6. Sisapronil

First draft prepared by

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Identity

International Non-proprietary name (INN; proposed): Sisapronil

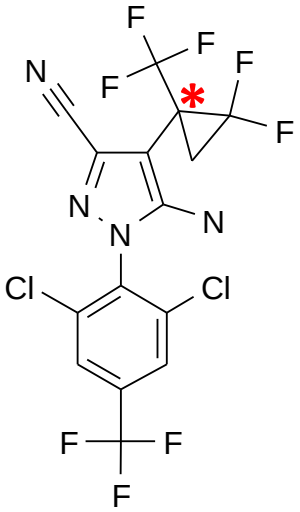
Synonyms: PF-00241851, PF-0241851, PF-241851, BRIN PF-241851, Arylpyrazole

IUPAC name: 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[2,2-difluoro-1-(trifluoromethyl) cyclopropyl]-1H-pyrazole-3-carbonitrile

Chemical Abstract Service Number: 856225-89-3

PubChem number: 172232505

Structural formula:



Molecular formula: $C_{15}H_6Cl_2F_8N_4$

Molecular weight: 465.1282 (average), 463.98416 (monoisotopic)

Other information on identity and properties

Pure active ingredient: Sisapronil is a racemic mixture containing one asymmetric carbon atom (*)

Appearance: White to off-white solid

Melting point: 185-186°C (Form A and Form B)

Log P: 5.1

Solubility:	0.002 g/L water, 20 g/L long-chain triglyceride oils, 100 g/L medium-chain triglyceride oils, 400 g/L short-chain triglyceride oils, 150 g/L benzyl benzoate, 130 g/L ethanol
UV_{max}:	> 220 nm

Residues in food and their evaluation

Conditions of use

Sisapronil is a member of the phenylpyrazole class of antiparasitics. It is a long-acting subcutaneous injectable ectoparasiticide for control of cattle ticks. It also aids in the control of bot fly larvae, hornfly and screwworm. Sisapronil binds tightly to ligand-gated chloride channels, in particular those gated by the neurotransmitter gamma-aminobutyric acid (GABA), blocking the pre- and post-synaptic transference of chloride ions through cell membranes in insects or mites, exposed through ingestion or contact. This mechanism of action results in hyperexcitability of the central nervous system and death of the parasites. Sisapronil has been registered for use in Brazil with a withdrawal period of 120 days.

Dosage

The recommended dose is a single subcutaneous injection of 1 mL *per* 50 kg body weight (BW), equivalent to 2 mg sisapronil/kg BW. The label includes a warning that the product is not indicated for use in dairy cattle.

Pharmacokinetics and metabolism

Test material used in radiolabel pharmacokinetic and metabolism studies

Pharmacokinetic and metabolism studies were conducted with [^{14}C]-sisapronil, which was synthesized using [^{14}C] diazomethane, incorporating the radiolabel into the cyclopropyl ring of the molecule.

Specific activity: 39mCi/mmol

Purity: 99.2% (by HPLC)

Pharmacokinetics in laboratory animals

Studies examining the pharmacokinetics of sisapronil in laboratory animals were conducted as part of the toxicology program, therefore doses were administered primarily *via* the oral route.

Rats

In a non-GLP compliant dose range finding study (Gagnon, 2012a), six groups of 2 male Wistar rats/group received a single oral dose of vehicle or 100, 250, 500, 1000, or 1500 mg/kg sisapronil. Sisapronil concentrations in plasma increased with increasing dose from 100 to 500 mg/kg and began to plateau from 500 to 1500 mg/kg. Mean concentrations in plasma at 6 days post dose were 3380, 6865, 33800, 27600, 30900 ng/mL for doses of 100 (n=2), 250 (n=2), 500 (n=1), 1000 (n=1) and 1500 (n=1) mg/kg BW, respectively.

In a single dose study (Ryan, 2011), rats were administered a single oral dose of 100, 500 or 1000 mg/kg sisapronil. Mean concentrations in plasma at 3 days post dose were 1210 ± 233, 9440 ± 5670, and 20500 ± 5250 ng/mL for 100 (n=20), 500 (n=20), and 1000 (n=14) mg/kg BW, respectively.

In a non-GLP compliant study (Hu, 2009), Sprague Dawley rats were dosed orally with 0.1, 1, or 10 mg/kg BW/day for 28 days. Blood samples were taken at 4, 8, and 24 hours post dose on days 1, 14 and 28. An additional group was given a single dose (0.5 mg/kg BW) with samples collected at 4, 8, and 24 hours after the first dose and on study days 4, 7, 14, 21, 28, 35, 38, and 42. In the 28-day treatment groups, substantial accumulation was observed in all dose groups with C_{max} 5-7 times higher at day 14 than at day 1; however, accumulation had reduced substantially between day 14 to 28 with C_{max} approximately 1.3 times higher at day 28 than at day 14. After the first dose, the AUC_{0-24h} was 142, 1460, and 10920 h ng/mL and C_{max} was 9.35, 77.4, and 580 ng/mL for doses 0.1, 1 and 10 mg/kg, respectively. Concentration of sisapronil in plasma appears to have increased in a slightly less than dose proportionate manner. The mean C_{max}/dose values at day 1 were 93.5, 77.4 and 58.0 ng/mL for doses 0.1, 1, and 10 mg/kg BW respectively. In the single dose animals, sisapronil was rapidly absorbed achieving a C_{max} of 43.5± 6.3 ng/mL at 8h post dose. The terminal disposition phase appeared to begin on study day 6 post dose and the calculation of $T_{1/2}$ was performed using data for study days 6-42, resulting in a plasma elimination half-life of 13.1 days and a mean residence time (MRT) of 19.0 days. Exposure was high in the single dose rats, with an $AUC_{0-\infty}$ of 583 ng day/mL.

In a GLP compliant study (Rodríguez Gómez, 2012), 10 Wistar rats/sex/group were dosed orally once daily with 0.1, 0.3, 1, or 10 mg sisapronil/kg BW/day for 13 weeks. Blood samples were collected 4, 8, and 24 hours after the first dose and analysed using LC-MS/MS. At day 90 of the study, blood was collected at a single time point from 6 animals/sex/group 4 hours post final dose. Due to the limited data available, the pharmacokinetic parameters were calculated combining data of male and female rats. Exposure increased with dose with the exception of the 0.1 and 0.3 mg/kg BW/day dose groups on day 1. Mean AUC_{0-24h} was 129, 103, 339, and 3,496 ng h/mL, for the 0.1, 0.3, 1.0, and 10 mg/kg BW/day dose groups, respectively. Mean C_{max} was 8.9, 6.4, 17.8, and 208.7 ng/mL for the 0.1, 0.3, 1.0, and 10 mg/kg BW/day dose groups, respectively. The sisapronil concentrations in plasma at 4 hours after the last dose (90^{th} day group) were 53, 110, 268, and 1046 ng/mL for the 0.1, 0.3, 1.0, and 10 mg/kg BW/day dose groups, respectively. These concentrations in plasma were 6, 17, 18 and 11 times higher than those at 4 hours after the 1st dose.

In another repeated dose study (non-GLP compliant), rats were administered 0.1, 0.3, 1, or 10 mg sisapronil/kg BW/day orally for 52 weeks (Rodríguez Gómez, 2013a, 2013b). In general, sisapronil concentrations in plasma increased with dose and treatment duration. At day 1, average concentrations at 4-hour post dose were 8.81, 7.53, 23.8, and 248 for 0.1, 0.3, 1, and 10 mg/kg BW/day dosing groups, respectively, and at day 362, the means were 333, 539, 1088, and 2203 ng/mL, respectively.

A strain comparison study was conducted to examine the observed study-to-study variability in concentrations of sisapronil in plasma (Gagnon, 2012b). No difference in C_{max} or AUC_{0-4d}

was found between sonicated and non-sonicated formulations or between Sprague Dawley and Wistar rats after oral dosing at 10 mg/kg. Mean C_{max} values ranged from 669 to 808 ng day/mL and t_{max} ranged from 4 to 8 hours.

Dog

Two studies were conducted examining the oral pharmacokinetics of sisapronil in dogs. In one study (Heward, 2011), beagle dogs (4/sex/group) were dosed orally with unlabelled sisapronil daily for 28 days at 0, 1, 5, or 25 mg/kg BW/day. Single blood samples were collected from all animals on study days 1, 8, 15, and 28 at 8 hours post dose, and plasma samples were analysed for unchanged sisapronil using a LC-MS/MS method (Table 6.1).

Table 6.1. Mean sisapronil concentration in plasma in ng/mL (%CV) determined 8 hours after oral dosing on days 1, 8, 15, and 28 (Heward, 2011).

Dose (mg/kg BW)	Concentrations of sisapronil in plasma (ng/ml) by study day			
	1	8	15	28
1	14.9 (115)	141 (48)	230 (46)	479 (57)
5	35.5 (95)	424 (62)	730 (67)	1500 (57)
25	375 (112)	2050 (47)	3980 (51)	7020 (41)

In the second study (Heward, 2012), beagle dogs (4/sex/group) were dosed orally with unlabelled sisapronil daily for 90 days at 0, 0.3, 1, or 10 mg/kg BW. Single blood samples were collected from all animals on study days 1, 30, 60, and 90 at 8 hours post dose. Plasma samples were analysed for unchanged sisapronil using a validated LC-MS/MS method, with a validated range of 0.500 to 500 ng/mL (Table 6.2).

Table 6.2. Mean sisapronil concentration in plasma in ng/mL (%CV) determined 8 hours after oral dosing on days 1, 30, 60, and 90 (Heward, 2012).

Dose (mg/kg BW)	Concentrations of sisapronil in plasma (ng/mL) by study day			
	1	30	60	90
0.3	5.92 (91)	166 (27)	345 (36)	485 (36)
1	15.1 (85)	503 (49)	920 (56)	1230 (47)
10	120 (110)	4670 (16)	8800 (17)	11000 (18)

Both studies showed a dose dependent increase in sisapronil concentrations in plasma over time, which did not appear to reach steady state.

Monkey

A non-GLP compliant study investigated the pharmacokinetics of sisapronil in monkeys following intravenous (IV) or oral administration (Stuhler, *et al*., 2012). Fasted male and female monkeys (2 of each sex *per* group) were administered sisapronil once either IV (0.5 mg/kg) or orally (2 mg/kg). Blood samples were taken at 1, 2, 4, 8, 24, 96, 168, 240, 336, 504, 672, 840, 1008, 1344, 1680, 2160, and 2880 hours following dosing. The terminal elimination half-life following IV dosing was 12.4 days. Absorption was moderately slow following oral dosing with mean T_{max} of 24 hours, and the oral bioavailability was low (6.5%). Following the 2 mg/kg oral dose, C_{max} was 16.8 ng/mL and $AUC_{0\text{-}70d}$ was 7152 h ng/mL.

Pharmacokinetics in food-producing Animals

Cattle

In a non-GLP compliant study (Boucher, 2012), four male and four female cross-bred cattle were administered a single subcutaneous injection of 2 mg/kg unlabelled sisapronil. Blood samples were collected prior to administration and at 1, 3, 5, 7, 14, 21, 56, 70, 84, 98, 112, 126, and 140 days post dose. In plasma, sisapronil reached a mean peak concentration of 74.8 ng/mL at 15.8 days post dose. The mean terminal half-life was 23.0 days, mean residence time was 48.3 days, and the extent of exposure (AUC) was 3950 day ng/mL.

In another study, groups of 10 cattle were treated with a single injection of sisapronil at 2.0 mg/kg SC or 2.0 mg/kg IV (Merritt, 2011 ref?). Following a single IV dose, mean clearance of sisapronil was very low (0.87 L/kg/d, or 0.6 mL/kg/min), mean volume of distribution was very high (24 L/kg), and the mean terminal half-life was 19 days. Following a single SC dose, mean C_{max} was 72 ng/mL at a mean T_{max} of 12 days. Based upon parallel comparison of mean AUCs for SC and IV treatments, the bioavailability after SC administration was near 100%. The mean terminal half-life was 19 days, and the mean residence time (MRT) was 32 days.

A GLP-compliant study with nine groups of three beef cattle (2 male and 1 female or 1 male and 2 female) averaging 207 kg BW received a single dose of [^{14}C]-sisapronil *via* subcutaneous injection, at a target dose rate of 2.0 mg/kg BW on study day zero (Walker, 2011). Plasma samples were collected from the final two sacrifice groups at study days 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, and 90 (final group only) post dose.

Radioactivity in plasma peaked at 5 days post-dose reaching a mean of 343 µg eq/kg (80 days post-dose), and 300 µg eq/kg (90 days post-dose). Radioactivity in plasma declined to a mean of 42 µg eq/kg at 80 days post-dose and 26 µg eq/kg at 90 days post-dose.

Metabolism in Laboratory Animals

In a GLP-compliant study, two groups of Sprague Dawley rats were administered [14C]-sisapronil by oral dose (Lineham, 2012). One group of eight rats (4/sex; group 1) received a daily oral dose of [^{14}C]-sisapronil at a dose rate of 50 mg/kg BW for four consecutive days. A second group of eight rats (4/sex; group 2) received a single oral dose of [^{14}C]-sisapronil at a dose rate of 50 mg/kg BW. Excreta and cage wash samples were collected daily for four days post first dose for group 1 (multiple dose) rats and daily for 6 days post-dose for group 2 (single

dose) rats. Livers were collected 24 hours post last dose (96 hours post 1st dose) for group 1 rats and 144 hours post dose for group 2 rats. Samples were analysed for total radioactive residues (TRR), and the nature of the radioactivity present in the excreta and liver tissues was investigated by HPLC profiling with off-line radioactivity detection using liquid scintillation counting (LSC).

The primary route of excretion of [^{14}C]-sisapronil derived radioactivity was in the faeces, with approximately 9% (group 1, multiple dose) and 28% (group 2, single dose) of the administered radioactivity excreted within the first 24 hours post the first dose. The percent recovery of total radioactivity over the entire study period was higher in the single dose group, with approximately 49% compared to approximately 31% in the multiple dose group.

The excretion of radioactive residues was gradual with the majority of radioactivity excreted via the faeces. For group 1 (multiple dose) male rats, 27.3 % of total dosed radioactivity was excreted *via* the faeces and 0.8% of total dosed radioactivity was excreted via the urine. For group 1 female rats, 22.6% of total dosed radioactivity was excreted via the faeces and 1.7 % of total dosed radioactivity was excreted via the urine. For group 1 rats, g> 97% of the excreted radioactivity from males and >93% from females partitioned into the faeces. For group 2 (single dose) male rats, 46.5% of total dosed radioactivity was excreted via the faeces and 0.87% of total dosed radioactivity was excreted via the urine. For group 2 female rats, 41.2% of total dosed radioactivity was excreted via the faeces and 2.3% of total dosed radioactivity was excreted via the urine. For group 2 rats, > 98% of the excreted radioactivity from males and >94% from females partitioned into the faeces.

Profiling results demonstrated that intact sisapronil was the primary residue in faeces from both treatment groups, representing >91% of TRR in males and females in both groups from 0-24 hours. The percentage of sisapronil gradually decreased over time, with a more rapid decline in group 2 rats.

Mean residues in liver represented 4.86 and 3.85% of the total radioactive dose administered, respectively, for multiple dose group 1 male and female rats and 3.25 and 3.41% of the total dose, respectively, for single dose group 2 male and female rats. [^{14}C]-sisapronil was the primary residue from group 1 and group 2 rats. One metabolite designated in the test site report as metabolite E correlates with the significant metabolite observed in bovine liver.

Metabolism in food-producing animals

Cattle

In a GLP-compliant study with nine groups of three beef cattle (2 male and 1 female or 1 male and 2 female) averaging 207 kg BW, the cattle each received a single dose of [^{14}C]-sisapronil *via* subcutaneous injection, at a target dose rate of 2.0 mg/kg BW on study day zero (Walker, 2011). Urine and faeces were collected on a total of 12 days, study days 10-12, 30-32, 60-62 and analysed for total radioactive residues (TRR. Bile was also collected at slaughter from each of the nine groups over the 90 day in-life period.

Radioactivity was excreted primarily via the faeces with greater than 97% of excreted residues present in the faeces. For study days 10-12, 2-4% of total dosed radioactivity was excreted

daily via the faeces with ≤0.1% of total dosed radioactivity excreted daily via the urine. By study days 60-62, ≤0.45% of total dosed radioactivity was excreted daily via the faeces with ≤0.01% of total dosed radioactivity excreted daily via the urine. For all days over which excreta and cage wash samples were collected, 14.58% (day 80 animals) and 16.29% (day 90 animals) of total dosed radioactivity was excreted via the faeces representing 97.3 and 97.5%, respectively, of total excreted radioactively for the two treatment groups collected during these study intervals. In bile, a maximum mean TRR concentration of 2409 µg eq/kg was measured at 10 days withdrawal, with a steady decrease in concentration to 84 µg eq/kg at 90 days post-dose.

HPLC fractionation with off-line radioactivity detection was performed for analysis of tissue extracts (Zielinski, 2010; Lu and Wang, 2012). Intact sisapronil was the primary residue in faeces, and typically represented < 40% of the residues in urine. Results in urine showed two significant co-eluting metabolites accounting for 15-62% of the TRR, with intact sisapronil representing 3-42% of the TRR. These two metabolites in urine were characterized as having undergone both an oxidation as well as a conjugation with glucuronic acid.

Tissue residue depletion studies

Radiolabelled residue depletion studies

Cattle

In a GLP compliant study, 27 beef cattle (14 male and 13 female) were treated with the recommended label dose consisting of a single subcutaneous injection of 2.0 mg/kg [^{14}C]-sisapronil (Walker, 2011). Cattle were killed starting at study day 10 and every ten days through study day 90. Loin muscle, injection site muscle (injection site core), surround injection site muscle (injection site ring), fat (omental & renal), small intestine (contents removed), liver (gall bladder removed), bile, kidneys, diaphragm, heart and lungs were collected and analysed for total radioactive residues (TRR).

For edible tissues, fat samples contained the highest concentrations of TRR at all time points, followed by liver, small intestine, kidney, and loin muscle. Concentrations were highest at 10 days withdrawal, and were detected in each of these tissues through 90 days withdrawal (Figures 6.1 and 6.2, Table 6.3). It was noted that homogenization of injection site samples proved difficult, which likely contributed to the inconsistent analytical results from the injection sites.

HPLC fractionation with off-line radioactivity detection was performed for analysis of tissue extracts (Zielinski, 2010; Lu and Wang, 2012). Intact sisapronil was the primary residue in fat, liver, kidney, loin muscle, and injection site muscle. Parent sisapronil accounted for 94-99.6, 86.0-99.6, and 90.2-100% of the TRR in fat, kidney, and loin muscle, respectively. One significant metabolite accounting for 19-45% of the TRR was observed in liver but was not identified. This metabolite comprised a smaller percentage of the TRR (around 20%) at the earlier withdrawal times and increased in percentage over the withdrawal times. Based on

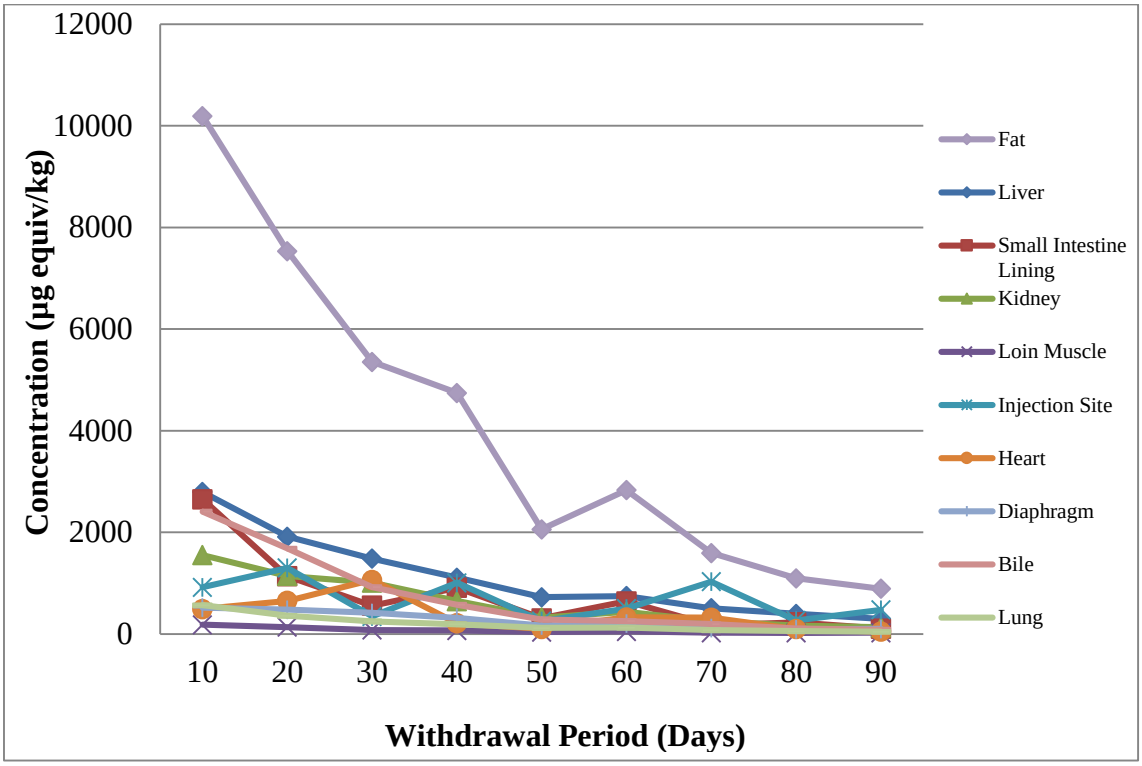


Figure 6.1. TRR depletion (group mean values) in tissues of cattle (n=27) following a single subcutaneous dose of [^{14}C] sisapronil at a rate of 2.0 mg/kg (Walker, 2011).

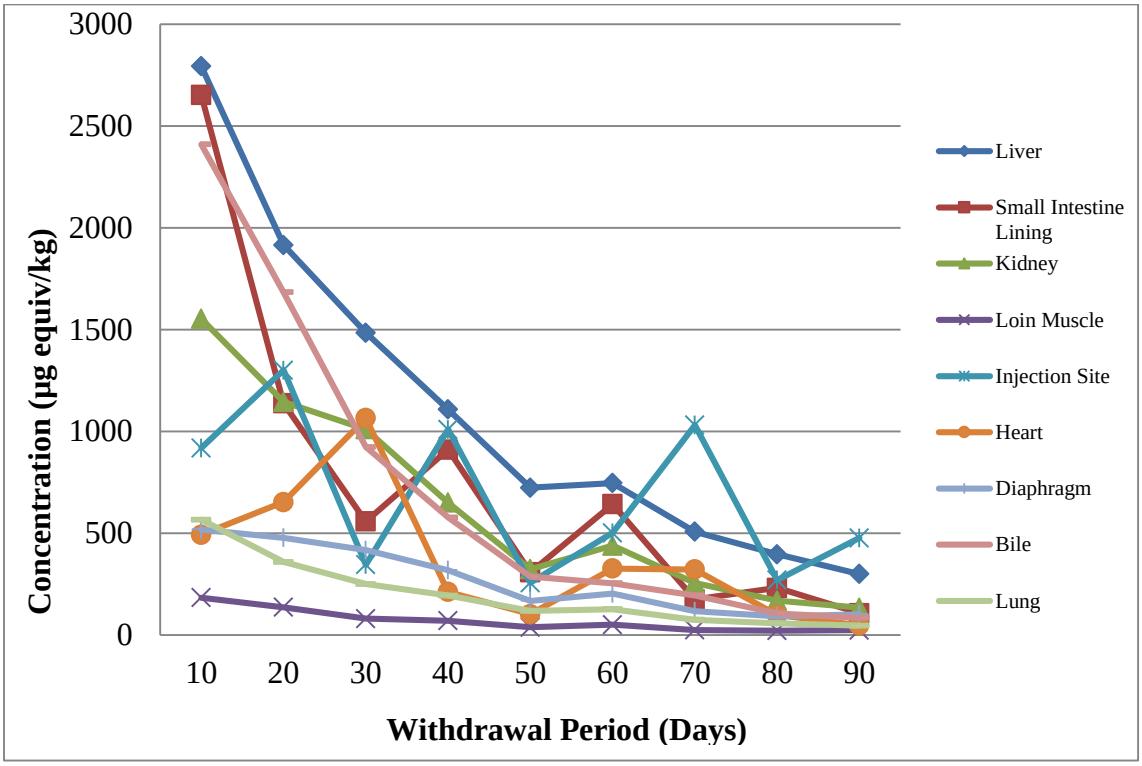


Figure 6.2. TRR depletion (group mean values) in tissues (with the exception of fat) of cattle following a single subcutaneous dose of [^{14}C] sisapronil at a rate of 2.0 mg/kg (Walker, 2011).

Table 6.3. Mean concentration of total radioactivity in tissues following subcutaneous administration of 2.0 mg/kg [^{14}C] sisapronil to cattle (Walker, 2011).

Withdrawal Time	Concentration of sisapronil in tissues reported as µg eq/kg				
	Fat	Liver	Small Intestine Lining	Kidney	Loin Muscle
10 days	10195 ± 763	2793 ± 226	2651 ± 291	1552 ± 564	183 ± 47.1
20 days	7534 ± 1234	1915 ± 588	1137 ± 314	1144 ± 329	136 ± 4.7
30 days	5355 ± 877	1484 ± 182	557 ± 110	1010 ± 357	79.5 ± 2.2
40 days	4743 ± 996	1107 ± 104	910 ± 80.9	650 ± 190	69.6 ± 11.8
50 days	2061 ± 592	723 ± 53.4	307 ± 138	323 ± 70.9	37.8 ± 11.9
60 days	2832 ± 730	746 ± 6.2	642 ± 281	438 ± 114	50.0 ± 13.2
70 days	1595 ± 418	507± 153	174 ± 36.5	255 ± 127	24.3 ± 4.8
80 days	1097 ± 693	396 ± 56.0	231 ± 242	168 ± 83.8	20.8 ± 12.8
90 days	891 ± 517	299 ± 55.0	107 ± 75.9	133 ± 67.3	< LOD

LOD = 30 dpm above background.

These results, parent sisaspronil was identified as the marker residue The mean ratios of parent sisapronil to TRR in liver at various timepoints following administration are presented in Table 6.4.

Table 6.4. Mean ratio of parent sisapronil marker residue (MR) to total radioactive residues (TRR) in liver tissue following subcutaneous administration of [^{14}C] sisapronil to cattle (Zielinski, 2010; Lu and Wang, 2012).

Withdrawal Time (days)	Sample	Mean MR / TRR
10	Liver	0.73
20	Liver	0.64
30	Liver	0.61
40	Liver	0.63
50	Liver	0.57
60	Liver	0.57
70	Liver	0.50
80	Liver	0.64
90	Liver	0.56

Residue depletion studies with unlabelled drug

Cattle

In a GLP-compliant study (Zielinski, 2011), 36 cattle (18 males, 18 females), weighing 236-342 kg at dosing, were treated with a single subcutaneous injection of sisapronil at a mean dose rate of 2.1 mg/kg BW. Two male and two female animals were killed after 30, 60, 90, 120, 150, 180, 210 and 240 days withdrawal post-dose. Hind quarter muscle, primary injection site muscle, surround injection site muscle, liver, kidney, fat (peri-renal) and small intestine (contents removed) were collected from each animal and submitted for analysis of sisapronil using a validated LC-MS/MS method with a Limit of Quantitation (LOQ) of 5 µg/kg.

Sisapronil residues depleted gradually from all tissues over the 240 day study period. Measurable concentrations were still detectable at greater than the LOQ at 240 days withdrawal in all tissues. Fat samples declined from a mean value of 7520 µg/kg at 10 days withdrawal to a mean of 564 µg/kg at 240 days withdrawal. Residues in all individual animal fat residues were below 1900 µg/kg by 120 days withdrawal. All hind quarter muscle, small intestine and kidney samples contained residues which were below 125 µg/kg by 120 days withdrawal, and all liver samples were below 225 µg/kg by 120 days. Table6.5 summarizes the mean tissue residue data.

Table 6.5. Mean concentration of sisapronil (parent drug, marker residue) in edible tissues of beef cattle (n=36) administered sisapronil by subcutaneous injection at a dose rate of 2.0 mg/kg BW (Zielinski, 2011).

Withdrawal Time (days)	Mean Concentration of Sisapronil Parent Drug (marker residue) in µg/kg				
	Fat	Liver	Kidney	Hind Quarter Muscle	Small Intestine
30	7520 ± 1240	759 ± 136	465 ± 136	172 ± 15	232 ± 36
60	3760 ± 741	385 ± 123	249 ± 150	110 ± 40	143 ± 42
90	2450 ± 782	264 ± 92	120 ± 39	93.9 ± 33	132 ± 24
120	1450 ± 429	158 ± 40	97.1 ± 19	49.4 ± 17	40.8 ± 9.2
150	1240 ± 158	133 ± 30	50.1 ± 8.7	61.5 ± 26	55.4 ± 16
180	1160 ± 308	117 ± 27	54.9 ± 13	62.0 ± 45	45.8 ± 12
210	825 ± 284	89.6 ± 30	42.4 ± 14	38.6 ± 15	86.3 ± 54
240	564 ± 211	60.3 ± 21	43.2 ± 22	32.4 ± 18	45.5 ± 24

Residues in the core injection site (~500 g) were greater than residues from their respective samples of tissue ringing the injection site samples (~300 g) for all individual animals through withdrawal day 90. At day 120, residues from 3 of 4 injection site muscle samples were greater than their corresponding surrounding tissue samples and at day 150, 2 of 4 injection site muscle samples had residue concentrations greater than their corresponding samples of surrounding tissue. Beginning at withdrawal day 180 and through the end of the study (day 240), sisapronil concentrations were higher in the samples of tissue surrounding the core injection site for all individual animals. Table 6.6 summarizes residue concentrations in core injection site muscle and concentrations in tissue surrounding the injection site, with an additional column summarizing results for injection site muscle with inclusion of concentrations in tissue surrounding the injection site at days 180, 210 & 240.

Table 6.6. Mean concentration of sisapronil residues for injection site muscle and muscle tissues immediately surrounding the injection site of beef cattle administered sisapronil by subcutaneous injection at a targeted dose rate of 2.0 mg/kg BW (Zielinski, 2011).

Withdrawal Tine (days)	Mean Concentration of Sisapronil Parent Drug (marker residue) in µg/kg	
	Primary Injection Site	Muscle Surrounding Injection Site
30	29650 ± 29662	4007 ± 4223
60	5376 ± 8386	350 ± 206
90	574 ± 625	313 ± 219
120	129 ± 24	159 ± 148
150	277 ± 360	107 ± 30
180	87.4 ± 43	165 ± 92
210	70.7 ± 27	99.5 ± 44
240	76.4 ± 49	119 ± 77

Methods of analysis for residues in tissues

Main principles of the analytical method

A high performance liquid chromatography tandem mass spectrometry method (Zielinski *et al*., 2012) was used to determine the marker residue (parent sisapronil) in bovine edible tissues. The target residue is extracted from 1 g tissue twice with 1% trifluoroacetic acid in (9:1 CH_3CN:H_2O, v/v) (1:7, v/v). After agitation and centrifugation, the supernatant is transferred to a HPLC vial. No additional purification step is performed on the extract. All reagents used

during the analysis were analytical grade or better. The mobile phase was 0.027% formic acid in 2 mM ammonium acetate (v/v) (A) and acetonitrile (B). Injected volume was 5 µL and flow rate was set at 0.5 mL min-1. The stationary phase was a 5 µm C18 100A 2 x 50 mm column equipped with a guard column (2 x 4 mm C18). The gradient was set as follow: 45%A at 0 min, 5%A from 0.6 to 2.4 min, 45%A from 2.5 to 6.5 min. The internal standard used (PF241851) was sisapronil labeled at three positions (13C2-15N). Standard curves were generated using simple linear regression. A 1/x weighting was required during validation in order to span the 5 to 1000 µg/kg analytical range.

Validation of the analytical method

The Committee assessed the validation data against the analytical requirements as published in the Codex guidelines for analytical methods for residue control, CAC/GL 71-2009 (FAO/WHO, 2014).

The method selectivity was proved by comparison of control samples with spiked samples. The non-presence of significant interfering substances eluting at or near the retention time of sisapronil was used as a criteria to demonstrate the method specificity.

The intra-day mean accuracy (defined as the % recovery of the actual concentration) for bovine muscle was 84.9-105%, 98.0-109% for liver, 96.2-108% for kidney, 97.0-110% for fat and 104-107% for small intestine. The inter-day mean accuracy for bovine muscle was 94.1-102%, 103-105% for liver, 100-101% for kidney and 103-105% for fat, meeting the criteria in CAC/GL 71-2009. Small intestine was assayed on just one day and thus inter-day accuracy was not applicable (Zielinski *et al*., 2012).

The intra-day precision (expressed as a coefficient of variation) for bovine muscle was 1.5-11.8%, 1.9-12.5% for liver, 2.9-17.5% for kidney, 1.1-14.9% for fat and 0.8-4.2% for small intestine. The inter-day precision for bovine muscle was 3.4-12.4%, 4.6-8.7% for liver, 5.1-11.8% for kidney and 3.4-9.3% for fat, meeting the criteria in CAC/GL 71-2009. Small intestine was assayed on just one validation day and thus inter-day precision was not applicable (Zielinski *et al*., 2012).

The calculated assay limit of detection (LOD) was established by analysing 20 samples of each control bovine matrix (from each of 6 different cattle) and determining the level of mean background noise in each sample. The LOD was calculated by determining the response of the peak or background at the retention time of the analyte peak and expressing this as the mean plus 3x standard deviations of the background data, meeting the criteria in CAC/GL 71-2009. The calculated assay LODs (rounded to 1 digit) were 0.2, 0.6, 0.6, and 0.3 µg/kg, for bovine muscle, liver, kidney, and fat, respectively (Zielinski, 2013).

The calculated assay limit of quantitation (LOQ) was first established by determining the response of the peak or background at the retention time of the analyte peak and expressing this as the mean plus 10x standard deviations of the background data, meeting the criteria in CAC/GL 71-2009. The calculated assay LOQs (rounded to 1 digit) were 0.9, 0.9, 0.8, and 0.7 µg/kg, for bovine muscle, liver, kidney, and fat, respectively (Boner, 2011). However, after implementation of the validated method, frequent failures of QC samples prepared near the method LOQ were observed. The LC system has thus been changed from an HPLC system to

an UPLC system prior to MS/MS measurement. A second validation study was then initiated (Zielenski, 2013) with main aim to re-evaluate the method performance (accuracy and precision in terms of repeatability and reproducibility) at an estimated LOQ of 5 µg/kg. Calculated assay LOQs (rounded to 1 digit) were 0.6, 1.6, 1.7 and 0.8 µg/kg for bovine muscle, liver, kidney, and fat, respectively. The new assay limit of quantification for sisapronil in bovine edible tissues was set at 5 µg/kg as precision and accuracy fitted within expected ranges at this concentration level.

- The stability of sisapronil was demonstrated in the following experiments:
- In spiking solutions, e.g. acetonitrile/water (50:50, v/v) stored at *ca* 1-8 °C for at least 104 d and 1% trifluoroacetic acid in acetonitrile/water (90:10, v/v) stored at *ca* 1-8 °C for at least 76 d.
- In fortified tissues at -10 °C for at least 80 d in fat/liver, and at least 91 d in muscle/kidney.
- In incurred tissue samples during up to 3 freeze/thaw cycles, as well as at least 18 h in all tissues at ambient temperature, -10°C for at least 193 d for all edible tissues.
- In final sample extracts stored at controlled room temperature for at least 3 d for all edible tissues.

Appraisal

Sisapronil has not been previously reviewed by the Committee. Sisapronil is a long-acting injectable phenylpyrazole ectoparasiticide for control of cattle ticks, and aids in the control of bot fly larvae, hornfly and screwworm. It is registered for use in cattle at a recommended dose of a single subcutaneous injection of 2.0 mg/kg BW. Sisapronil accumulates primarily in fat and is slowly released through the circulatory system and skin, providing prolonged ectoparasitic control.

A radiolabelled study in cattle demonstrated that parent sisapronil is the marker residue and that it remains predominantly unmetabolized (Walker, 2011; Zielinski, 2010). The ratios between the marker residue and the total residues remained steady through 90 days withdrawal, and have been determined in cattle as 0.90 in muscle, 0.50 in liver, 0.96 in kidney, 0.97 in fat. Fat and liver have been identified as prinicpal target tissues. Marker to total ratios in fat, kidney and muscle were farily consistent over time. However, it was noted that there were some fluctuations in recovery of TRR. Therefore, the Committee concluded that a conservative approach to assignment of the marker residue: total residue of pharmacological concern (MR:TR) would be appropriate, and chose the lowest values reported for each tissue.

Residue data were obtained using a validated HPLC-MS/MS method to quantify sisapronil in bovine edible tissues – muscle, liver, kidney, fat and small intestine. The method is applicable in the range of 5.00-1000 µg/kg for all tissues.

Maximum Residue Limits

MRLs could not be recommended by the Committee, as an ADI could not be established.

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