METABOLIC FATE OF PHENOTHIAZINE IN THE MARMOSET (*Callithrix jacchus*)

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SUMMARY

The fate of [^{35}S]-phenothiazine, a veterinary anthelmintic, has been investigated in the adult male marmoset (*Callithrix jacchus*) following oral administration. A near complete recovery of radioactivity (*c.* 95%) was achieved in 0-3 days, with just over one-third of the dose (*c.* 37%) being present in the urine and the remainder (*c.* 58%) being accounted for in the faeces. The majority of the urinary radioactivity (*c.* 91%) was present as conjugates, tentatively identified as phenothiazine *N*-glucuronide and leucophenothiazone sulphate. Smaller amounts of phenothiazone, thionol, phenothiazine sulphoxide and unchanged phenothiazine were also identified. The only compound identified in the faeces was unchanged phenothiazine.

KEY WORDS

marmoset, metabolism, phenothiazine, sulphoxide, anthelmintic

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INTRODUCTION

During the last 50 years many heterocyclic compounds that have been introduced into clinical practice to treat ailments as diverse as nausea and vomiting, coronary insufficiency and psychoses, have contained the tricyclic phenothiazine nucleus. Indeed, the widespread usage and extensive popularity of these phenothiazine derivatives has all but erased from memory that during the middle of the last century the parent compound itself was employed as a bacteriocide, fungicide, insecticide and vermifuge. At that time, its phenomenal worldwide success as a veterinary anthelmintic ranked it alongside DDT and penicillin for its impact on mankind /1/. More recently, phenothiazine has been investigated for its efficacy against resistant strains of sheep nematodes /2,3/ and, surprisingly perhaps, it is still regarded as the 'drug of choice' for treating caecal nematode infestations in poultry /4/. Consequently, information, albeit limited, is available within the literature concerning the metabolic fate of phenothiazine in a variety of animals. Opportunity has been taken to add to this compendium of knowledge by examining the biotransformation of phenothiazine in the marmoset, a species once advocated as an ideal model for investigating biological processes in man.

MATERIALS AND METHODS

Chemicals

Phenothiazine (thiodiphenylamine; dibenzothiophene) was obtained from Sigma-Aldrich Ltd (Dorset, UK) and purified by recrystallisation from diethyl ether /5/ then aqueous ethanol to give light tan crystals, m.p. 181-183°C, UV absorption peaks at 254 and 317 nm, mass spectrum M^+ *m/z* 199 (mol. wt. 199) /6,7/. Radio-labelled phenothiazine was synthesised by refluxing a 50% molar excess of elemental [^{35}S]-sulphur (Nycomed-Amersham, Amersham, UK) with diphenylamine at 180°C in 1,2-dichlorobenzene for 1 h under an atmosphere of nitrogen, 1% (w/w) iodine being added as a catalyst /8,9/. The evolved [^{35}S]-hydrogen sulphide was carried away via the nitrogen stream and trapped by bubbling through an aqueous solution of $FeCl_3$ (1 M). The refluxing solvent was removed by distillation, the unreacted diphenylamine precipitated in anhydrous

diethyl ether by dry HCl gas, the solution filtered and the [^{35}S]-phenothiazine recovered via solvent evaporation. Re-crystallisation twice from aqueous ethanol gave light yellow crystals of specific activity 189.4 mCimol^{-1}, radiochemical purity >98% (thin-layer chromatography [t.l.c.]), mixed melting point 181-183°C, with spectral properties as above.

Phenothiazine sulphoxide (phenothiazine 5-oxide) was prepared by reaction of 3-chloroperbenzoic acid with phenothiazine in cold dry acetone /10/. Light green crystals were obtained, m.p. 247-248°C, UV absorption peaks at 229, 271, 303 and 336 nm, IR sharp band at 1070 cm^{-1}, mass spectrum M^+ *m/z* 215 (mol. wt. 215) /7/.

Phenothiazone (phenothiaz-3-one) was synthesized by the oxidation of phenothiazine with ferric chloride in hot water /11,12/. The crude residue was extracted with hot ethanol and a crystalline product obtained by chilling /13/. Further purification by elution from aluminium oxide columns with chloroform followed by preparative t.l.c. (2.0 mm, silica gel G; carbon tetrachloride/acetone, 4/1, v/v), gave red crystals, m.p. 161-163°C, UV/vis absorption peaks at 236, 258, 272, 368 and 508 nm, mass spectrum M^+ *m/z* 213 (mol. wt. 213) /7/.

Thionol (7-hydroxyphenothiaz-3-one) was prepared by refluxing phenothiazine with sulphuric acid (80% v/v) at 160-165°C for 6 h, the residue being poured into excess iced-water and filtered /14,15/. The crude product was purified by elution with 10% (v/v) aqueous NH_4OH from aluminium oxide columns, followed by preparative t.l.c. (2.0 mm, silica gel G; carbon tetrachloride/acetone, 4/1, v/v) to give red/purple crystals, m.p. >300°C, UV/vis absorption peaks at 232, 276 and 592 nm, mass spectrum M^+ *m/z* 229 (mol. wt. 229).

Leucophenothiazone sulphate was isolated via precipitation by cooling urine obtained from guinea pigs previously fed phenothiazine. Subsequent re-crystallisation from hot water furnished translucent needles that turned purple on heating and decomposed without melting (*c.* 250°C) /16/. Samples of impure phenothiazine *N*-glucuronide were obtained by cold (4°C) butan-1-ol extraction of pooled bile obtained from similarly dosed guinea pigs /16/.

Animals

Marmosets (cotton-eared marmoset, white-tufted-ear marmoset; *Callithrix jacchus*; male, body weight approximately 300 g), were kept

in a warm (20-21°C) humid (relative humidity >65%) environment with a constant light/dark cycle. They were fed once a day with 'Mazuri Primate Diet' (BP Nutrition UK Ltd, Stepfield, Witham, Essex, UK), augmented with meat and liver (Pedigree Petfoods, Melton Mowbray, Leics, UK) and diluted sweetened condensed milk. Chopped fresh fruit (apple, orange, banana, grape) sprinkled with a vitamin-mineral supplement (SA37, Intervet Laboratories Ltd, Bar Hill, Cambs, UK) was also given daily together with water *ad libitum*.

Animals were dosed orally via syringe with [^{35}S]-phenothiazine (150 mg/kg body wt.; dose *c.* 45 mg, 43 μCi) as a suspension in 'Cytacon' vitamin B_{12} syrup (Glaxo Lab. Ltd, Greenford, UK). Accurate dosings were calculated gravimetrically by weighing the syringes before and after phenothiazine administration. The dosing suspensions were prepared immediately before use, and after their administration the animals received a further 1.0-1.5 ml of the dosing vehicle from a clean syringe.

During the experiments the animals were housed in glass metabolism cages (R.B. Radley & Co. Ltd, Sawbridgeworth, Herts, UK) that permitted the separate collection of urine and faeces. Although air was continuously drawn through the system no expired gases were collected.

Quantification of radioactivity

Aliquots of urine and HPLC eluant (0.1 to 1.0 ml) and areas of silica from thin-layer plates were added directly to vials containing scintillation fluid ('Ecoscint', National Diagnostics, NJ, USA) and analysed by liquid scintillation spectrometry using a Packard model 385 scintillation counter (Ambac Industries Inc., Downers Green, IL, USA), efficiency being determined by automatic external standardisation and previously prepared quench-correction curves. Control samples containing known amount of [^{35}S]-material were interspersed with experimental samples to indicate radioactive decay (radioactive half-life of [^{35}S] is 87.4 days). Radioactive decay values were also checked mathematically. Faeces were lyophilised, ground to a mixed powder, weighed and triplicate samples (about 50 mg) combusted in oxygen (Harvey Biological Material Oxidiser, ICN Tracer Labs, High Wycombe, Surrey, UK), the [$^{35}SO_2$] so produced being trapped in an alkaline scintillation mixture containing 2-phenylethylamine and then analysed as above.

Chromatography

Thin-layer chromatography (t.l.c.) was carried out in nitrogen-flushed tanks at 4°C in the absence of light using plates coated with silica gel G (20 x 20 cm, 0.4 mm thick; Merck, Darmstadt, Germany). Three developing solvents were employed: A (chloroform/acetone, 9/2, v/v), B (carbon tetrachloride/acetone, 4/1, v/v) and C (hexane/acetone, 3/2, v/v). Phenothiazine and its derivatives were detected initially under UV light and then by spraying the dried plates with one of several chromogenic reagents: (1) 50% v/v aqueous H_2SO_4, (2) 1% (w/v) I_2 in methanol, (3) 0.8% (w/v) HgI_2 in ethanol (with gentle warming), (4) 5% (w/v) $AgNO_3$ in 10% (v/v) aqueous NH_4OH, or (5) 1% (w/v) $PdCl_2$ in 60ml 0.1 M HCl plus 40 ml ethanol /17/.

High-performance liquid chromatography (h.p.l.c.) was undertaken using a Perkin-Elmer LC sample processor ISS200 (PerkinElmer Analytical Instruments, Bucks, UK) connected to a Dionex quaternary gradient pump (Dionex Ltd, Camberely, Surrey, UK) in line with an LDC SpectroMonitor 5000 photodiode array detector (LDC/Milton Roy, Riviera Beach, FL, USA) and a fraction collector (LKB Bromma; Pharmacia LKB, Piscataway, NJ, USA). Data acquisition was by Chromoquest chromatography software (ThermoFinnigan, Hemel Hempstead, Herts, UK). A reverse-phase Zorbax Rx-C8 (5 μ, 250 x 4.6 mm) analytical column (Phenomenex UK Ltd, Macclesfield, Cheshire, UK) was used together with a gradient elution programme at an overall solvent flow rate of 1 $ml.min^{-1}$. The mobile phase consisted of aqueous acetic acid (0.1% v/v) with its acetonitrile component increasing linearly from an initial 5% to 90% over 15 min, thereafter rapidly returning to 5% during the next 3 min and remaining at these proportions for another 7 min (a total run time of 25 min).

Investigation of metabolites

Urine was examined both neat (concentrated by lyophilisation where necessary) and following incubation with a β-glucuronidase/sulphatase mixture (type H-1 from *Helix pomatia*, Sigma-Aldrich) in acetate buffer (equal vol.; 0.05 M, pH 5) at 37°C for 18 h (to hydrolyse any conjugates present). Incubations with standard phenolphthalein glucuronide or *p*-nitrophenol sulphate confirmed the hydrolysing activity of this enzyme preparation. Lyophilized powdered faeces were thrice extracted with cold methanol (3 x 1 ml) and the

combined extracts filtered and decreased in volume by rotary evaporation at room temperature under reduced pressure. The extracted faecal residues were lyophilised and combusted as described above to detect any residual radioactivity. Known amounts of [^{35}S]-phenothiazine added to control faeces and then extracted gave recoveries of 91.7 ± 4.4% (n = 6) and the results obtained were corrected for this.

Fluids (urine, incubates, extracts) were examined, as spots (10-50 µl) or streaks (100-500 µl), by t.l.c. in the three solvent systems. When developed and dried, the thin-layer plates were scanned directly for radioactivity using a Berthold Series 272 computerized radiochromatogram scanner (Berthold Instruments Inc, Aliquippa, PA, USA) or otherwise cut into strips (0.3 cm) and analysed for radioactivity as described above. Quantification was also undertaken by adding the relevant eluant fraction obtained from h.p.l.c. examination to scintillation fluid and counting as above. Authentic metabolites, dissolved in urine or methanolic extracts of faeces, were co-chromatographed by both t.l.c. and h.p.l.c. to provide provisional identification. Radioactive areas removed from preparative plates (2 mm thick) were extracted with methanol, concentrated, dissolved again in minimum quantities of methanol and, after filtration, examined by UV and mass spectral techniques. Similarly, the collection of relevant h.p.l.c. eluant fractions, with their lyophilisation and reconstitution in methanol, provided further samples for spectral analysis.

Spectrometry

UV and visible spectra were recorded in methanolic solution on a Pye-Unicam SP 1800 UV spectrophotometer (Pye-Unicam, Cambridge, UK). IR spectra were measured using a potassium bromide disc and a Pye-Unicam SP 200 IR spectrophotometer (Pye-Unicam). Electron impact mass spectrometry was carried out on a Kratos MS80 instrument (Kratos Ltd, Manchester, UK) with Kratos D555 (data generator) computerized display and printout facilities. The samples were inserted directly into the ionization chamber at 70 eV with a source temperature of 200°C.

RESULTS

Excretion balance study

All three male marmosets showed similar results. Just over one-third (*c.* 37%) of the administered radioactivity was recovered from the urine (0-72 h) with the majority (31% dose; *c.* 85% of 0-72 h urinary recovery) being voided during the first day. Over half of the dose (*c.* 58%) was recovered from the faeces during the 0-72 h period. In total, a reasonable recovery (*c.* 95%) of the administered radioactivity was recovered during the three days following oral administration (Table 1). The remainder of the radioactivity presumably was still within the animals awaiting excretion, probably via the faeces.

Metabolism

Examination of the first day (0-24 h) urine by t.l.c. showed the same qualitative pattern for all three animals. Five areas of radioactivity were detected at various distances along the t.l.c. plate. The majority of the radioactivity on the plate (and hence within the urine) (*c.* 90%) remained on the t.l.c. plate origin. Elution of material from the four areas that moved off the t.l.c. plate origin and examination by h.p.l.c. (co-chromatography) and spectral analysis, confirmed their identity as the expected metabolites, phenothiazine suphoxide, phenothiazone, thionol and unchanged phenothiazine (Fig. 1; Tables 1 and 2). Together, these four compounds accounted for less than 10% of the urinary radioactivity.

Incubation with β-glucuronidase/sulphatase of material eluted from the t.l.c. origin and re-chromatography by t.l.c demonstrated two radioactive areas corresponding to phenothiazone and phenothiazine, with no radioactivity now remaining on the origin. Examination of urine that had previously undergone β-glucuronidase/sulphatase treatment before chromatography displayed no radioactivity remaining on the origin and a relative enhancement of radioactive content within two (phenothiazone, phenothiazine) of the four radioactive areas detected on the plates. The presence of these two compounds within the t.l.c. plate origin material was also confirmed by h.p.l.c. Isolated crude conjugates taken through this enzyme hydrolysis procedure behaved in an identical manner. Although not proven, it was presumed

TABLE 1

Physical and chromatographic properties of phenothiazine and its derivatives

Compound/metabolite	Phenothiazine	Phenothiazine sulphoxide	Phenothiazone	Thionol
Physical properties				
Melting point (°C)	181-183	247-248	161-163	>300
Absorption maxima				
Ultraviolet	254, 317	229, 271, 303, 336	236, 258, 272, 368	232, 276
Visible			508	592
Mass spectral fragmentation (*m/z*)				
Molecular ion (base peak; M^+)	199	215	213	229
Other diagnostic ions *	198, 167, 154	199, 198, 186, 167, 154	199, 197, 185/6, 135	215, 213, 199, 186, 167

Compound/metabolite	Phenothiazine	Phenothiazine sulphoxide	Phenothiazone	Thionol
Chromatographic properties				
High pressure liquid chromatography *Rt* (min)	13.7	4.0	7.4	5.8
Thin layer chromatography *Rf* value **				
solvent A	0.96	0.35	0.87	0.26
solvent B	0.87	0.13	0.77	0.19
solvent C	0.91	0.37	0.75	0.42

* These daughter ions may be explained as follows: the loss of a fragment to yield phenyl sulphide ($C_{12}H_{10}S$, mol. wt. 186), carbazole ($C_{12}H_9N$, mol. wt. 167) and diphenyl ($C_{12}H_{10}$, mol. wt. 154); all degradation products observed during chemical reaction in the presence of metal ions. The ion at *m/z* 135 observed with phenothiazine may represent the loss of a benzene moiety (M^+, 213 - C_6H_6, 78 = 135).

** Chromogenic thin layer chromatography spray reagents produce distinctive and diagnostic colours with phenothiazine: green with ammoniacal $AgNO_3$, blue with $PdCl_2$ and brown with I_2 and HgI_2.

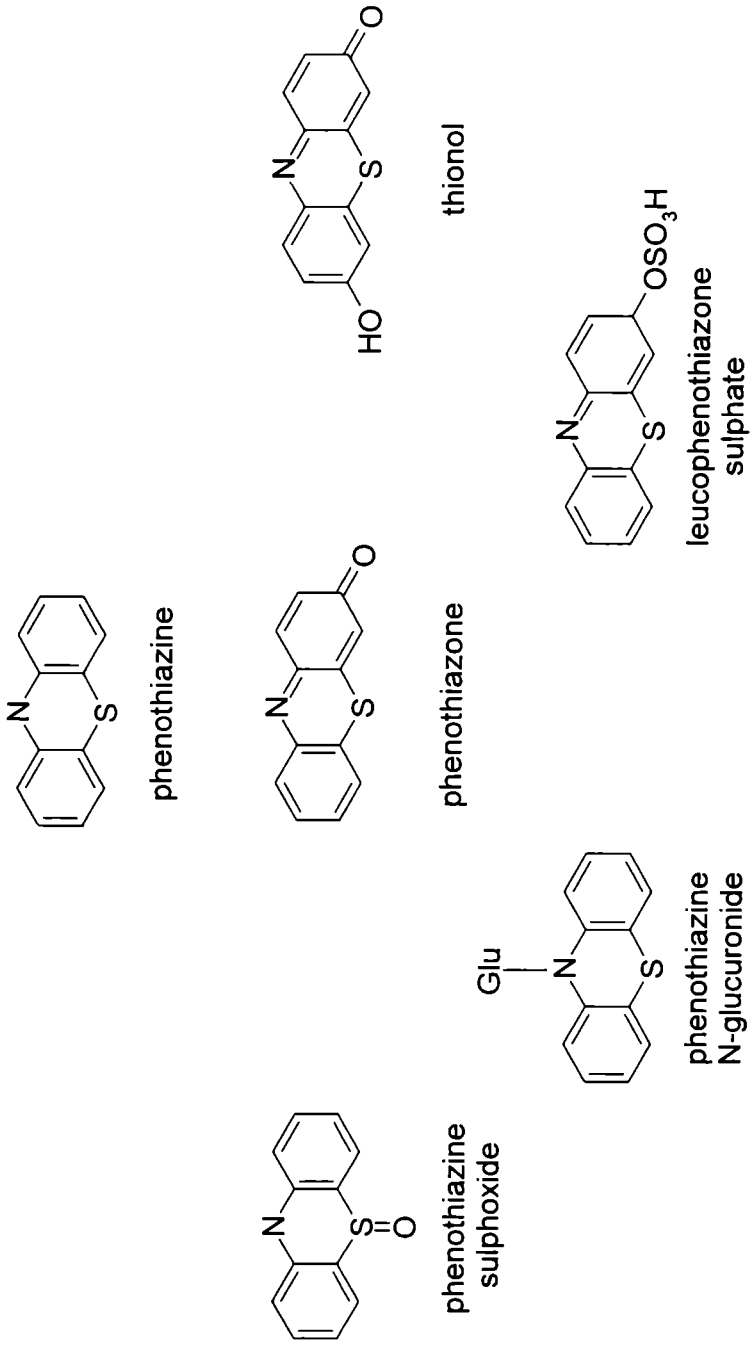

Fig. 1: Structures of phenothiazine and its metabolites.

TABLE 2

Mass balance study and metabolite profile following oral administration of [^{35}S]-phenothiazine to male marmosets

	Animal 1		Animal 2		Animal 3		Mean value	
Radioactive mass balance study (0-3 days)								
Urine								
Day 1	34.6		28.9		30.0		31.2	
Day 2	4.1		6.6		3.6		4.7	
Day 3	0.6		1.6		0.8		1.0	
Total	39.3		37.1		34.4		36.9	
Faeces								
Days 1 to 3	55.5		53.5		63.7		57.6	
Total (0-3 Days)	94.8		90.6		98.1		94.5	
Urinary metabolites (0-24 h)*								
Phenothiazine	3.1	(1.1)	1.5	(0.4)	2.5	(0.8)	2.4	(0.7)
Phenothiazine sulphoxide	1.8	(0.7)	1.1	(0.3)	0.8	(0.2)	1.2	(0.4)
Phenothiazone	4.6	(1.7)	4.1	(1.2)	3.8	(1.1)	4.2	(1.3)
Thionol	2 5	(0.9)	1.2	(0.4)	1.2	(0.4)	1.6	(0.5)
Total free	12.0	(4.4)	7.9	(2.3)	8.3	(2.5)	9.4	(2.9)
Phenothiazine conjugate**	25.2	(9.2)	23.5	(6.8)	38.3	(11.5)	29.0	(9.1)
Phenothiazone conjugate**	62.8	(22.8)	68.6	(19.8)	53.4	(16.0)	61.6	(19.2)
Total conjugated	88.0	(32.0)	92.1	(26.6)	91.7	(27.5)	90.6	(28.3)
Total metabolites	100	(36.4)	100	(28.9)	100	(30.0)	100	(31.2)

* Values given represent the percentage of the amount of radioactive material excreted in the urine in 0-24 h whereas values in brackets relate to the percentage of administered dose.

** These conjugates have been tentatively assigned the structures of phenothiazine *N*-glucuronide and leucophenothiazone sulphate.

that the radioactive material originally remaining on the origin of the t.l.c. plates was conjugated material and consisted of a mixture of phenothiazine *N*-glucuronide (*c.* 29%) and leucophenothiazone sulphate (*c.* 62%) (Tables 1 and 2).

The only radioactive component found in methanolic extracts of faeces showed chromatographic and spectral properties identical to those of the parent compound, phenothiazine (Tables 1 and 2). Methanolic extraction, although more efficient in removing [^{35}S]-phenothiazine added to control faeces (91.7 ± 4.4%), only removed 84.4 ± 5.6% of the radioactivity associated with faeces. Hence, the chemical identity of about 15% of the radioactive material within the faeces (0-72 h) remained unknown.

DISCUSSION

The recovery of a large proportion of an orally administered dose within the faeces reflects the efficiency of this compound to remain unabsorbed within the gut lumen and undertake its role as an anthelmintic agent, removing gastrointestinal nematodes during its transit. This faecal route of elimination may be enhanced by biliary excretion; phenothiazine oxidation products have been observed in bile collected from chicken, cow, dog, guinea pig, human, rabbit, rat and sheep /18/. However, when totalled, these metabolites only accounted for a few percent of the administered dose. In a radiolabel study involving the guinea pig, only 2.7% of the dose was collected in the entire first day bile output, although for other non-cannulated animals traces of phenothiazone and phenothiazine sulphoxide (presumed from bile) were identified in faeces /16/. In the present investigation, only phenothiazine was identified in faeces in the marmoset (although the chemical identity of 15% of the radioactivity was unknown), and this situation has been reported for several other species, including cows, horses, humans, rabbits and sheep /18/. Unfortunately, that part of the dose which is absorbed from the gastrointestinal tract is undoubtedly a hindrance to the desired anthelmintic therapy and is presumably responsible for many of the adverse reactions and toxic episodes that have been observed /1,19/.

The collection of metabolites found in the marmoset excreta is in general agreement with that observed for other species examined and there is nothing atypical or unexpected in the marmoset profile. All of

the phenothiazine metabolites presently reported, that involve sulphur and carbon oxidation and conjugation with glucuronic acid or sulphate, have been described before in the literature /1,18,19/.

Information accrued so far indicates that all species studied excrete the majority of their urinary products in a conjugated form (>80%), mainly as phenothiazine *N*-glucuronide or leucophenothiazone sulphate, although thionol glucuronide is important in some species (rabbit, rat) /20,21/. In terms of comparative metabolism, the underlying difference appears to reside with the percentage drug undergoing C-oxidation and not if conjugation occurs with glucuronic acid or sulphate. Accurate quantitative data are not available for many species, but nevertheless they may be placed into two groups; those in which C-oxidation predominates (dog, horse, sheep, cow, rabbit, gerbil, rat, mouse, marmoset) and those in which it accounts for a third or less of the urinary output (man, pig, guinea pig, hamster). This division is not reflected in the spectrum of the remaining unconjugated metabolites /18/.

Owing to their relatively small size, marmosets are often used in toxicological testing and as an experimental model in drug metabolism studies /22,23/. In particular, they have been advocated as an alternative animal model for human drug glucuronidation /24/. Investigations into the expression (and amino acid sequence) of marmoset hepatic cytochromes P450 indicated that they showed about 90% homology with humans, 3% lower than the cynomolgus monkey but 17% greater than the rat. It was concluded that, '*the marmoset stands at a midpoint between human and non-primate experimental animals*' /25,26/. In the present investigation, with respect to the metabolism of phenothiazine, the marmoset appears to dwell within the larger group of species studied in which C-oxidation predominates, and hence does not necessarily reflect the metabolism profile obtained for man. However, many factors, not least the dose rate, need to be taken into consideration before any sound judgments of this nature may be made.

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