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L-2-Chloropropionic acid metabolism and disposition in male rats: relevance to cerebellar injury

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Abstract L-2-Chloropropionic acid (L-CPA) produces selective necrosis to the granule cell layer of the rat cerebellum. As part of a study to understand the mechanism of selective toxicity we have investigated the metabolism and disposition of [2-^{14}C]L-CPA in the rat, with particular emphasis on the brain. Following a single oral non-toxic dose of 250 mg/kg or a neurotoxic dose of 750 mg/kg or 250 mg/kg per day for 3 days, L-CPA is very rapidly absorbed from the gastrointestinal tract into the blood stream. Peak plasma concentrations of 2 mM (250 mg/kg) and 6 mM (750 mg/kg) L-CPA occurred within 1 h of dosing, and the compound was readily cleared from the plasma with a half-life of 2.6 h. The only metabolite detected in the plasma was 2-S-cysteinylpropanoic acid, presumably derived from the glutathione conjugate. About 60% of the dose is excreted in the urine in the first 24 h as unchanged L-CPA, with a smaller amount excreted as the mercapturate, 2-S-N-acetylcysteinylpropanoic acid. Little radiolabel from L-CPA is excreted in the faeces; however, ~18% of a 250 mg/kg dose of L-CPA is eliminated as carbon dioxide. The radiolabel from [2-^{14}C]L-CPA present in the cerebellum, forebrain and liver at all time intervals examined was L-CPA. There was some indication of retention of L-CPA in the brain relative to the plasma with a small but consistently higher concentration found in the cerebellum. Whole body autoradiography studies indicated some selective retention of radiolabel in the cerebellum after the third dose of 250 mg/kg [2-^{14}C]L-CPA. Our findings indicate that the initial insult to the cerebellum following L-CPA administration is probably due to the parent compound however, the prolonged presence of 2-S-cysteinylpropanoic acid in the plasma and concomitant depletion of glutathione in the cerebellum may also play a role in the toxicity. The relevance of the slightly greater retention of L-CPA in the cerebellum to the selective neurotoxicity of L-CPA requires further study.

Key words L-2-Chloropropionic acid · Glutathione conjugation · Cerebellar granule cell necrosis

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Introduction

The ability to identify why a specific population of neurons are vulnerable to various chemicals might lead to a greater understanding of the basis to discrete neuronal cell necrosis, which occurs in neurological diseases such as Huntington's chorea and Parkinson's disease. The specific lesion obtained following an oral dose of 750 mg/kg L-2-chloropropionic acid (L-CPA) or 250 mg/kg L-CPA daily for 3 days is characterized by a marked loss of cerebellar granule cells, which begins as a multifocal lesion, and rapidly spreads to encompass the entire granular layer of the cerebellum; there may be resulting destruction of up to 70–80% of granule cells (Simpson et al. 1996). Other cell types in the cerebellum are largely unaffected by L-CPA although a small proportion of Purkinje cells appear damaged (Simpson et al. 1996; Jones et al. 1997). The effect of a selective loss in cerebellar granule cells is a severe impairment of normal locomotor activity, which develops by 36 h after L-CPA administration.

Little is known about the metabolism and disposition of L-CPA in the rat; we have reported that L-CPA produces a rapid and time-dependent depletion of liver non-protein sulphydryl (NP-SH) content, mainly glutathione (GSH), while in the cerebellum and forebrain there is a slower time-dependent decrease in GSH (Wyatt et al. 1996a). The decrease in cerebral GSH is not accompanied by a concomitant increase in oxidized GSH (Wyatt et al. 1996a). There is also no evidence of increased activity of the pentose phosphate pathway in slices of cerebellum removed from rats treated with 750 mg/kg L-CPA and examined 24 h after dosing at a time when GSH depletion is maximal (Lock et al. 1995).

Nor is there alteration in ascorbic acid content in the cerebellum (Widdowson et al. 1997), indicating that oxidative stress is not occurring prior to the onset of granule cell neurosis.

The decrease in liver GSH following L-CPA treatment appears to be due to the formation of 2-S-glutathionyl propanoic acid (the GSH conjugate of L-CPA), this reaction being catalyzed by a theta class glutathione-S-transferase. However, no conjugate formation could be detected using homogenates or cytosolic fractions of cerebellum (Wyatt et al. 1996a). Studies with cerebellar slices have shown that the cysteine conjugate of L-CPA (L-2-cysteinyl propanoic acid) can inhibit cystine uptake into ells, which may be a contributory factor accounting for the depletion of cerebellar GSH, by reducing the availability of cysteine as an essential intermediate for GSH synthesis (Wyatt et al. 1996b). The aim of this work was to investigate the distribution, excretion and metabolic fate of [2-^{14}C]L-CPA in male rats following oral administration of a single non-toxic dose of 250 mg/kg and toxic doses of 750 mg/kg, or 250 mg/kg per day for 3 days, with particular emphasis on the site of toxicity, namely the cerebellum. Preliminary observation of this work has been reported in an abstract (Wyatt et al. 1996c).

Materials and methods

Chemicals

L-[2-^{14}C]Chloropropionic acid (98.5% pure; 26 mCi/mmol) was purchased from Amersham International, Little Chalfont, Bucks, UK. L-CPA (96.1% pure) was supplied by Zeneca Specialities, Manchester, the main impurities being D-CPA (2.1%) and 2,2′-dichloropropionic acid (1.0%). 2-S-Glutathionyl propanoic acid (DL-CPA glutathione) was synthesized as described by Wyatt et al. (1996a) and 2-S-L-cysteinyl propanoic acid (L-CPA cysteine) was prepared by the method of Mamalis et al. (1960). 2-S-(N-Acetylcysteinyl) propanoic acid (L-CPA-NAC) was prepared by an adaptation of the method of Mamalis et al. (1960).

Essentially, N-acetylcysteine (2.45 g) was added with clean dry sodium to liquid ammonia (~100 ml) until the blue colour was maintained. Ammonium chloride (0.3 g) followed by L-2-chloropropionic acid (1.63 g) was added dropwise during 10 min, the mixture allowed to evaporate slowly, and residual ammonia being removed under vacuum. Attempts to re-crystallize the residue from aqueous solutions were unsuccessful. The residue was taken up in methanol saturated with hydrogen chloride gas and stirred at ambient temperature for 2 h. The methanol was removed and the residue taken up in water and extracted with ethyl acetate. The evaporated ethyl acetate extract was purified by 'flash' chromatography on silica eluting with 10% methanol in chloroform. The pure diester was de-esterified with dilute aqueous sodium hydroxide to give, after extraction from the acidified solution, pure 2-S-(N-acetylcysteinyl)-propanoic acid. Proton NMR (CD_3OD) and negative ion electrospray mass spectrometry were consistent with the expected structure.

Cellulose tri-acetate filters for micro-centrifuge tubes (mol. wt. cut-off 12 000 Da) and a Bondapak C_{18} HPLC column of 10 μm particle size (3.9 mm × 150 mm) were purchased from Whatman International, Maidstone, Kent. The scintillation cocktails Ultima Gold, Hionic-Fluor and Ultima Flo 17, tissue oxidizer ingredients Carbo-Sorb and Permafluor and tissue solubilizer Soluene 350 were purchased from Packard, Pangbourne, Berks. Glass metabolism chambers were supplied by Jencons Scientific, Leighton Buzzard, Beds. Halothone was obtained from Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK. All other chemicals were of analytical grade and were supplied by Sigma or Fisons Scientific Equipment, Loughborough, Leicester.

Animals

Male Alderley Park rats (200–250 g body weight) were housed in groups under controlled humidity (40–50%), temperature (20 ± 2°) and a 12 h light/dark cycle (lights on at 0600 hours). Animal care and monitoring were carried out in accordance with strict guidelines issued by the UK Home Office. All animal procedures and treatments were performed according to approved animal licences and guidelines. The animals had free access to water at all times and were allowed food (Porton combined diet) ad libitum apart for 18–23 h prior to dosing.

Tissue distribution of radiolabelled [2-^{14}C]L-CPA

A stock solution of neutralized L-CPA (100 mg/ml) in deionized water was prepared and animals were orally dosed at 10 ml/kg with either 250 mg/kg or 750 mg/kg [2-^{14}C]L-CPA and 250 μCi/kg. Groups of four rats per time interval were killed with an overdose of halothane 1, 2, 4, 8, 12, 24 and 48 h after dosing. In another study, rats were dosed orally with 250 mg/kg [2-^{14}C]L-CPA and 200 μCi/kg per day for 3 days; groups of four animals were killed with an overdose of halothane at 2, 8 and 24 h after the 2nd dose and at 1, 2, 4, 8, 12 and 48 h after the 3rd dose. Blood was taken from the heart into lithium heparin tubes using the S-monovette system supplied by Sarstedt (Beaumont Leys, Leicester, UK) and stored on ice prior to centrifugation to obtain the plasma. The liver, kidneys, lung, cerebellum and forebrain were removed and homogenized in ice-cold 0.25 M sucrose, 5.4 mM EDTA, 20 mM TRIS, pH 7.4, to give 25% (w/v) homogenates, which were stored on ice. Samples of plasma (0.1 ml), in duplicate were added directly to vials containing scintillation fluid. Duplicate samples of tissue homogenates (0.5 ml) were digested in Soluene 350 (2 ml) and added to Hionic-Fluor scintillant (20 ml). The amount of radioactivity present in each sample was determined using a Packard 2500 TR scintillation counter. The radioactivity present in the tissues and plasma was converted to μg equivalents of L-CPA/g wet weight of tissue or ml of plasma using the specific activity of the dosing solutions. The remaining tissue homogenates and plasma were stored at −20°.

Excretion studies

The animals used for the 48 h tissue distribution study with 750 mg/kg L-CPA were individually housed in glass metabolism cages and the urine and faeces collected into containers at −70° at 0–12, 12–24, 24–36 and 36–48 h and a final cage wash made at 48 h. At post-mortem the gastrointestinal tract was removed, homogenized and samples, in duplicate, taken for determination of radioactivity. In another study, rats were given a single oral dose of 250 mg/kg L-CPA and 250 μCi/kg and placed singly in glass metabolism cages. Expired air was monitored for exhaled volatiles (0–12, 12–24 h) and carbon dioxide (0–12, 12–24, 24–36, 36–48 and 48–72 h) using *n*-hexane at −70° and sodium hydroxide traps respectively. Urine and faeces were also collected at −70° at 12-h intervals over 72 h and a final cage wash made at 72 h. Duplicate aliquots of urine, hexane and sodium hydroxide were added to Hionic Fluor scintillant (10 ml) and radioactivity was determined by scintillation counting. A 20% (w/v) homogenate of faeces in methanol was prepared and then centrifuged at 2000 g and 4 °C for 20 min; 1 ml of supernatant, in duplicate, taken for determination of radioactivity. The methanol-extracted faecal pellet was allowed to air dry, weighed and radioactivity present was determined in two aliquots of known weight using a Packard sample oxidizer B 307 with collection in carbo-sorb E and Permafluor E$^+$. All fractions were stored at −20°.

Whole body autoradiography

Two rats were dosed orally with 250 mg/kg [2-^{14}C]L-CPA and 200 μCi/kg; one was killed 24 h after the first dose and the other 24 h after the third dose and the distribution of the radioactivity determined by whole-body autoradiography. Following termination, 2% (w/v) carboxymethylcellulose (CMC) was injected into the external nasal passages, the rats were frozen immediately in a mixture of haxane/dry-ice and embedded in 2% (w/v) CMC using the same coolant. Longitudinal sagittal sections of 30 μm in thickness were taken, mounted on adhesive tape and freeze-dried for 48 h. Autoradiographs were prepared by contact with X-ray film (Hyperfilm-βmax; Amersham) and exposed for 6 weeks.

HPLC analysis of L-CPA and its metabolites in urine, plasma and tissues

Samples of liver, forebrain and cerebellar homogenate were thawed and centrifuged at 12 000 g for 20 min at 4 °C. Aliquots of urine, plasma or tissue supernatant (0.4 ml) were placed in microfilter centrifuge tubes (mol. wt. cut-off 12 000 Dalton) and centrifuged at 9700 g for 90 min at 4 °C. The filtrate was analysed by HPLC using the following conditions based on the method of Patel et al. (1994). L-CPA and its glutathione-derived metabolites were separated by injecting 50 μl of sample onto a Bondapak C_{18} column (3.9 mm × 150 mm, 10 μm particle size) coupled to a Shimadzu LC-10 diode-array detector operating at 205 nm. The column was eluted at a flow rate of 1 ml/min, with a gradient of solvent A (0.1 M potassium phosphate, pH 3.0) and solvent B (40% methanol in water) as follows: 0–10 min 100% A; 10–30 min linear increase to 100% B; 30–35 min returned to 100% A and maintained for up to 30 min.

The column effluent was monitored at 205 nm and for radioactivity using a Radiomatic Flo-one beta (Packard) with a 3 ml flow cell detector pumping Ultima-Flo 17 scintillant. Elution times were 1.8 min for 2-S-cysteinyl propanoic acid, 5.5 min for L-CPA, 9.0 min for 2-S-glutathionyl propanoic acid, and 14 min for 2-S-(N-acetylcysteinyl) propanoic acid. For animals dosed with 750 mg/kg [2-^{14}C]L-CPA, plasma, urine and tissues were analysed, whilst only plasma samples were analysed from the single and multiple dose studies at 250 mg/kg [2-^{14}C]L-CPA.

A portion of the urine collected 24 h after administration of 750 mg/kg [2-^{14}C]L-CPA was incubated with β-glucuronidase (1000 Units) for 2 h at 37 °C and submitted to HPLC analysis as described above. Pharmacokinetic analysis of the plasma concentration was performed using the pharmacokinetic data analysis program PHASAR (Zeneca Pharmaceuticals, version 1.3). The area under the curve of plasma concentration versus time (AUC) was calculated using the linear trapezoidal rule to the last time point in the profile with a plasma concentration value judged to be significantly above the background value. The elimination rate constant (λ_z) was calculated by log-linear regression of those data points considered to represent the elimination phase. The half-lives were calculated using the equation $t_{1/2} = 0.693/\lambda_z$.

Results

Tissue distribution and excretion of [2-^{14}C]L-chloropropionic acid

The distribution of radiolabel from [2-^{14}C]L-CPA in the rat, 24 h after a single oral dose of 250 mg/kg or after 250 mg/kg per day for 3 days was broadly similar as detected by whole body autoradiography (Fig. 1). The organs containing the highest radiolabel were the pancreas, thymus and the stomach, including both the mucosa and contents (Fig. 1). The concentration of radiolabel was marginally higher in the cerebellum than in other brain regions after three doses of L-CPA, but otherwise the radioactivity was generally distributed throughout all tissues. The concentration of radiolabel from [2-^{14}C]L-CPA in the plasma peaked ~1 h after administration of 250 mg/kg L-CPA at a concentration of 230 μg/ml (2.1 mM) and at a similar time after 750 mg/kg L-CPA at a concentration of 700 μg/ml (6.5 mM) and was rapidly cleared from the plasma (Figs. 2, 3).

The peak concentration of radiolabel in the brain, both forebrain and cerebellum regions, was very similar at approx. 100 μg/g wet weight (0.93 mM) after 250 mg/kg L-CPA and 250 μg/g wet weight (2.3 mM) after 750 mg/kg L-CPA (Figs. 2, 3). The radiolabel then declined, more slowly than from the plasma, with some retention in both brain regions at 24 and 48 h after dosing (750 mg/kg L-CPA, 24 h: ratio of forebrain/plasma, 1.50 ± 0.20, cerebellum/plasma, 2.13 ± 0.28; 48 h: forebrain/plasma, 3.57 ± 0.30, cerebellum/plasma, 4.65 ± 0.59). The retention of radiolabel was always greater in the cerebellum than in the forebrain at all doses examined with ratios starting at unity during the first 2 h after dosing, and increasing to ~1.1 by 4 h, 1.2 by 12 h and 1.4 by 24 h after 750 mg/kg L-CPA. The data indicate slightly more retention in the cerebellum than in the forebrain. In the liver, kidney and lung the profile of radiolabel following either dose was similar to that of the brain, although the peak concentrations were higher and some retention was observed in the liver relative to other tissues 24 h after dosing (Figs. 2, 3). Administration of 250 mg/kg per day for 3 days led to elimination of radiolabel from plasma, brain and other tissues similar to that seen after a single dose (Fig. 3). There was no obvious indication of accumulation of the chemical in the site of injury, namely the cerebellum.

Excretion of radioactivity, following a dose of 250 mg/kg [2-^{14}C]L-CPA, into the urine was rapid with almost 50% of the dose being cleared within 12 h of dosing, followed by a further 18% in the next 12 h, with a total of 71% being eliminated over 3 days (Table 1). A similar pattern was seen after 750 mg/kg L-CPA except more of the dose was cleared between 24 and 48 h giving a total of 76% elimination in urine over 48 h (Table 1). Excretion in the faeces was small at 3.2% and 8.8% at 250 and 750 mg/kg respectively (Table 1). Following administration of 250 mg/kg L-CPA no exhaled volatile material was detected (< 0.005% of the dose); however, L-CPA was metabolized to CO_2 as shown by the collection of ~11% of the dose over the first 24 h and a total of 16% overall (Table 1). The overall recovery of radiolabel following 250 mg/kg L-CPA was 90 ± 8% compared with 89 ± 3% following 750 mg/kg L-CPA where expired carbon dioxide was not determined.

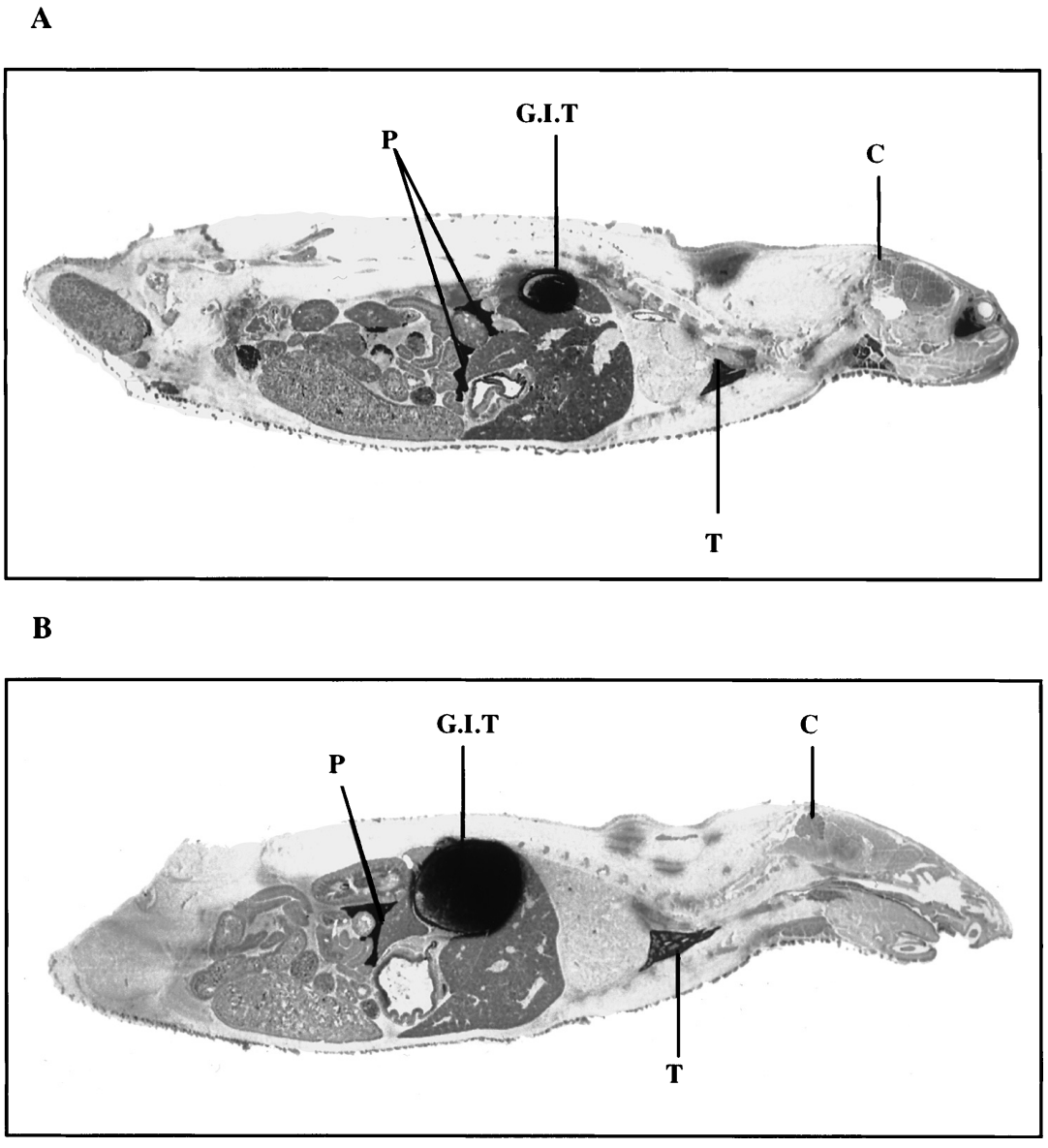
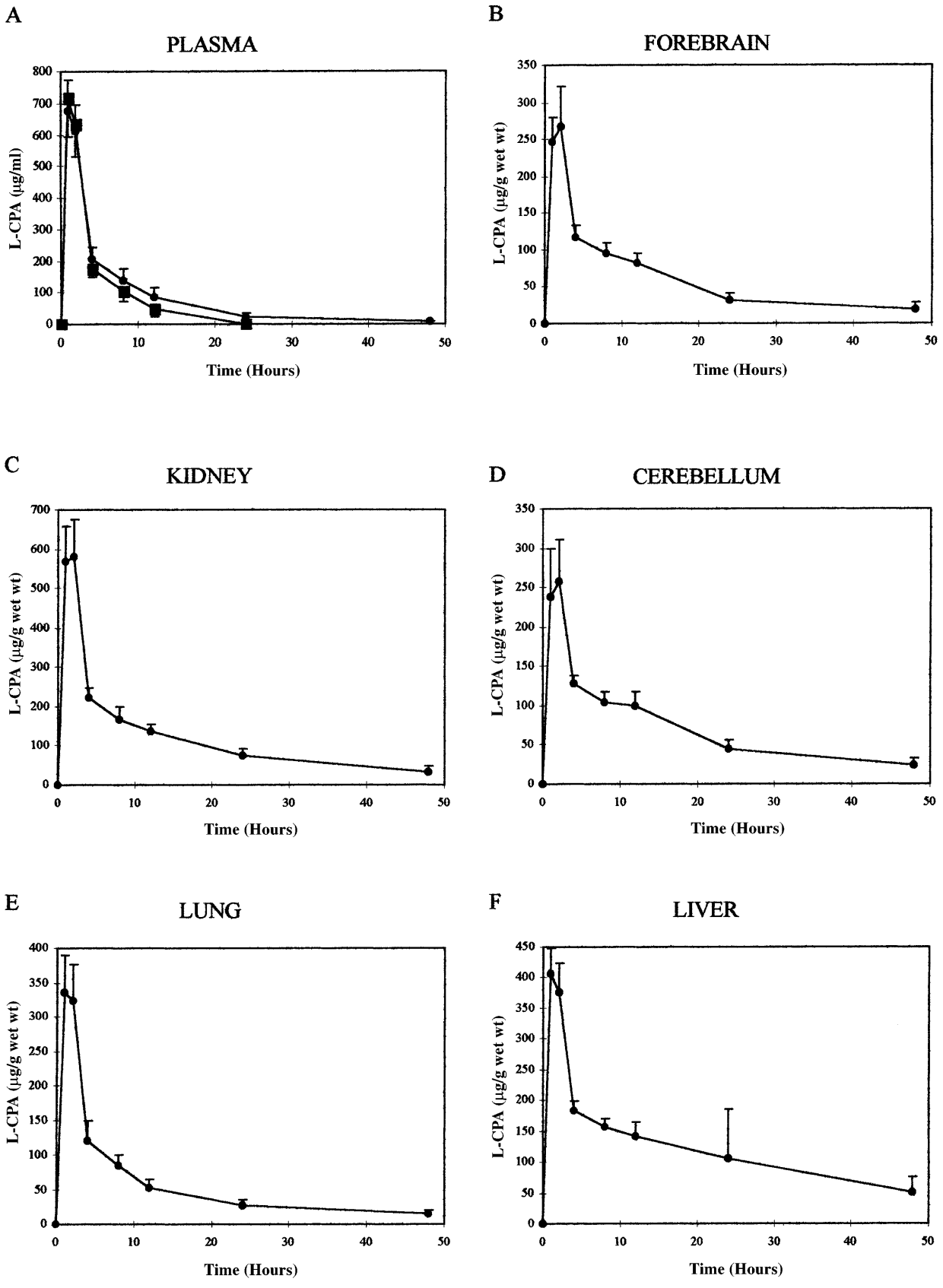


Fig. 1 Whole body autoradiograms of rats dosed orally with [2-^{14}C] L-chloropropionic acid, **A** 24 h after 250 mg/kg and **B** 24 h after three daily doses of 250 mg/kg. Retention of radioactivity observed in pancreas (*P*), thymus (*T*) and gastrointestinal tract (*G.I.T.*) and, after the third dose, some retention in the cerebellum (*C*)

Tissue and urinary concentrations of L-CPA and metabolites

Analysis of the plasma from rats treated with either 250 or 750 mg/kg L-CPA showed the presence of the parent compound and the cysteine conjugate derived from GSH conjugation. The loss of L-CPA from the plasma followed first-order kinetics, the peak concentration being 297 μg/ml after 250 mg/kg and 860 μg/ml after 750 mg/kg (Fig. 4). The area under the curve of plasma versus time and the half-life for elimination from plasma were 1015 μg^{-1} h and 2.62 h for 250 mg/kg L-CPA and 3031 μg^{-1} h and 2.63 h for 750 mg/kg L-CPA. The concentration of 2-S-cysteinyl propanoic acid in plasma following a single dose of 250 mg/kg L-CPA was ~7 μg/ml after the first time interval examined, 1 h after dosing, and remained at this concentration for ~12 h; however, the level was below the limit of detection at 24 h (Table 2). A similar profile of plasma 2-S-cysteinyl propanoic acid was seen after the third dose of 250 mg/kg L-CPA although the concentration was higher than after a single dose at all time points examined, suggesting some increase in the formation of the cysteine conjugate (Table 2). The plasma concentration of 2-S-cysteinyl propanoic acid after a single dose of 750 mg/kg L-CPA was higher than after a single dose of 250 mg/kg L-CPA, but the increase was not proportional relative to the plasma concentration of L-CPA (Table 2).

HPLC radiochemical analysis for L-CPA metabolites in tissues following 750 mg/kg showed that L-CPA was the only material detected in the cerebellum, forebrain


A
PLASMA
L-CPA (µg/ml)
Time (Hours)
B
FOREBRAIN
L-CPA (µg/g wet wt)
Time (Hours)
C
KIDNEY
L-CPA (µg/g wet wt)
Time (Hours)
D
CEREBELLUM
L-CPA (µg/g wet wt)
Time (Hours)
E
LUNG
L-CPA (µg/g wet wt)
Time (Hours)
F
LIVER
L-CPA (µg/g wet wt)
Time (Hours)

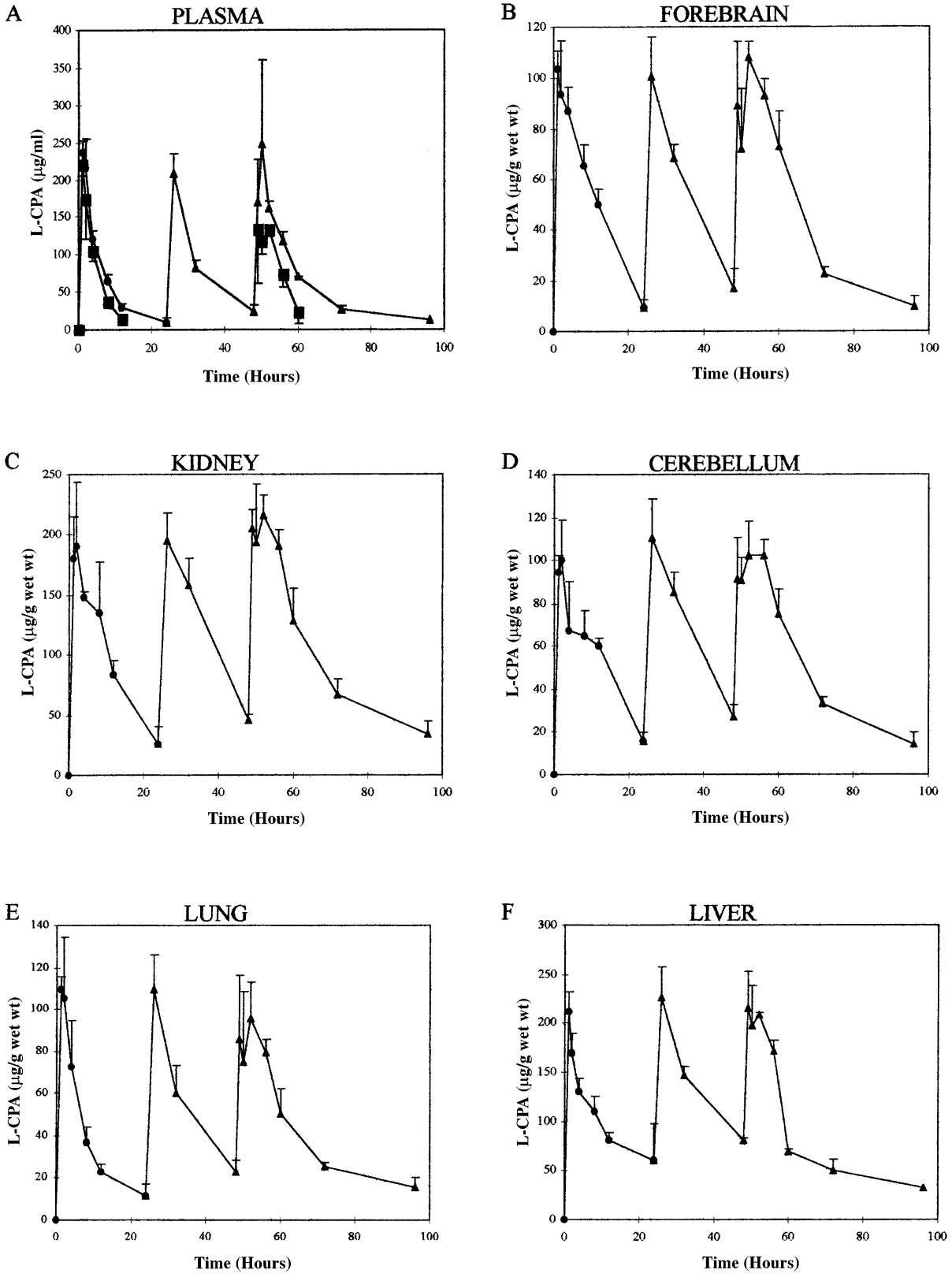


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Fig. 2A–F Distribution of radioactivity from a single oral dose of 750 mg/kg [2-^{14}C]L-chloropropionic acid (L-CPA) in selected tissues during the first 48 h after dosing. Results are of mean ± SEM with four animals per time point. ●–●, Total radioactivity in each tissue expressed as µg equivalent of L-CPA; ■–■, plasma concentration of L-CPA

Fig. 3A–F Distribution of radioactivity following daily oral dosing of 250 mg/kg [2-^{14}C]L-chloropropionic acid for 3 days in selected tissues at various times after dosing. Results are of mean ± SEM with four animals per time point. See legend to Fig. 2 for explanation of symbols

Table 1 Excretion of radioactivity from [2-^{14}C]L-chloropropionic acid following oral dosing of 250 mg/kg or 750 mg/kg to rats. Results are of mean ± SD with four animals per dose (*ND* Not determined)

Treatment	Time after dosing (h)	Urine (% dose)	Faeces (% dose)	GI tract (% dose)	CO_2 (% dose)	Total recovery (%)
250 mg/kg	0–12	47.4 ± 7.2	0.3 ± 0.6	N.D	7.3 ± 2.3	90.4 ± 7.9
	12–24	18.1 ± 6.3	1.8 ± 1.5	N.D	4.2 ± 1.2	
	24–36	1.9 ± 0.5	0.4 ± 0.3	N.D	1.7 ± 0.5	
	36–48	1.2 ± 0.4	0.3 ± 0.1	N.D	1.2 ± 0.3	
	48–72	2.5 ± 0.3[a]	0.2 ± 0.0	N.D	1.7 ± 0.3	
	Total	71.4 ± 5.5	3.2 ± 1.5	–	15.6 ± 0.8	
750 mg/kg	0–12	43.7 ± 14.7	5.4 ± 1.6	N.D	N.D	88.9 ± 2.5
	12–24	14.2 ± 4.0	0.7 ± 0.3	N.D	N.D	
	24–36	6.6 ± 4.6	1.5 ± 2.1	N.D	N.D	
	36–48	11.2 ± 6.4[a]	1.3 ± 0.3	4.4 ± 2.6	N.D	
	Total	75.7 ± 4.0	8.8 ± 2.6	4.4 ± 2.6	–	

[a] Includes cage wash

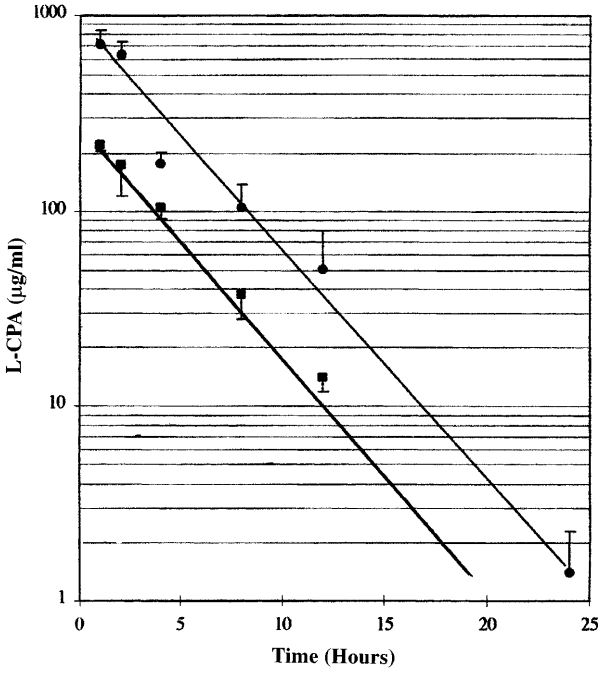

Fig. 4 Clearance of L-2-chloropropionic acid from plasma of rats following a single oral dose of either 250 mg/kg or 750 mg/kg. The *solid circles* are results from the 750 mg/kg dose. Results are of mean ± SEM with four animals per time point

Table 2 Plasma concentration of 2-S-cysteinyl propanoic acid following oral dosing of [2-^{14}C]L-chloropropionic acid (L-CPA) to rats. Results are of mean ± SD with four animals per time point

Treatment	Time after dosing (h)	2-Cysteinyl propanoic acid (µg/ml plasma)
First dose of 250 mg/kg L-CPA	1	6.8 ± 2.1
	2	7.3 ± 2.1
	4	7.2 ± 1.8
	8	9.8 ± 1.8
	12	5.8 ± 0.4
	24	Not detected
Third dose of 250 mg/kg L-CPA	1	10.4 ± 1.9
	2	16.4 ± 4.6
	4	20.9 ± 1.3
	8	13.3 ± 6.5
	12	12.4 ± 3.7
	24	1.9 ± 0.7
750 mg/kg L-CPA	1	14.2 ± 4.3
	2	17.7 ± 5.1
	4	9.4 ± 0.7
	8	13.0 ± 2.8
	12	8.9 ± 1
	24	3.0 ± 2.0

and liver. The concentration of L-CPA in the cerebellum 2 and 8 h after dosing was 192 ± 48 and 91 ± 40 µg/g wet weight respectively, while the concentration of radiolabel expressed as µg equivalents of L-CPA was 203 ± 69 and 114 ± 15 at 2 and 8 h respectively, showing that 95 and 80% of the material in the brain at these times is the parent compound.

HPLC radiochemical analysis of the urine from rats dosed with 750 mg/kg L-CPA showed the presence of two major peaks, representing unchanged L-CPA and its mercapturic acid. During the first 12 h, ~85% of the radioactivity present in the urine was excreted as the parent compound and 9% as the mercapturate; overall ~93% of the radioactivity present in the urine was present as these two metabolites (Table 3). Some urine samples had material that chromatographed with a retention time of 2 min and 9 min, similar to the 2-S-cysteinyl and 2-S-glutathionyl propanoic acid respectively. However, such material was not consistently detected in all animals at each time point and was < 2% of the material in the urine. There was also a small amount of radioactivity consistently found over the first 24 h with a retention time of 17–18 min, accounting for ~4.5% of the radiolabel in urine which had not been identified. Treatment of the urine was β-glucuronidase did not alter the HPLC profile of metabolities, suggesting that conjugation of L-CPA with glucuronides is not a major pathway of metabolism.

Table 3 Main metabolites detected in urine following dosing of rat with 750 mg/kg [2-^{14}C]L-chloropropionic acid (L-CPA). Results are of mean ± SD with four animals per time point

Time after dosing (h)	Total radioactivity in urine (% of dose)	L-CPA in urine (% of dose)	2-S-N-acetyl-cysteinyl propanoic acid in urine (% of dose)
0–12	43.7 ± 14.7	37.0 ± 14.4	4.1 ± 0.5
12–24	14.2 ± 4.0	7.8 ± 2.2	4.9 ± 2.1
24–36	6.6 ± 4.6	3.0 ± 2.4	2.9 ± 1.8
36–48	2.4 ± 1.3	1.3 ± 0.7	1.1 ± 0.7

Discussion

Following oral administration L-CPA is rapidly absorbed from the gastrointestinal tract of the rat. Peak plasma values occurred ~1 h after dosing. L-CPA was readily cleared from the plasma with a half-life of 2.6 h, for both the non-toxic (250 mg/kg) and toxic (750 mg/kg) dose. The bulk of the radioactivity present in the plasma at early times after dosing was L-CPA, although a low concentration of the cysteine conjugate of L-CPA was present. The parent compound and its mercapturic acid are subsequently readily excreted in the urine with the bulk of the radiolabel being eliminated within the first 24 h after dosing. Thus, L-CPA is readily absorbed and enters the plasma.

Calculation of the initial plasma concentration from the plasma decay curves (Fig. 4) gives initial values of 860 μg/ml and 297 μg/ml for 750 and 250 mg/kg respectively, which is close to the expected value if the L-CPA had distributed into total body water. Both the peak plasma concentration and the area under the curve of plasma versus time was three times greater for the 750 mg/kg dose compared to the 250 mg/kg dose. As three doses of 250 mg/kg will produce neurotoxicity analogous to a single dose of 750 mg/kg, the area under the curve of plasma versus time rather than the peak plasma concentration is likely to be important with regard to the neurotoxicity. The radioactivity from L-CPA present in the brain appeared to be mainly the parent compound, no glutathione-derived metabolities being detected. Presumably L-CPA can enter the brain via a transport system such as that for propionic or lactic acid (Oldendorf 1973). The concentration of L-CPA in both the cerebellum and forebrain was very similar at all time points examined, although the amount present in the cerebellum was consistently higher than in the forebrain. The concentration ratio of L-CPA in the cerebellum or forebrain relative to plasma was ~0.4, 1 h after dosing. As the L-CPA was cleared from the plasma the brain/plasma ratio increased being ~1.0 at 8 h, 2.0 at 12 h and ~4.0 at 48 h after a toxic dose. Thus there appeared to be some retention of L-CPA in the brain relative to the plasma, the retention being slightly higher in the cerebellum compared to the forebrain. Whole body autoradiography demonstrated that radiolabel from L-CPA was retained to a significant extent in the thymus and pancreas, but was not associated with histological damage in either of these tissues (M. Simpson, personal communication). However, thymic lymphoid necrosis has been reported in rats given a diet containing 1% (w/v) D,L-2-chloropropionic acid (O'Donoghue 1985). There was also some indication from the autoradiogram of the rat given three daily doses of 250 mg/kg, of localization of radiolabel over the granule cell layer in the cerebellum. Thus there may be some, albeit small, selective retention of L-CPA to the granule cell layer of the cerebellum, the site of injury.

We have derived a hypothesis to explain the selective toxicity of L-CPA for the granule cell layer of the cerebellum. The cerebellar lesion and the cascade of subsequent neurochemical events (Widdowson et al. 1996a; Jones et al. 1997) might be initiated by L-CPA-induced activation of a particular subtype of N-methyl-D-aspartate (NMDA) receptor (NR2A and C) located primarily in the cerebellum (Ebert et al. 1991; Akazawa et al. 1994; Kadotani et al. 1996). Confirmation of the involvement of the NMDA receptor in L-CPA-induced neurotoxicity comes from the findings that non-competitive and competitive NMDA receptor antagonists afford full protection against the neurotoxicity (Widdowson et al. 1996b, c; Lock et al. 1997).

The metabolism of L-CPA appears to occur primarily via conjugation with GSH. In the liver, extensive depletion of the non-protein sulphydryl content was seen following doses of either 250 mg/kg or 750 mg/kg L-CPA (Wyatt et al. 1996a). The glutathione conjugate formed was then presumably converted to the cysteine conjugate by peptidase and γ-glutamyltransferase action with clearance into urine following acetylation to the mercapturate. 2-S-N-Acetyl-cysteinyl propanoic acid was detected in the urine, while the only metabolite detected in plasma was 2-S-cysteinyl propanoic acid. No metabolites of L-CPA were detected in the brain or liver within the limits of sensitivity of our assay. L-CPA was also eliminated unchanged in the urine with a larger amount being excreted at the earlier times after dosing. In addition, some 16% of the administered L-CPA was oxidized to carbon dioxide, presumably by β-oxidation analogous to propionic acid forming a 2-chloropropionyl-CoA ester. The latter is further metabolized to methylmalonyl-CoA and then succinyl-CoA, which can enter the Krebs cycle and undergo metabolism to produce carbon dioxide. This is presumably a detoxification pathway for L-CPA, although metabolism of certain xenobiotic compounds via CoA esters can lead to their incorporation into lipid metabolism with some pharmacological and toxicological consequences (Dodds 1995).

The concentration of 2-S-cysteinyl propanoic acid in the plasma remained fairly constant for ~12 h after dosing at approx. 100 μM following the third dose of 250 mg/kg L-CPA or a single dose of 750 mg/kg L-CPA. 2-S-Cysteinyl propanoic acid has been shown to inhibit the transport of cystine into slices of rat cerebellum with a K_i of 60 μM (Wyatt et al. 1996b), cystine

being the source of cysteine for cerebral glutathione synthesis. As we could find no evidence for enzyme-mediated conjugation of L-CPA with cerebellar cytosol or whole homogenate to account for the depletion of cerebellar GSH seen in vivo, the depletion of cerebellar GSH could be explained by inhibition of cystine transport into the brain. Immature cortical neurons in culture have been reported to be uniquely sensitive to glutamate toxicity when cystine uptake is inhibited (Murphy et al. 1990). Therefore, we cannot currently exclude the possibility that 2-S-cysteinyl propanoic acid may be a contributory factor to toxicity of L-CPA in cerebellar granule cells. Further studies are needed to determine whether the slightly greater retention of L-CPA in the cerebellum and the depletion of cerebellar glutathione are relevant to the mechanism of selective neurotoxicity of L-CPA to the rat.

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