with methanol. The primary stock solution B (4000 ng/ml) was prepared by diluting 0.90009 ml of stock solution A to 50 ml with MeOH. The solutions were stored at ≤−20°C.

2.7.2. Working stock solutions for standards (SS1–SS8)

The working stock solutions SS1–SS8 (0.5 to 2000 ng/ml) were prepared by diluting the stock

Table 1
Preparation of working stock solutions for standards and quality control samples

From stock solution concentration (ng/ml)	Take (ml)	QS with MeOH (ml)	Working stock solution concentration (ng/ml)	Standard No.	QC No.
4000	12.5	25	2000	SS8	QCS5
4000	6.25	25	1000	SS7	–
4000	1.875	25	300	SS6	–
4000	0.625	25	100	SS5	QCS4
4000	0.1875	25	30	SS4	–
4000	0.0625	25	10	SS3	QCS3
100	1.25	25	5	SS2	QCS2
5.0	1.0	10	0.5	SS1	QCS1

solutions with methanol as shown in Table 1. The solutions were stored at ≤−20°C.

2.7.3. Internal standard stock solutions (I.S.-A and I.S.-B)

The primary stock solution I.S.-A (180 μg/ml) was prepared by carefully dissolving 9.0 mg of Co 200222 in 50 ml methanol with the aid of a volumetric flask. The working stock solution I.S.-B (500 ng/ml) was prepared by diluting 0.139 ml of stock solution I.S.-A to 50 ml with MeOH. The solutions were stored at ≤−20°C.

2.7.4. Primary stock solutions QC-A and QC-B for quality control samples

The primary stock solution QC-A (277.4 μg/ml) was prepared by carefully transferring 13.8 mg of Co 102862 to a 50-ml volumetric flask and diluting to volume with methanol. The primary stock solution QC-B (4000 ng/ml) was prepared by diluting 0.721 ml of stock solution QC-A to 50 ml with MeOH. The solutions were stored at ≤−20°C.

2.7.5. Working stock solutions for quality control samples (QCS1–QCS5)

The working stock solutions for quality control samples QCS1–QCS5 (0.5 to 2000 ng/ml) were prepared by diluting the stock solutions with methanol as shown in Table 1. The solutions were stored at ≤−20°C.

2.8. Mouse pharmacokinetics

Male naive NSA mice (Charles River, CA, USA) weighing between 20 and 25 g body weight and 4 weeks old were used for the study. The mice ($n=5$ per time point) were dosed p.o. via gavage at doses of 10, 50, 100, 500 or 1000 mg/kg of Co 102862 in a nanoparticulate suspension formulation. The animals were maintained under a standard 12 h light–dark cycle with food and water provided ad libitum throughout the study. The concentrations and dosing volumes of the formulations for each dose group are listed in Table 2. Blood samples (≤0.5 ml) were collected after p.o. dosing from each mouse by cardiac puncture (under anesthesia) at pre-dose, and 1, 2, 3, 4, 6 and 8 h post-dose in powdered K_2-EDTA Vacutainer tubes. The tubes were centrifuged at 1500 *g* for 10 min between 0 to 4°C. For each of the dose groups, plasma samples at each of the time-points (100 μl from each of the tubes) were pooled and transferred to a 2.0-ml cyrostorage tube and stored at ≤−20°C until analyzed.

2.9. Rat pharmacokinetics

Male naive Sprague Dawley rats (Charles River Canada, Québec, Canada), weighing between 225 to 283 g (approximately 8–10 weeks old) were used for the study. Two days prior to treatment the rats were catheterized in the femoral vein and allowed to recover. Co 102862 was administered in the rats i.v. (via tail vein, as a 10% HPβCD solution) at 2 mg/kg or p.o. (by gavage, as a nanoparticulate suspension) at doses of 10, 20, 50, 100 and 500 mg/kg. The animals were maintained under a standard 12 h light–dark cycle with food and water provided ad libitum throughout the study. The concentrations and dosing volumes of the formulations for each dose group are listed in Table 2. Before the onset of

Table 2
Formulations and dosing regimens for pharmacokinetic studies

Species	Formulation	Route	Dose (mg/kg)	Concentration (mg/ml)	Dosing volume (ml/kg)
Mouse	Nano-suspension	p.o	10	1	10
	Nano-suspension	p.o	50	5	10
	Nano-suspension	p.o.	100	5	20
	Nano-suspension	p.o.	500	50	10
	Nano-suspension	p.o.	1000	50	20
Rat	10% HPβCD	i.v.	2	1	2
	Nano-suspension	p.o.	10	5	2
	Nano-suspension	p.o.	20	5	4
	Nano-suspension	p.o.	50	5	10
	Nano-suspension	p.o.	100	50	2
	Nano-suspension	p.o.	500	50	10
Monkey	50% HPβCD	i.v.	0.4	1	0.4
	Nano-suspension	p.o.	2	2	1
	Nano-suspension	p.o.	50	5	10
	Nano-suspension	p.o.	100	50	2
	Nano-suspension	p.o.	500	50	10
	Nano-suspension	p.o.	1000	100	10
	Nano-suspension	p.o.	2000	200	10
Dog	10% HPβCD	i.v.	0.4	1	0.4
	10% HPβCD	p.o.	2	1	2
	50% HPβCD	p.o.	2	2	1
	MethCell-suspension	p.o.	2	2	1
	PEG–Tween	p.o.	2	2	1
	Nano-suspension	p.o.	2	2	1
	Nano-suspension	p.o.	10	5	2
	Nano-suspension	p.o.	20	5	4
	Nano-suspension	p.o.	50	50	1
	Nano-suspension	p.o.	100	50	2

sample collection from treated rats, blood was collected from four to six donor non-treated rats into tubes containing heparin as anticoagulant by cardiac puncture under anesthesia and kept in a water bath (37°C) for a maximum of 4 h.

Serial blood samples (approximately 0.5 ml) were collected after p.o. dosing using the catheter at pre-dose, and 30 min, 1, 2, 4, 6, 8, 12, 24 and 30 h post-dose and after i.v. dosing at; pre-dose, and 2, 5, 15, 30 min, 1, 2, 3, 4, 6, 8 and 10 h post-dose. Following each blood sampling (except after the last sampling time point), each animal was administered via the cannula a volume of blood from the donor rats, equal to the volume of blood withdrawn. The blood sample was collected in powdered K_2-EDTA Vacutainer tubes and centrifuged at 1500 *g* for 10 min between 0 to 4°C. The plasma was then transferred to a 2.0-ml cyrostorage tube and stored at ≤ -20°C until analyzed.

2.10. Monkey pharmacokinetics

Cynomolgus monkeys (ITR, Québec, Canada) of both sexes (10 males and six females, 4 monkeys/dose group, two males and two females where applicable) weighing between 3 to 7 kg (approximately 3–9 years old) were used for the study. The monkeys used in the study were not naive, but had not received any test article for at least 5 weeks before dosing with Co 102862. A 2-week wash-out period was observed between the i.v. (0.4 mg/kg, as a 50% HPβCD solution) and p.o. (2.0 mg/kg, as a nanoparticulate suspension) doses. Only male monkeys were dosed at 50, 100 and 500 mg/kg doses.

The animals were maintained under a standard 12 h light–dark cycle with food and water provided ad libitum throughout the study. The animals were fasted overnight and fed approximately 2 h after dosing. The concentrations and dosing volumes of the formulations for each dose group are listed in Table 2.

Serial blood samples (approximately 1 ml) were collected after p.o. dosing at the following timepoints; pre-dose, 15, 30, 45 min, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60 and 72 h post-dose (except for 2 mg/kg dose in which 36, 60 and 72 h samples were not taken and instead a sample was withdrawn at 32 h) and after i.v. dosing at; pre-dose, 2, 5, 15, 30 min, 1, 2, 4, 6, 8, 10, 12, 24, 32 and 48 h post-dose. The blood sample was collected in powdered K_2-EDTA Vacutainer tubes and centrifuged between 0 to 4°C at 1500 *g* for 10 min. The plasma was then transferred to a 2.0-ml cyrostorage tube and stored at ≤−20°C until analyzed.

2.11. Dog pharmacokinetics

Naive male Beagle dogs (Marshall Research Labs., North Rose, NY, USA or Covance Research Products, USA) weighing between 7 to 10 kg (approximately 4–6 months old) were used for the study. A 2-week wash out period was observed between doses. The animals were dosed Co 102862 in various formulations, i.v. (cephalic vein, $n=2$) at 0.4 mg/kg and p.o. (gavage, $n=2$ or 4) at 2, 10, 20, 50 and 100 mg/kg. The animals were maintained under a standard 12 h light–dark cycle with food and water provided ad libitum throughout the study. The animals were fasted overnight and fed approximately 2 h after dosing. The corresponding formulations, concentrations and dosing volumes of the formulations for each dose group are listed in Table 2.

Serial blood samples (approximately 2–3 ml) were collected after p.o. dosing at the following time points; pre-dose, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24, 30, 48, 56 and 72 h post-dose; and after i.v. dosing at; pre-dose, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 12 h post-dose. The blood sample was collected in powdered K_2-EDTA Vacutainer tubes and centrifuged at 1500 *g* for 10 min between 0 to 4°C. The plasma was then transferred to a 2.0-ml cryogenic tube and stored at −70°C until analyzed.

2.12. Sample preparation and extraction procedure

2.12.1. Preparation of quality control (QCP1–QCP5) plasma samples

The blank plasma was tested for interference prior to preparing standards or quality control samples. The plasma was stored frozen ≤−20°C until use. Transfer 5.0 ml of working stock solutions for QC samples (QCS1–QCS5) at concentrations of 0.5 (monkey only), 5, 10, 100 and 2000 ng/ml, into 5-ml volumetric flasks and evaporate to dryness under a nitrogen steam (Turbo Vap LV evaporator, Zymark). Reconstitute with 5.0 ml of blank rat, monkey and dog plasma and sonicate for 5 min and vortex (Multi-tube vortexer, Model 12-810, VWR Scientific) for 1 min. Store the QC plasma samples (QCP1–QCP5) in disposable screw top glass tubes as 200-μl aliquots at −20°C until required for analysis.

2.12.2. Preparation of plasma calibration standards (SP1–SP8)

Plasma standards SP1–SP8 (in duplicate) were prepared freshly before each run by adding 200-μl aliquots of the working stock solutions (SS1–SS8) into separate 125×16 mm disposable screw-cap glass test tubes (VWR Scientific) with PTFE-lined caps (Fisher Scientific, Pittsburgh, PA, USA) and evaporating off the solvent in a ≤40°C water bath under a stream of nitrogen. Blank plasma (200 μl) from one species and 100 μl of I.S.-B working solution was added to each of these tubes and each set consisted of standards at concentrations of 0.5, 5, 10, 30, 100, 300, 1000 and 2000 ng/ml. Different sets of standards were prepared and run on three consecutive days for the assay validation.

2.12.3. Preparation of plasma blanks

Additionally, each standard curve included three blank samples. The plasma blank was prepared by transferring 200 μl of blank plasma to a disposable screw-cap glass test tube. The I.S. blank was prepared by transferring blank plasma (200 μl) and 100 μl of I.S.-B working solution to each of these tubes. Lastly, the drug blank was prepared by spiking the tube with 200 μl aliquot of the working stock solution (SS8, 2000 ng/ml), evaporating it to dryness and then adding 200 μl of blank plasma.

2.12.4. Preparation of study samples

The study samples and a set of individually frozen aliquots of QC samples (QCP1–QCP5) were thawed at room temperature for 1 h. Study samples (200 μl) were transferred into disposable screw-cap glass test tubes. In the case where sample size was insufficient they were appropriately diluted with blank plasma. I.S.-B working solution (100 μl) was spiked of into each tube.

2.12.5. Plasma extraction procedure

To each of the plasma calibration standards, plasma blanks, study samples and QC samples was added 300 μl of distilled water and the tubes vortex-mixed for 5 min. TBME (5 ml) was added to each tube, the tubes were shaken (Eberback Reciprocal Shaker, two-speed, VWR Scientific) for 15 min at high speed and then centrifuged (Marathon bench top refrigerated centrifuge, Model 12KBR, Fisher Scientific) for 10 min at 3000 rpm. The tubes were then placed into a dry ice–methanol bath for 3 min and the bottom water layer frozen. The supernatant was transferred into properly labeled 100×13 glass culture tubes (VWR Scientific) and evaporated to dryness under a nitrogen stream at ≤40°C. The residue was reconstituted with 200 μl of mobile phase, vortex-mixed for 1 min and then transferred to autosampler vials (autosampler glass vials with 200 μl conical glass inserts and snap caps, Pesce Lab Sales, Kennett Square, PA, USA) and arranged on the autosampler tray for LC–MS–MS analysis. Injections of 10 μl were made on the HPLC for analysis.

2.13. Precision, accuracy, specificity, sensitivity, recovery and stability

2.13.1. Specificity

Specificity was determined in blank plasma from five different lot numbers of each species that was extracted and analyzed on LC–MS–MS.

2.13.2. Sensitivity

Using 200 μl of plasma sample, the limits of quantification (LOQ), and detection (LOD) and signal-to-noise ratio were determined for Co 102862.

2.13.3. Precision and accuracy

QC samples from at four concentration levels (5, 10, 100 and 2000 ng/ml) (QCP2–QCP5) were prepared for the determination of inter-day ($n=2$) and intra-day ($n=5$) reproducibility. The samples were extracted and analyzed on the same day during each of three consecutive runs and the peak area ratios were compared to an independent standard curve for inter- and intra-day precision and accuracy (during intra-day run).

2.13.4. Recovery

The extraction recovery of Co 102862 and Co 200222 was calculated by comparing the peak areas of extracted Co 102862 and Co 200222 plasma standard to the corresponding peak areas of un-extracted standards of identical concentration in the same matrix. Recovery was performed on the second lowest standard (10 ng/ml) and the highest standard (2000 ng/ml), each in duplicate.

2.13.5. Stability

Bench top stability of Co 102862 rat plasma standards was established at room temperature, over 96 h. Two sets of reconstituted standards were injected on the same day they were prepared. After injection, these samples were left on the bench for 4 days before being injected a second time.

Freeze–thaw stability for Co 102862 was done on the QC samples that had gone through at least three freeze–thaw cycles and analyzed in two runs.

Long-term freezer storage stability of Co 102862 in dog, monkey and rat plasma was assessed in this study. The stability samples were prepared and kept in a −20°C freezer. Stability was determined by comparing the QC samples analyzed on the first day of preparation vs. being stored in a freezer.

2.14. Calibration

The peak area ratios of Co 102862 to I.S. were correlated with the standard concentration over the range of 5 to 2000 ng/ml in mouse, rat, monkey and dog plasma. During one monkey plasma sample analysis, the assay range at the low end was extended to 0.5 ng/ml, in order to quantify samples with concentrations in the range of 0.5 ng/ml to 5 ng/ml. After analysis, a standard curve for the compound was constructed by quadratic regression analysis with $1/x^2$ of weighting of peak area ratio (y-axis) and compound concentration (x-axis) using MassLynx v3.0 software (Micromass).

Table 3
Regression analysis

Species	LOD (ng/ml)	LOQ (ng/ml)	Mean quadratic equation (weighted $1/x^2$)	R^2
Mouse	0.4	5	$y=0.00x^2+0.00675x-0.00333$	0.998358
Rat	0.4	5	$y=3.33\cdot10^{-7}x^2+0.00497x-0.00117$	0.999552
Monkey	0.4	0.5	$y=5.00\cdot10^{-7}x^2+0.00541x-0.00408$	0.999596
Dog	0.4	5	$y=0.00x^2+0.00515x-0.00168$	0.999568

2.15. Pharmacokinetic data analyses

Descriptive pharmacokinetic parameters [C_{max}, T_{max}, $T_{1/2}$, $AUC_{(0-\infty)}$, CL, Vss and F] were determined by standard model independent methods [9] based on the plasma concentration–time data, where; C_{max}=maximum plasma concentration, T_{max}=time of C_{max}, $T_{1/2}$=terminal half-life, $AUC_{(0-\infty)}$=area under the plasma concentration–time curve (from 0 to infinity), CL=total body clearance, Vss=volume of distribution at steady-state, and F=absolute bioavailability. Results are presented as mean and standard deviation (SD), except $T_{1/2}$ which is expressed as harmonic mean and pseudo standard deviation based on jackknife variance [10]. All statistical analyses were performed using Microsoft Excel Version 7.0 (Redmond, WA, USA) and the pharmacokinetic analyses were performed using Microsoft Excel Version 5.0.

3. Results

3.1. Linearity and calibration standard range

The peak area ratios of Co 102862 to I.S. were correlated with the standard concentration over the range of 5 to 2000 ng/ml in mouse, rat, monkey and dog plasma. During one monkey plasma sample analysis the assay range at the low end was extended to 0.5 ng/ml, in order to quantify samples with concentrations in the range of 0.5 to 5 ng/ml. The coefficient of determination (R^2) for the regression was greater than 0.998 for mouse, rat, monkey and dog plasma (Table 3).

Linearity of the calibration curves was demonstrated by calculating the mean absolute %difference and RSD and both being $\leq\pm15\%$ considered as evidence of linearity. Excellent linearity was observed for each of the runs for mouse, rat, monkey and dog plasma, with mean absolute differences ranging from 0.6 to 8.7% and RSDs from 0.8 to 7.9% (Table 4).

3.2. Specificity

LC–MS–MS analysis of blank plasma from five different lot numbers showed no endogenous peaks that interfered with the quantification of Co 102862 and its respective internal standard. Representative chromatograms of extracted blank dog plasma, with internal standard, and with drug and internal standard are shown in Fig. 2.

Table 4
Linearity of calibration curves based on mean absolute %difference and RSD of back-calculated values of calibration standards

Spiked (ng/ml) (SP1–SP8)	Mouse		Rat		Monkey		Dog	
	Abs. mean %diff.	RSD (%)	Abs. mean %diff.	RSD (%)	Abs. mean %diff.	RSD (%)	Abs. mean %diff.	RSD (%)
0.5	–	–	–	–	1.9	2.0	–	–
5	1.1	–	5.8	7.9	6.2	7.3	2.9	3.8
10	4.1	–	3.8	4.1	5.0	6.2	2.8	2.9
30	3.6	–	2.5	4.3	1.6	1.7	1.7	2.9
100	8.7	–	2.6	1.9	1.6	2.1	1.6	1.9
300	5.1	–	1.6	1.9	1.3	1.1	0.6	0.8
1000	4.2	–	2.2	2.8	3.4	4.4	1.6	1.9
2000	2.3	–	0.8	1.4	1.4	1.9	1.0	1.4

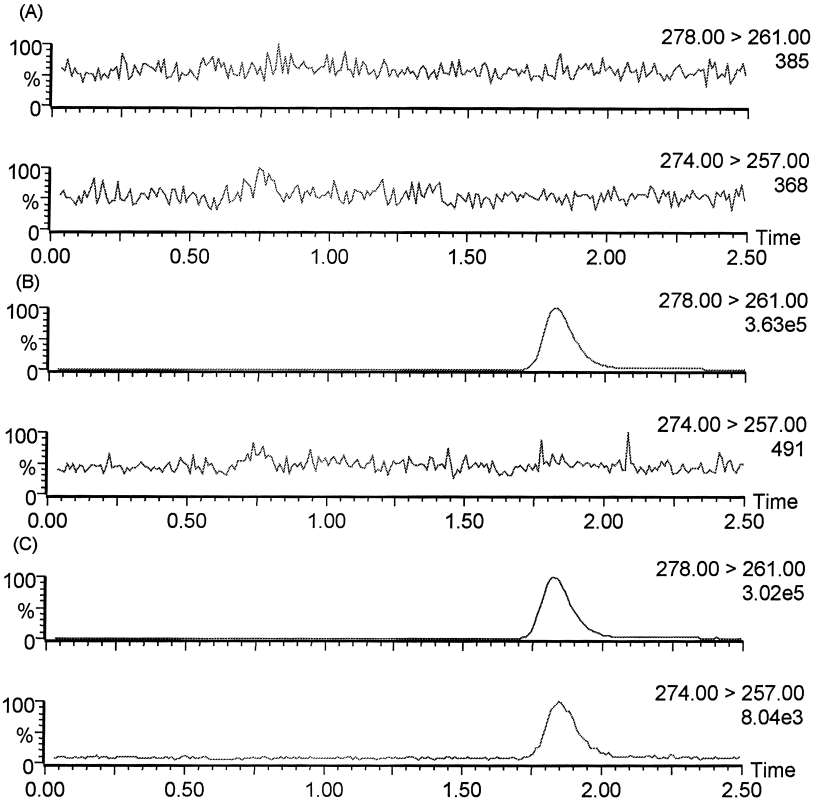

Fig. 2. Representative LC–MS–MS chromatograms from extracted blank dog plasma (A), extracted blank dog plasma with I.S. (B), and extracted dog plasma with I.S. and Co 102862 (5 ng/ml) (C). Internal standard and Co 102862 were monitored from *m/z* 278.00 to 261.00 and 274.00 to 257.00, respectively.

3.3. Sensitivity

The LOQ for Co 102862 using 200 μl of dog plasma sample was 5 ng/ml, with a signal-to-noise ratio of approximately 36.8. The LOD for this study was approximated to be 0.4 ng/ml, based on a ratio of three to one. Concentrations that were calculated to be below 5 ng/ml for Co 102862 calibration standard curve range 5–2000 ng/ml were reported as below quantifiable limit (bql) and below 0.5 ng/ml for calibration standard curve with LOQ of 0.5 ng/ml (Table 3).

3.4. Reproducibility

3.4.1. Inter-day precision and accuracy determination

Analysis of the peak area ratios from QC samples ($n=2$) for Co 102862 during each of three consecutive runs when compared to an independent standard curve gave RSDs that ranged from 6.0–10.1%, 6.4–9.7%, and 3.5–8.9% for rat, monkey and dog plasma, respectively (Table 5). The inter-day accuracy was determined as the mean absolute difference between the spiked and found concentrations for Co 102862 that ranged from 7.6–10.7%, 5.3–12.0% and 5.9–11.4% for rat, monkey and dog plasma, respectively. Assay reproducibility was observed (values$\leq \pm 15\%$) over the range of 5 to 2000 ng/ml (0.5 to 2000 ng/ml for monkey plasma).

3.4.2. Intra-day precision and accuracy determination

The intra-day precision for Co 102862 was evaluated by analysis of plasma QC samples ($n=5$) along with an independent standard curve for quantification. The results summarized in Table 6 show RSDs that ranged from 2.8–5.3%, 0.6–5.7% and 1.0–2.3%

Table 5
Inter-day precision and accuracy for the quantification of Co 102862 in plasma ($n=2$)

Spiked (ng/ml) (QCP2–QCP5)	Rat				Monkey				Dog			
	Found		Abs. mean %diff.	RSD (%)	Found		Abs. mean %diff.	RSD (%)	Found		Abs. mean %diff.	RSD (%)
	Mean	SD			Mean	SD			Mean	SD		
5	5.0	0.5	7.6	10.1	5.5	0.5	12.0	9.6	5.1	0.3	5.9	6.4
10	10.6	0.8	8.8	7.7	10.2	0.9	8.2	9.2	11.1	0.4	11.4	3.5
100	110.3	6.7	10.7	6.0	95.9	6.2	5.3	6.4	109.3	8.1	10.6	7.4
2000	2184.4	165.5	10.7	7.6	1957.6	189.3	8.0	9.7	2149.2	190.9	10.6	8.9

for rat, monkey and dog plasma, respectively. The accuracy of the assays for Co 102862 was determined during the intra-day precision determination (Table 6). The mean absolute difference between the spiked and found concentrations for Co 102862 ranged from 2.2–5.1%, 2.7–12.1% and 10.1–14.9% for rat, monkey and dog plasma, respectively. Assay reproducibility was observed (values $\leq \pm 15\%$) over the range of 5 to 2000 ng/ml.

3.5. Recovery

The mean recovery (Table 7) for Co 102862 ranged from 83.7 to 110.5% while that for Co 200222 ranged from 83.0 to 111.8% in rat, monkey and dog plasma. No concentration-dependent recovery was evident.

3.6. Stability

3.6.1. Bench top stability

There was no significant difference ($\leq \pm 15\%$) between the responses of standards at time zero and after 96 h in terms of %difference (−6.8 to 7.8), indicating stability of Co 102862 in mobile phase at room temperature over 96 h (Table 8).

3.6.2. Freeze–thaw stability in plasma

The mean absolute percent difference between the spiked and found concentrations for Co 102862 in

Table 6
Intra-day precision and accuracy for the quantification of Co 102862 in plasma ($n=5$)

Spiked (ng/ml) (QCP1–QCP5)	Rat				Monkey				Dog			
	Found		Abs. mean %diff.	RSD (%)	Found		Abs. mean %diff.	RSD (%)	Found		Abs. mean %diff.	RSD (%)
	Mean	SD			Mean	SD			Mean	SD		
0.5	–	–	–	–	0.5	0.0	3.4	2.7	–	–	–	–
5	5.1	0.3	3.5	5.3	5.6	0.3	12.1	5.7	5.5	0.1	10.1	2.3
10	10.5	0.3	5.1	2.8	10.7	0.4	7.4	3.9	11.5	0.1	14.9	1.0
100	101.5	2.9	2.3	2.8	102.7	0.6	2.7	0.6	114.5	1.3	14.5	1.2
2000	2009.2	57.8	2.2	2.9	2144.2	20.7	7.2	1.0	2250.4	23.5	12.5	1.0

Table 7
Mean absolute recovery of Co 102862 and I.S. in plasma ($n=2$)

Spiked (ng/ml)	Co 102862			I.S.		
	Rat	Monkey	Dog	Rat	Monkey	Dog
10	110.5	100.0	110.1	105.2	100.0	86.7
2000	83.7	97.6	103.8	83.0	111.8	79.7

Table 8
Bench top stability for Co 102862 in plasma ($n=2$)

Spiked (ng/ml) (SP2–SP8)	Response (peak area ratio of Co 102862/I.S.)		
	Injection at time zero	Injection after 96 h	%Diff. (96 h and 0 h)
5	0.028	0.028	−2.5
5	0.025	0.026	6.0
10	0.057	0.057	−1.2
10	0.055	0.052	−5.1
30	0.186	0.173	−6.8
30	0.169	0.164	−3.1
100	0.561	0.602	7.3
100	0.552	0.555	0.5
300	1.761	1.830	3.9
300	1.712	1.781	4.0
1000	6.003	6.211	3.5
1000	5.860	6.036	3.0
2000	13.149	13.793	4.9
2000	12.851	13.848	7.8

Table 9
Freeze–thaw stability for Co 102862 in plasma ($n=2$, 3 cycles)

Spiked (ng/ml)	Mean absolute %difference (3 and 0 cycles)		
	Rat	Monkey	Dog
10	4.8	13.8	0.3
2000	3.5	11.4	2.4

QC samples that had gone through at least three freeze–thaw cycles ranged from 3.5–4.8%, 11.4–13.8% and 0.3–2.4% for rat, monkey and dog plasma, respectively indicating freeze–thaw stability (Table 9).

3.6.3. Frozen stability

The mean absolute percent difference between the spiked and found concentrations for Co 102862 in QC samples that had been stored in the freezer ranged from 0.3–4.4% over 116 days, 1.4–9.0% over 57 days, and 0.8–14.7% over 65 days for rat, monkey and dog plasma, respectively indicating frozen stability (Table 10).

3.7. Pharmacokinetic study in mouse, rats, monkeys and dogs

The pharmacokinetics and absolute bioavailability of Co 102862 was determined in male dogs, male

Table 10
Frozen stability for Co 102862 in plasma ($n=2$)

Species	Spiked (ng/ml)	Stability (days)	Found mean (ng/ml)	%Diff. (last day and day 1)
Rat	10	61	9.0	
		116	8.6	4.4
	2000	1	1889.9	
		76	1883.9	0.3
Monkey	10	7	11.1	
		57	10.1	9.0
	2000	7	1611.2	
		57	1633.6	1.4
Dog	10	1	8.9	
		65	10.3	14.7
	2000	1	1830.0	
		65	1815.8	0.8

Table 11
Intravenous pharmacokinetic parameters of Co 102862 in male animals, mean±SD

Parameter	Rat[a]	Monkey[b]	Dog[a]
Dose (mg/kg)	2	0.4	0.4
$AUC_{(0-\infty)}$ (ng h/ml)	527±71	931±217	1504±220
CL (l/h/kg)	3.8±0.5	0.5±0.1	0.3±0.0
Vss (l/kg)	3.6±0.5	2.2±0.5	1.6±0.1
$T_{1/2}$ (h)[c]	0.7±0.1	4.5±0.8	4.1±0.4

[a] 10% HPβCD in water formulation.
[b] 50% HPβCD in water formulation.
[c] Expressed as harmonic mean and pseudo SD based on jackknife variance.

mice, male rats and male and female monkeys after intravenous (not in mice) and oral dosing of Co 102862 at different doses and using different formulations.

The pharmacokinetics of Co 102862 in dogs was characterized by low systemic clearance of 0.27 l/h/kg. The steady-state volume of distribution was 1.6 l/kg and the terminal half-life was 4.1 h (Table 11, Fig. 3A). Co 102862 was readily absorbed into the systemic circulation at lower doses but variability in absorption was observed at doses above 10 mg/kg. Absorption of Co 102862 was vehicle dependent as the absolute bioavailability ranged from ~13% in methylcellulose to over 100% in HPβCD (Table 12). The oral absorption of Co 102862 in nanosuspension formulation was studied from 2 to 100 mg/kg. Absorption at lower doses was greater than 23.1% (Fig. 3B) while absorption at high doses was less than 8.4% (Table 13) which was indicative of absorption saturation (in terms of C_{max} and AUC) and prolongation (Fig. 4D).

In mice, Co 102862 was readily absorbed into the systemic circulation at lower doses (Table 13, Fig. 3B) but absorption appears to be saturated (in terms of C_{max} and AUC) at doses above 100 mg/kg (Fig. 4A). At 100 mg/kg, the maximum plasma concentration was 1621 ng/ml and the terminal half-life was 1.6 h.

The pharmacokinetics of Co 102862 in rat was characterized by high systemic clearance of 3.8 l/h/kg. The steady-state volume of distribution was 3.6 l/kg and the terminal half-life was 0.7 h (Table 11,

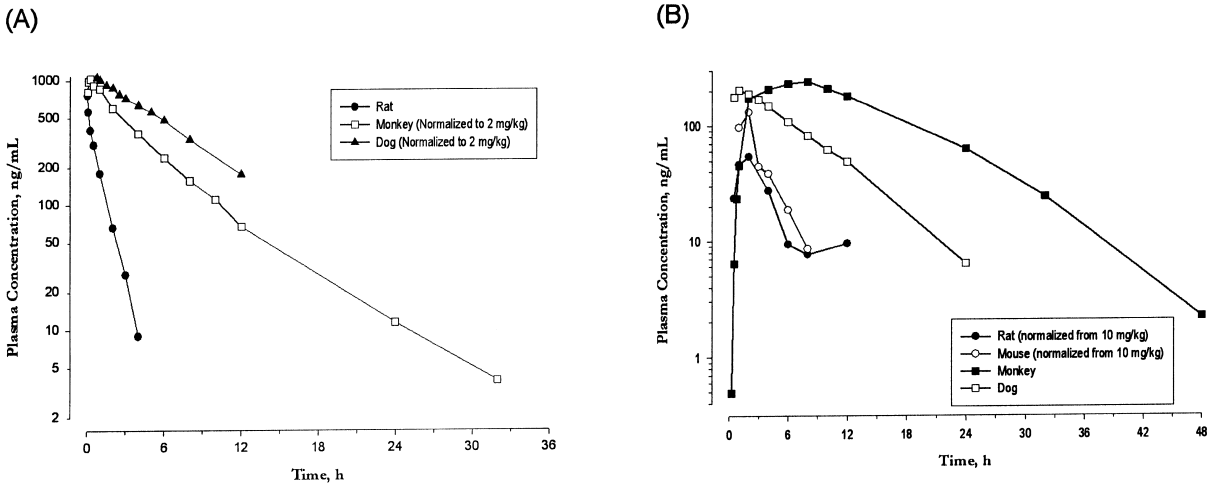

Fig. 3. Mean intravenous (A) and oral (B) plasma concentration–time profiles of Co 102862 in male animals at a 2 mg/kg dose.

Table 12
Oral pharmacokinetic parameters of Co 102862 in male dogs after a 2 mg/kg dose of Co 102862 in various formulations, mean±SD

Parameter	Formulation[a]				
	A	B	C	D	E
C_{max} (ng/ml)	1048±93	788±84	126±31	735±70	206±11
T_{max} (h)[b]	1.0 (1-1)	1.0 (1-1)	1.5 (1-2)	1.0 (1-1)	1.0 (1-2)
$AUC_{(0-\infty)}$ (ng h/ml)	8403±1666	5621±880	949±198	5645±700	1739±366
$T_{1/2}$ (h)[c]	5.0±1.1	3.9±0.3	4.6±0.9	4.6±0.3	4.0±1.2
F (%)[d]	111.8	74.8	12.6	75.1	23.1

[a] A=10% HPβCD in water. B=50% HPβCD in water. C=1% Methylcellulose aqueous suspension. D=95% PEG300–5% Tween 80. E=1.2% HPMC aqueous nanosuspension.
[b] Expressed as median and range.
[c] Expressed as harmonic mean and pseudo on jackknife variance.
[d] Calculated by $AUC_{oral}/AUC_{i.v.}$ normalized for dose.

Fig. 3A). Co 102862 appeared to be well absorbed at low doses (Fig. 3B). Maximum plasma concentration of 271 and 407 ng/ml were achieved, and the absolute bioavailability was 58 and 75% after 10 and 20 mg/kg oral doses, respectively (Table 13). Absorption was saturated (in terms of C_{max} and AUC) and prolonged at doses above 50 mg/kg (Fig. 4B).

The pharmacokinetics of Co 102862 in monkey was characterized by low systemic clearance of 0.45 l/h/kg. The steady-state volume of distribution was 2.2 l/kg and the terminal half-life was 4.5 h (Table 11, Fig. 3A). There was no sex difference in the pharmacokinetics of Co 102862 in monkeys. Co 102862 appeared to be well absorbed at low doses (Fig. 3B). Maximum plasma concentration of 251 ng/ml was achieved after a 2 mg/kg oral dose (Table 13). Absorption was saturated (in terms of C_{max} and AUC) and prolonged at doses above 500 mg/kg (Fig. 4C).

4. Conclusions

The liquid–liquid extraction method gave excellent recoveries of Co 102862 and the I.S. and provided clean extracts. HPLC–MS–MS with ESP in the positive mode of detection appeared to be a very sensitive and selective method for the determination of Co 102862 and possibly its metabolites in mouse, rat, monkey and dog plasma. This technique combines the universality of liquid chromatographic separation with the sensitivity and selectivity of MS detection.

Acknowledgements

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Table 13
Oral pharmacokinetic parameters of Co 102862 in mice, rats, monkeys and dogs after a 2 mg/kg dose of Co 102862, mean±SD

Parameter	Mice[a]	Rats[a]	Monkeys	Dogs
C_{max} (ng/ml)	131	54±15	251±75	206±11
T_{max} (h)[b]	2	2.0 (2-2)	8.0 (4-8)	1.0 (1-2)
$AUC_{(0-\infty)}$ (ng h/ml)	399	275±43	4271±1448	1739±366
$T_{1/2}$ (h)	1.8	2.1±0.5[c]	4.7±0.7[c]	4.0±1.2[c]
F (%)[d]	NA	57.8	91.8	23.1

[a] Normalized to 2 mg/kg from 10 mg/kg.
[b] Expressed as median and range.
[c] Expressed as harmonic mean and pseudo SD based on jackknife variance.
[d] Calculated by $AUC_{oral}/AUC_{i.v.}$ normalized for dose.
NA=Not applicable.

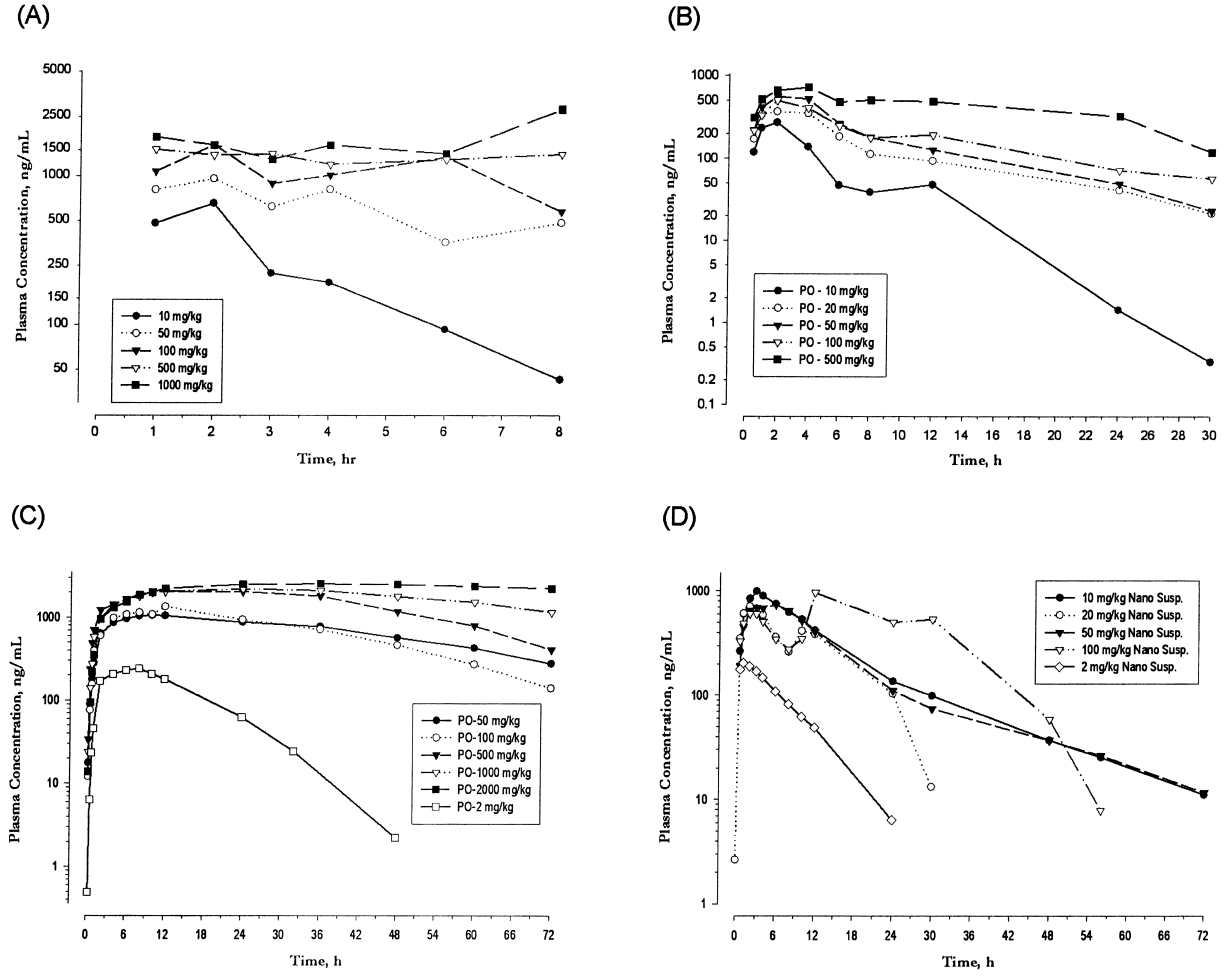

Fig. 4. Mean oral plasma concentration–time profiles of Co 102862 in mice (A), rats (B), monkeys (C) and dogs (D) after a single dose.

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